Fast Phase Dispersion Microscope: A New Instrument for Cellular Biology

by

Andrew In-Kyun Ahn

Submitted to the Department of Electrical Engineering and Computer Science
in partial fulfillment of the requirements for the degree of

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Abstract

This thesis describes the design, implementation, and preliminary results of an instrument, the Fast Phase Dispersion Microscope (FastPDM), that uses the dispersion properties of biological materials as a means of achieving contrast in a microscope image. We use a Mach-Zender interferometer with two harmonically related wavelengths of light to measure phase changes due to dispersion. By using harmonically related light, we can correct for motional jitters which would otherwise make phase measurements impossible. A microscope image is formed by using a translation stage to scan the sample between two high numerical aperture microscope objective lenses, and phase measurements are made using analog phase detectors. The optics and electronics systems will be described in detail, and phase dispersion images of an onion skin and a human carotid artery will be shown. Future refinements to the FastPDM will also be discussed.

Thesis Supervisor: Michael S. Feld
Title: Professor of Physics
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Chapter 1

Introduction

In the presentation speech to announce the award of the 1953 Nobel Prize in Physics to Frits Zernike, for the invention of the phase contrast microscope, the Nobel selection committee wrote,

"It would scarcely be an exaggeration to claim that the microscope is one of our most important instruments of research. Every improvement, even a slight sharpening of this eye towards the micro-cosmos, may pave the way to great advances in the natural sciences, medicine, and the technical sciences."

Since the advent of the compound microscope first allowed investigators to view the structure and dynamics of tiny biological structures that are invisible to the naked eye, refinements and innovations in microscope technology have continued to advance the field of cell biology, and have been a vital tool in understanding the natural world we live in. The origins of the light microscope began nearly 800 years ago, when glass was manipulated to make lenses. Three hundred years later, in the sixteenth century, the compound microscope was invented, allowing scientists to peer upon objects that had previously been invisible to the naked eye. Robert Hooke, in the seventeenth century, peered through a microscope and coined the term "cell". In the 1820's, Robert Brown used a microscope to discover Brownian motion. [10, pp 2-5].
From the use of compound microscopes to discover cells as the basic unit of life, to the use of high-energy electrons to image sub-cellular structures and even individual atoms, the implementation of new microscopy techniques has allowed scientists to probe smaller structures more clearly and to see structures that have never been seen before. Such refinements have led to an increase in the knowledge of the subtle structures and behavior of the microscopic world.

This thesis describes the evolution of a new microscopy technique, known as phase dispersion microscopy. This new instrument uses the physical and optical properties of dispersion in biological material to distinguish structures in cells and tissues, without the need of exogenous staining, and is able to image objects that otherwise appear transparent to standard microscopy techniques, such as phase contrast microscopy. The phase dispersion microscope uses interferometry to quantitatively measure nanometer optical length changes, and uses two common-path laser beams to eliminate interferometer noise. The phase dispersion microscope is novel because it uses the natural dispersion property of biological objects as a contrast agent.

In Chapter 2, a brief overview of current and historically important microscopy techniques will be presented. This discussion will include Zernike’s famous phase contrast microscope, as well as newer techniques such as Nomarski interferometry and optical coherence tomography. The standard techniques and limitations of light amplitude-based microscopy will be discussed. This chapter can be by no means inclusive, and only a subsection of the relevant microscopy techniques will be introduced, particularly those related to measurements of phase.

In Chapter 3, the principles of interferometry will be discussed, as well as the use of overlapping harmonic wavelengths as a stabilization method. Finally, we will introduce the principle of phase dispersion, and will derive the appropriate equations to show that this technique can be used to stabilize an interferometer, as well as act as a contrast agent to produce images without the need for staining. The proof-of-principle phase dispersion microscope will be discussed, and the need and the potential applications for a new instrument based on phase dispersion microscopy will be presented.
The two subsequent chapters will deal with the issues involved in designing and building the new instrument. In Chapter 4, the experimental optical setup for this new instrument will be presented, as well as a discussion of the design evolution and alternative designs. We will discuss the basic principles of optics, as well as the intricacies of dealing with harmonic wavelengths. Chapter 5 will discuss the experimental electronics design and implementation for scanning control and phase measurement, as well as the data acquisition and imaging software. Issues with laser noise, and alternative implementations for the phase measurement and control systems will be discussed.

Chapter 6 will present the preliminary results of this new instrument, from the initial calibration measurements to the first biological samples. We will show that the new phase dispersion microscope is capable of measuring nanometer-scale optical length changes in non-biological and biological samples, as well as image interesting pathology samples.

Finally, in Chapter 7, the potential for this new instrument in pathology and histology will be discussed, as well as improvements that will be necessary for the instrument to be fully utilized.

This thesis research was performed at the George R. Harrison Spectroscopy Laboratory of the Massachusetts Institute of Technology, and would not have been possible without the generous financial support of the MIT Laser Biomedical Research Center and Hamamatsu Corporation.
Chapter 2

Microscopy Tools for Cellular Biology

The microscope has been the most vital tool in the study of cells, in understanding basic cellular architecture, subcellular structures, and in determining the interactions between groups of cells and tissues and the dynamic behavior of cells. Without it, the modern understanding of cells and tissues would not be possible. Over the past centuries, technological innovations have allowed investigators to study structures that are ever smaller and subtler than before. There are numerous types of microscopes, ranging from simple compound microscopes to instruments that use X-rays, acoustic waves, and electron beams to probe a biological sample. We will begin our discussion by overviewing microscopes that are particularly useful in cellular biology.

2.1 Important Criteria for Microscopy

There are numerous characteristics by which various microscopy techniques are judged. Depending on the type of sample and the type of investigation, different types of microscopes will be appropriate.

The optical principle for a conventional light microscope is described in Figure...
2-1. There are three planes normal to the optical axis, the object plane (O), the intermediate image plane (O'), and the eye plane (O''). The objective lens \( L_{ob} \) projects a magnified image of the object at the intermediate image plane \( O' \), and the ocular lens \( L_{oc} \) magnifies the intermediate image \( O' \) into the retina. The result is an inverted, magnified image into the retina of the observer. The total transverse magnification, \( M_{tot} \), is the product of the ratios of the front and back distances of both sets of lenses, such that

\[
M_{tot} = \frac{b}{a} \times \frac{25\text{cm}}{f_{oc}}
\]  

(2.1)

where 25cm is the near distance from the observer's eye to the ocular and \( f_{oc} \) is the focal length of the ocular. [3, pp 17.4-5]

![Figure 2-1: Optical principle setup for conventional light microscope.](image)

In addition to the transverse magnification, the important criteria for microscopy is the lateral resolution, which is limited by diffraction. Assuming ideality of the objective lens and a small source in the object plane, the projection of the point source onto the intermediate objective plane is a diffraction image known as Fraunhofer diffraction. The irradiance pattern of the diffraction is given by the first order Bessel function, and includes a central bright disk known as the Airy disk, with a radius proportional to the wavelength of light and inversely proportional to the numerical
aperture of the objective lens,

\[ x_{\min} > \delta = \frac{\lambda}{2NA} \]  

(2.2)

where \( \delta \) is the radius of the Airy disk, \( \lambda \) is the wavelength of light, and \( NA \) is the numerical aperture of the lens, defined as

\[ NA = \frac{D}{2f}, \]  

(2.3)

where \( f \) is the focal length of the lens and \( D \) is the diameter. The numerical aperture defines the maximum cone angle of light accepted or emitted by an optical system. [2, p11-30] It is not possible to resolve images of two points on the object plane if they are closer together than the radius of the Airy disk, a condition known as the Rayleigh criterion, or the diffraction limit. This is seen in Figure 2-2, where two overlapping Airy disks can be clearly resolved (the separation is greater than \( \delta \), (a) in Figure 2-2) and where the two overlapping Airy disks cannot be clearly resolved (the separation is equal to \( \delta \), (b)). [3, pp 17.7-8]

Thus, important considerations for designing a microscope include the light source, which determines the wavelength of light being used; the type of lens, which determines magnification, and the radius of the Airy disk; and the imaging modality, whether it is viewed by the eye, recorded onto film, or displayed electronically. There are other constraints that need to be considered, such as the minimum allowable depth of focus. Additionally, a vital characteristic of a microscope, in addition to the magnification and the resolution, is how it generates contrast.

### 2.2 Contrast Generation in Light Microscopy

Light from a physical standpoint has a dual nature as a particle (photon) and wave (through electric and magnetic fields that are mutually orthogonal to each other and to the direction of light propagation). When considering the amount of light that
Figure 2-2: The Rayleigh criterion. If the Airy disks are spaced by greater than the radius of the Airy disk, then they can be clearly resolved (a). If the spacing of the two disks is equal or smaller than the radius of the Airy disk (b), we cannot clearly resolve the two separate disks [3, p 17.7]

passes through a solid, non-transparent object, it may be adequate to consider only the amplitude of the light as a photon, although this would not explain the diffraction limiting effects described in the previous section. As the designs of microscopes get increasingly complex, the photon view is insufficient and the wave view of light, and particularly the amplitude and phase of the electric field, become particularly important.

Contrast is as important to the performance of a microscope as the resolving power of the instrument. [10, p 199] In typical biological microscopy applications, specimens being examined are thin, transparent samples. Because absorption varies exponentially with thickness, when a sample is sliced into thin sections for a microscope slide, very little of the light passing through the sample will be absorbed. The only optical difference across the sample will be slight differences in refractive index or thickness. [3, p 17.22] Thus, conventional light microscopy will fail to render a significant image for many interesting biological samples. Many methods of artificially generating contrast have been developed to make otherwise transparent objects visible. They can be categorized into two classes, amplitude methods and phase methods.
When developing the phase contrast microscope, Frits Zernike first compared a microscope image to a superposition of diffraction gratings (as theorized by Ernst Abbe), where diffraction is caused by the variation in amplitude of light passing through the sample, which will be considered an amplitude object, or by the variation in light path-length, a phase object. Samples of biological interest are generally considered to be phase objects, and so to be observable must be changed into amplitude objects.[14, p 346]

2.2.1 Amplitude Methods

In the absence of absorbing substances in a specimen, a transparent object contrast can sometimes be achieved by observing the object slightly out of focus. This is known as a Becke line, and occurs at a slightly out of focus boundary with a sharp gradient of refractive index. However, this effect worsens the transverse resolution and disappears when the boundary is exactly focused. [3, p 17.22] Other means have been proposed to adequately image a microscopic boundary.

Chemical Staining

Histological samples are prepared for use under a microscope by first fixing it in order to preserve the properties of the tissue. Generally this is done by chemically fixing, dehydrating, and embedding the sample in paraffin wax. [9, pp 313-4] In addition, most cellular components do not adequately absorb light and must therefore be stained using a chemical dye. These dyes bind to specific molecules that may be transparent but will absorb light when bound to the chemical stain. Depending on their chemical binding properties, different stains will dye different cell structures, which is useful if a specific cell structure needs to be discerned. [5, p142-3]

Dyes are specific to the structure that is being observed. Common dyes include benzidine, which binds to heme-containing proteins and nucleic acids. Cytochemical
staining involves catalyzing a reaction producing a colored precipitate from a precursor that is present in certain structures of cells. The most common stain used for routine histology is the haematoxylin and eosin (H and E) stain, which binds to DNA. [5, p143] Silver stain can highlight sub-cellular structures such as nucleolar organizing regions. Antibody staining, which increases the specificity of a stain for a particular protein in a cell or in the extracellular matrix, is common through immunoperoxidase. [9, p 316-7] In some situations, multiple stains can be used to highlight different structures in the same cell or tissue sample.

Staining has a number of limitations which may prohibit its use. First, an appropriate stain must be chosen, or else it may not bind to anything or will bind to the wrong structure. Because of the specificity of stains, it is not always clear whether a microscope image is yielding the desired view of a cell. Second, the addition of a stain will alter the chemical properties of the specimen. Also, staining is a lengthy and time-consuming process. The staining process can also denature proteins and nucleic acids, causing unnatural bonds which could alter the appearance of various cell structures. [5, p 142] If an accurate, dynamic and unaltered view of a cell or tissue is needed, staining is inadequate.

Fluorescence

A type of staining that merits its own discussion is fluorescent staining. Molecules are considered fluorescent if they absorb light at an excitation wavelength and emit at a specific, longer wavelength. In a fluorescence experiment, light at the excitation wavelength is shone onto a sample to induce fluorescence, and then filtered out such that only light that is emitted by the fluorescent object is detected.

Like staining with a light absorbing dye, fluorescence introduces exogenous factors into a cell or tissue culture, and thus has the same issues as staining. Nevertheless, fluorescence is a versatile and powerful technique for revealing specific proteins in cells. Fluorescent dyes, which may by themselves have low affinity for biological molecules, can be chemically coupled with antibodies for a desired macromolecule. When the
fluorescent dye is coupled with an antibody and added to cells or a tissue section, the complex will bind to the antigen and will emit light at a specific wavelength. This is known as immunofluorescence, and several different such dyes, known as tagged proteins, can be introduced to a single sample to divulge numerous different cell and tissue structures at once. In addition, immunofluorescent dyes can be added to living cells, which will not necessarily alter normal cell function. These dyes can be micro-injected into a cell, or possibly introduced using recombinant DNA as a metric for gene expression. One popular method uses a naturally fluorescent jellyfish protein known as green fluorescent protein (GFP), which can be cloned into the genome of a group of cells and used to identify and localize specific cells in a tissue section or even an entire animal. GFP can also be tagged to another protein of interest to reveal sub-cellular localization of that particular protein. Certain fluorescent dyes are dependent on ionic concentrations in a cell and can thus be used to monitor the chemical properties of a biological specimen. [5, p 142-14]

Fluorescent microscopes are often used in conjunction with confocal microscopy to yield clear optical sections, even if the specimen is relatively thick. [3, p17.40-41] In addition, there are natural fluorophores in cells and tissues that can allow microscope study and provide contrast.

Optical Coherence Tomography

It is possible to image the internal structure of biological systems in vivo without the addition of exogenous chemical or genetic agents. Techniques such as X-rays, magnetic resonance imaging and ultrasound are in common use in medical and commercial applications. Such imaging is also possible using visible light; one such application is known as optical coherence tomography (OCT), which is capable of imaging thick biological samples.

OCT is an example of an application of low coherence interferometry (LCI). Interferometry is dealt with in more detail in Chapter 3 of this thesis. The optical schematic setup for an OCT experiment is shown in Figure 2-3. The light source for
the OCT scanner is a low coherent light source, in this case a superluminescent diode (SLD). Low coherent light has a short coherence length, $l_c$, which is on the order of

$$l_c \sim \frac{\lambda^2}{\Delta \lambda}$$  \hspace{1cm} (2.4)$$

where $\lambda$ is the center wavelength of the light source and $\Delta \lambda$ is the width of the distribution of optical wavelengths of a particular light source. [6, p 73] All light sources have a finite coherence length ($\Delta \lambda > 0$). Let us consider a light source with a Gaussian spectrum profile, such that

$$E(\omega) = E(\omega_c) e^{-\frac{(\omega - \omega_c)^2}{\Delta \omega^2}},$$  \hspace{1cm} (2.5)$$

where $E(\omega)$ is the electric field amplitude at optical frequency $\omega$, and $\omega_c$ is the center optical frequency. The quantity $\Delta \omega$ is the characteristic frequency bandwidth of the light source, and relates

$$\Delta \lambda = \frac{\lambda^2}{2\pi \cdot c} \Delta \omega,$$  \hspace{1cm} (2.6)$$

where $\Delta \lambda$ is the characteristic wavelength bandwidth, and $c$ is the speed of light in a vacuum (roughly $3.0 \times 10^8 m/s$). Then we can evaluate the irradiance at the detector if this light source is used in an interferometry setup like the one described in Figure 2-3.
2-3. Consider that the arm of the interferometer labelled “Sample”. A beam that propagates through this arm must first be emitted from the laser, and be split by the 50-50 beamsplitter into the upper arm of the interferometer. This light is then reflected back to the beamsplitter where it is transmitted down to the detector. We will define the distance that this light must propagate from laser to sample to source as $x_s$. We can also define any phase shifts on this arm as $\phi$. Alternatively, light that is split by the beam splitter and travels down the reference arm and back to the detector will travel a distance $x_r$, with a constant phase $\phi_r$. The resulting interference signal, measured as the irradiance at the detector, is given by the integral

$$I_{\text{detector}} = E_r^2 + E_s^2 + 2E_rE_s \cos(2k(x_r-x_s)+\phi_r-\phi_r).$$

(2.8)

where $E_r$ and $E_s$ are the magnitudes of the electric fields of the light on the reference arm and sample arms, respectively. After integration,

$$I_{\text{detector}} = E_r^2 + E_s^2 + 2E_rE_s e^{-\frac{\pi \Delta \lambda (x_r-x_s)^2}{2\lambda^2}} \cos(2k(x_r-x_s)+\phi_r-\phi_r).$$

The term $k_o$ is equal to $\frac{\omega}{c}$, where $c$ is the speed of light in vacuum. [12][pp 22-25]

In order to detect the signal, a piezoelectric transducer (PZT) modulates the sample arm to produce a heterodyne signal at the detector. An interferometric signal is detected only when the delay in the sample and the reference arms are closely matched to within the coherence length defined by Equation 2.4, and by simultaneously recording the position of the reference mirror, the optical path that the beam in the sample arm traveled can be recorded. The modulation of the sample arm, which shows up mathematically in Equation 2.8 as a variation in $x_s$ (from the cosine term), with a Gaussian envelope as a result of the $e^{-\frac{\pi \Delta \lambda (x_r-x_s)^2}{2\lambda^2}}$ term. The generation of the heterodyne signal from the PZT is used to separate signal from noise by band-passing the recorded signal at the heterodyne frequency. [8] Thus, only the interference envelope is detected. The peak of the envelope will be at the point where $x_r = x_s$. Using this technique, OCT measures the amount of back-scattered or reflected light from
a given depth of tissue. Because both the interference envelope and the position of the reference mirror (which modulates $x_r$) are recorded, correlating the peak of the interference envelope with the position of the reference mirror will indicate at which depth the sample light was back-scattered or reflected.

This technique, while successful, is sensitive to vibrations in the arms of the interferometer, known as jitter. This noise affects the cosine component in Equation 2.8, so only the envelope of the interference signal can be measured. The phase information, which would provide more information on a finer length scale, is lost.

Variations to conventional OCT exists. Some biological tissues will yield poor contrast in OCT if only the intensity of the backscattered or reflected light is measured. However, these objects can also change other properties of light, such as the polarization or the phase, which can be used as a contrast mechanism, and can also be measured for quantitative analysis. A phase sensitive variant of low coherence interferometry will be discussed in a later section. [7]

### 2.2.2 Phase Methods

The index of refraction is defined as the ratio of the speed of light in a vacuum to its speed in a medium. The speed of light in a medium, $u$, is a function of the electromagnetic properties of the medium, namely the electric permittivity, $\epsilon$ and the magnetic permeability, $\mu$. We can define the index of refraction, $n$, as

$$n = \frac{c}{u} \quad (2.9)$$

where

$$u = \frac{1}{\sqrt{\mu\epsilon}} \quad (2.10)$$

and $c$ is the speed of light in vacuum. Furthermore, the index of refraction varies with the frequency of light passing through the medium, due to the phenomenon of
dispersion. An optical field that traverses a sample of a specific refractive
index will undergo a delay in phase, which is wavelength-dependent. The quantity
that describes this phenomenon is the optical pathlength, \( S \), defined as

\[
S(\lambda) = \int n(s, \lambda) ds
\]

(2.11)

where \( s \) is the distance of propagation. The phase delay accumulated can be expressed as

\[
\phi(\lambda) = k(\lambda)S(\lambda)
\]

(2.12)

\[
= \frac{2\pi}{\lambda}S(\lambda)
\]

(2.13)

where \( \phi \) is the phase shift, and \( k \) is the modulus of the wave vector and equals \( \frac{2\pi}{\lambda} \).

Measuring \( \phi \) associates with the mean wavelength of an optical field in each point of
an image is referred to as phase microscopy.

**Phase Contrast Microscopy**

Developed by Zernike in the 1930’s, phase contrast microscopy revolutionized the
study of cells because it converted phase changes into amplitude changes that could
be observed by the human eye. Zernike earned the 1953 Nobel Prize in physics for his
discovery, which he made while observing diffraction gratings. [14, p 345]. The optical
principle is described schematically in Figure 2-4. The specimen is illuminated with a
point light source that passes through a sample at the sample plane. Unscattered light
(dotted line) passes through the sample and to the objective. Scatter light (dashed
line), when it passes through the sample, will suffer a \( \lambda/4, \frac{\pi}{2} \) phase shift relative
to the undeviated wave. Thus, light traversing through a scattering phase object
will result in two spatially separate light beams with a \( \lambda/4 \) phase difference relative
to each other. This phase deviation by itself is not adequate enough to generate
contrast. In order to notice the phase deviation, a phase plate is placed at the back
focal plane of the objective. Unscattered light will pass through the back focal point of the objective, while scattered light will hit different parts of the phase plate. The phase plate is designed such that the unscattered light will pass through the plate without a phase change, while the scattered light will undergo a $\pi$ phase shift. A tube lens projects this image onto the image plane, where the phase contrast image is viewed.

Figure 2-4: Optical principle setup for phase contrast microscopy. Light passing through the sample will either be unscattered (dotted line) or scattered (dashed line), and will be phase shifted by the phase plate.

Phase contrast can also be understood mathematically. Considering the amplitude transmittance $T$ at a point $(x, y)$, as

$$T(x, y) \approx e^{i\phi(x, y)} \quad (2.14)$$

If the phase shift $\phi$ is small, less than a radian, then we can approximate the amplitude transmittance can be approximated as

$$T(x, y) \approx 1 + i\phi(x, y) \quad (2.15)$$

The real term in this equation refers to the unscattered component of the incident light (wave s in Figure 2-4) while the imaginary term refers to the weaker diffracted
light. The irradiance image produced under a conventional microscope is

\[
I_i \cong |1 + i\phi|^2 = \sqrt{1 + \phi^2} \cong 1
\]  

(2.16)

for small phase shifts. Thus, all phase information is lost under conventional microscopy. Zernike realized that this was because the scattered and unscattered light waves were out of phase by \(\frac{\pi}{2}\), or in phase quadrature, with one another. By modifying this relationship, such that the real part (the unscattered wave) is either phase shifted by \(\frac{\pi}{2}\) or \(\frac{3\pi}{2}\), the two waves will interfere more directly, such that for the \(\frac{\pi}{2}\) phase delay case,

\[
I_i = |e^{i\pi/2} + i\phi|^2 \\
= |i(1 + \phi)|^2 \\
\cong 1 + 2\phi
\]  

(2.17)

(2.18)

(2.19)

By doing so, the phase change that was invisible under a conventional microscope becomes visible under phase contrast.

Experimentally, the phase contrast microscope takes advantage of this diffracting phenomenon by introducing a phase plate, which introduces an additional \(\lambda/4\) phase delay to one of the waves, in order to create a sum total of \(\lambda/2\) phase difference between \(s\) and \(q\). If these two waves are recombined, they will interfere destructively, producing an interference image of the scattered versus unscattered light that images the object being examined. [3, p 17.25]. However, the information gathered under phase contrast is only quantitative for small phase shifts.

Phase contrast microscopy is quite useful for examining the structure and movement of large organelles in live cell cultures, and thus can be used in time-lapse microscopy, to study the same cell and its movements and dynamics over many hours. [5, p 147]

Although phase contrast has been an invaluable tool in the study of cells and particular in the dynamics of cell cultures, it is largely qualitative. Furthermore, if
the specimen itself does not scatter light or only weakly scatters light, the interference effect will not occur and no contrast will be achieved. This is because the magnitude of the s wave will be negligible compared to the q wave.

**Differential Interference Contrast**

Another commonly used phase microscopy method is differential interference contrast (DIC). DIC images regions of changing index of refraction, as would occur at a boundary between cell and cytoplasm, for instance. DIC utilizes a polarizing Wollaston prism, which splits an incident beam into two separate, parallel, and orthogonally polarized beams that are spatially separated by a small distance. The two beams pass through a biological sample at adjacent points, and then the beams are recombined using a second Wollaston prism. A relative phase difference between the two polarized beams is introduced if there is a gradient of thickness or refractive index. The resulting interference image will show bright (or dark) if there is a positive gradient of the index of refraction or thickness, and the reverse contrast for negative gradient. Though useful, DIC is limited because it cannot show a sharp image because two non-overlapped beams must traverse a sample to create a single point of interference in the image plane. Because it can only measure gradients in phase instead of measuring phase directly, it is not quantitative.

A modification of DIC is Nomarski interferometry, which operates using the same principle but modifies the optical setup. [3, p 17.32-3]

### 2.3 Other Types of Microscopy

#### 2.3.1 Electron Microscopy

By the diffraction limit, conventional light microscopy has a lateral resolution of 0.2 μm for visible light, when the numerical aperture is 1. If a light source is replaced
with an electron beam (and optical lenses made of glass are replaced with magnetic lenses), it is possible to decrease the magnitude of the limit of resolution by more than 3 orders of magnitude, to 0.1nm. Because of electron absorption in air, the sample and the beam path must be in an ultra-high vacuum. High voltage electrodes (with a potential difference of typically 50,000-100,000 volts) are placed in a vacuum tube and cause electrons to accelerate between the two electrodes. Lenses focus the electrons through a sample, which pass through onto a viewing screen or photographic film. In such applications, known as transmission electron microscopy (TEM), the electron wave properties have a wavelength of 0.005nm (compared to the wavelength of blue light, 450nm). According to the diffraction, the limit of resolution should be $\frac{\lambda}{2}$, or 0.05nm, but under real conditions this limit is actually 0.10nm.

The drawback of TEM is that the samples must undergo extensive preparations. Cell and tissue sections must be made extremely thin (on the order of 50-100nm, much smaller than the thickness of a single cell). Plating metals, such as gold, or evaporated metals such as platinum, must be applied to generate contrast. An alternative electron microscopy method, known as scanning electron microscopy, does not require sections, but requires that the entire sample be coated with a thin layer of evaporated heavy metal. An intense electron beam is scanned rapidly over the sample, which excites other electrons that can be detected by the microscope. The resolution is only 10nm compared to the 0.1nm resolution of TEM. [5, p 140, 147-8]

### 2.4 Developing a New Instrument

Given the current limitations of cellular microscopy techniques, a need for a new contrast mechanism for studying biological samples has arisen. In the subsequent chapter we will describe a new microscope that has been built in the George R. Harrison Spectroscopy Lab at the Massachusetts Institute of Technology. This microscope uses a laser to make quantitative phase measurements of dispersion between two harmonically-related wavelengths of light. The phase measurement will be sta-
bilized from noise, and will be able to image biological materials without the need of staining or other preparation.
Chapter 3

Interferometry using Harmonic Wavelengths

In this chapter, a new microscopy methodology that measures the dispersive properties of biological samples is described. First, the principles of interferometry will be discussed, followed by a review of the utilization of harmonic wavelengths as a stabilization method, and finally an introduction to phase dispersion microscopy.

3.1 Interferometry

3.1.1 The Wave Nature of Light

Visible light is a form of electromagnetic energy with characteristics of both a wave and a particle. The phenomenon of interference is understood through the wave description of light. The manner in which light is detected is evidenced through the photo-electric effect, which can only be understood using the particle nature of light. In a uniform medium, in the absence of scattering and absorption, light propagates in a direction as given by the $k$ vector. Perpendicular to the propagation direction are two fields, the electric field ($E$) and the magnetic field ($H$), which are mutually
orthogonal. [6, p 2-3, 22-23]

Under Poynting's theorem, the irradiance at a detector, \( I \), is given by

\[
I = \frac{1}{2} E_o H_o
\]

\[
= \frac{1}{2} \sqrt{\frac{\varepsilon}{\mu}} |E|^2
\]

where \( E_o \) and \( H_o \) are the magnitudes of the electric and magnetic field vectors at the detection point. The electric and magnetic field vectors are given by

\[
E = E_o e^{i(k \cdot r - \omega t)}
\]

where \( \omega \) is the optical frequency, \( k \) is the wave vector, and \( r \) is the observer position.

Interference is based on the principle of linear superposition, in particular to electric fields. For the purposes of this discussion, there will be only two interfering waves, though the principle can be applied to any number of fields. The two sources have electric fields \( \mathbf{E}_1 \) and \( \mathbf{E}_2 \), respectively. In a point in space, the total electric field is

\[
\mathbf{E} = \mathbf{E}_1 + \mathbf{E}_2
\]

where \( \mathbf{E}_1, \mathbf{E}_2 \), are the electric field vectors produced at the point \( r \) by two different light sources (of low intensity). The individual electric fields, \( \mathbf{E}_i \), are given by

\[
\mathbf{E}_i = |E_i| e^{i(k_i \cdot r - \omega t + \phi_i)}
\]

where \( \phi_i \) is a constant phase. [6, p25]

The total irradiance at the detector as a result of the two fields of parallel polarization interfering is given, in sinusoidal steady state, by

\[
I = |E|^2 = (\mathbf{E}_1 + \mathbf{E}_2) \cdot (\mathbf{E}_1 + \mathbf{E}_2)^* = I_1 + I_2 + 2 \sqrt{I_1 \cdot I_2} \cdot e^{i\theta}
\]
where

$$\theta = k_1 \cdot r - k_2 \cdot r + \phi_1 - \phi_2 + (\omega_1 - \omega_2)t. \tag{3.7}$$

and $I_1$ and $I_2$ are the scalar intensities from the two light sources. [6, p 59]

3.1.2 Heterodyne Interference

It is often desirable to artificially modify the $\theta$ term from Equation 3.6. For instance, if $\theta$ were made to vary linearly with time, the resulting irradiance would be detected as a sinusoid with an amplitude of $E_1 \cdot E_2$. This is particularly important in interferometry because modern photodetectors, usually based on a semiconductor material, detect light by detecting photons, rather than measuring the magnitude of the electric field. The reasons for this are discussed in Chapter 5. By modulating the signal at a lower frequency, many important measurements can be made.

Figure 3-1: Demonstration of heterodyne interference. Two cosine signals at 30Hz and 33Hz are summed; the heterodyne envelope is shown to be $(33-30)\text{Hz} = 3\text{Hz}$
This modulation, known as heterodyne generation, involves frequency shifting one of the fields while keeping the other constant. The frequency different between the two fields is known as the heterodyne frequency, and determines the modulation of the intensity of light detected by the photodetector. Ignoring the constant terms, the time dependence of the phase $\theta$ is

$$\theta = \Delta \omega \cdot t$$  \hspace{1cm} (3.8)

and so

$$I = I_1 + I_2 + 2E_1E_2e^{j(\Delta \omega \cdot t)} \hspace{1cm} (3.9)$$

This is demonstrated through MATLAB in Figure 3-1. Two cosine signals, at 30Hz and 33Hz, are summed and graphed. The expected result can be derived from Equations 3.6 and 3.7, where $\omega_1$ is $2\pi \cdot 30Hz$ and $\omega_2$ is $2\pi \cdot 33Hz$. The result is a heterodyne signal that has an envelope of $2\pi \cdot 3 rad/sec$, or 3Hz. When a silicon or InGaAs photodetector is used to detect the signal, only the envelope frequency will be detected, and can be converted into an electrical signal.

![Figure 3-2: Optical setup of the Michelson interferometer.](image-url)
3.1.3 Optical Configuration for Interferometry

There are numerous configurations which are used to observe interference. One common optical setup is known as the Michelson interferometer (Figure 3-2). In this setup, an incident beam of light is split into two beams using a beamsplitter, which partially reflects some of the incident light and transmits the remainder. The two beams travel through different parts of the interferometer, referred to as the sample arm and the reference arm, respectively. Mirrors are placed at the ends of both arms such that both beams return and recombine at the same beamsplitter, where they jointly travel down a third arm, known as the detector arm, and can be observed using a photodetector.

The intensity seen at the detector is a function of the electric field magnitudes for both arms as well as scaled by the cosine of the phase difference between the two beams, as shown in Equation 3.6. As referred to in Chapter 2, the phase difference is proportional to the optical path length difference, \( S \). A \( 2\pi \) phase difference between the two beams corresponds to an optical path length difference equal to the wavelength of the beam used. Thus, if the lengths of the sample and reference arms are closely matched, jitter on the order of fractions of a micron in the interferometer arms will widely modulate the intensity of the detected beam.

There are a number of other optical configurations that are commonly used, such as the Fabry-Perot and the Mach-Zender interferometer. The Mach-Zender will be introduced in detail in Chapter 4.

3.2 Principle - Harmonic Wavelengths

3.2.1 Motivation

Interferometers are capable of measuring optical shifts in the interferometer on the order of fractions of its wavelength, or several nanometers. While this is useful for
measuring or imaging small changes in a material, it also means that any phase jitter in one arm of the interferometer compared to another, as a result of air currents or mechanical vibrations, will result in a large fluctuation in the phase measurement. In order to correct for this phase noise, we can superimpose two light sources of different wavelengths. Any fluctuations that result in phase noise in one wavelength will also cause the same phase noise at the other wavelength. In particular, it is easy to correct for phase noise if the two light sources are harmonically related.

Two waves are considered harmonically related if their respective wavelengths are an integer multiple apart. The wave with the longer wavelength, $\lambda_1$, is known as the fundamental wave, while the integer multiple is referred to as the $m$th harmonic, where $m$ is the positive integer multiplication factor, thus,

$$\lambda_m = \frac{\lambda_1}{m}$$

(3.10)

The wave numbers of the two harmonic wavelengths are

$$k_m = m \cdot k_1$$

(3.11)

Because we will use the fundamental and second harmonic wavelengths, $\lambda_2 = \frac{\lambda_1}{2}$ and $k_2 = 2 \cdot k_1$. In frequency, $\omega_1 = k_1c$ and $\omega_2 = 2k_1c$.

If we analyze the heterodyne signal resulting from interference of the electric fields of two harmonically related light sources, $E_1$ for the fundamental and $E_2$ for the second harmonic, then

$$E_1(t) = E_1^0 e^{i(k_1 \cdot r - k_1ct + \phi_1)}$$

(3.12)

$$E_2(t) = E_2^0 e^{i(2k_1 \cdot r - 2k_1ct + \phi_2)}$$

(3.13)

By the principle of superposition, each wavelength can be treated separately. Using
Equation 3.9, the interference signal generated will be, for each wavelength \( \lambda \),

\[
I = I_{\text{sample}} + I_{\text{reference}} + 2E_{\text{sample}}E_{\text{reference}}\cos(\Delta \omega t + \phi_{\text{sample}} - \phi_{\text{reference}}) \quad (3.14)
\]

In the final result, we are only concerned with the time-varying component of the detected intensity, such that

\[
I_{\text{heterodyne}, \lambda} = 2E_{\text{sample}}E_{\text{reference}}\cos(\Delta \omega t + \phi_{\text{sample}} - \phi_{\text{reference}}) \quad (3.15)
\]

The phase of the signal, \( \theta \), is therefore

\[
\theta = \phi_{\text{sample}} - \phi_{\text{reference}} \quad (3.16)
\]

Because we are using two wavelengths, we will be measuring two separate phases, \( \theta \). The two phases, \( \theta_1 \) and \( \theta_2 \), dependent on the wave number and the optical path lengths of both arms of the interferometer, as shown in Equation 2.11. Suppose there is a jitter in the sample arm of \( \Delta x \), and each arm has an optical path length of \( S_{\text{sample}} \) and \( S_{\text{reference}} \), respectively. Then, for each wavelength, the phase component will be

\[
\theta(t) = k(S_{\text{sample}} + \Delta x(t) - S_{\text{reference}}) \quad (3.17)
\]

Note that in the absence of dispersion in the optical path of the sample and reference arms, \( S_{\text{sample}} \) and \( S_{\text{reference}} \) would be independent of wavelength. In reality, this is not the case because even air has a dispersion profile. However, to the first order, we can assume wavelength independence. The situation where a dispersive element is placed in the interferometer will be considered later in this chapter.

Extending Equation 3.17 to a situation with two harmonically related light sources,

\[
\theta_1(t) = k_1(S_{\text{sample}} + \Delta x(t) - S_{\text{reference}}) \quad (3.18)
\]
and

$$\theta_2(t) = k_2(S_{\text{sample}} + \Delta x(t) - S_{\text{reference}})$$  \hspace{1cm} (3.19)$$

As we can see, the noise factor $\Delta x(t)$ will be seen in both phase measurements. Furthermore, because we are considering second harmonic light sources, we know that $k_2 = 2k_1$. This provides an opportunity to stabilize the phase signal, by using the differential phase between the two wavelengths. In order to cancel the $\Delta x$ term, we must first double the phase of the fundamental wavelength, such that

$$\phi_{\text{diff}}(t) = 2\theta_1 - \theta_2$$  \hspace{1cm} (3.20)$$

$$= 2[k_1(S_{\text{sample,1}} + \Delta x(t) - S_{\text{reference,1}})] - [k_2(S_{\text{sample,2}} + \Delta x(t) - S_{\text{reference,2}})]$$  \hspace{1cm} (3.21)$$

$$= 2[k_1(S_{\text{sample,1}} + \Delta x(t) - S_{\text{reference,1}})] - 2[k_1(S_{\text{sample,2}} + \Delta x(t) - S_{\text{reference,2}})]$$  \hspace{1cm} (3.22)$$

$$= 2k_1[(S_{\text{sample,1}} - S_{\text{reference,1}}) - (S_{\text{sample,2}} - S_{\text{reference,2}})]$$  \hspace{1cm} (3.23)$$

It is evident from Equation 3.23 that measuring the differential phase $\phi_{\text{diff}}(t)$ will allow us to cancel out the interferometer noise term $\Delta x(t)$.

### 3.3 Phase Dispersion

From Equation 3.23, we will see a constant differential phase in the absence of dispersion. If a dispersive sample is placed in one of the arms of the interferometer, we will be able to measure this dispersion profile. Consider a sample with indices of refraction of $n_1$ and $n_2$ at the fundamental and second harmonic wavelengths, and a thickness of $L$. Using Equation 2.11, and assuming that the optical path length difference in the reference arm and the remainder of the sample arm, the phase
difference will be

$$\phi_{diff} = k_2(n_1 - n_2)L$$  \hspace{1cm} (3.24)

### 3.3.1 Proof of Principle Setup

The idea of using phase dispersion to create contrast in microscopy was first demonstrated in the Spectroscopy Lab and used to quantify minute differences in dispersion in various chemical and biological samples. [13] The experimental setup utilized a low-coherence Michelson interferometer, and is depicted in Figure 3-3.

![Optical setup of the Michelson interferometer-based phase dispersion microscope.](image)

Figure 3-3: Optical setup of the Michelson interferometer-based phase dispersion microscope. [12, p79]

The light source was a composite low coherent 400nm/800nm beam generated
from a Ti:sapphire laser pulsed at 150 fs and a full-width at half maximum (FWHM) beam diameter of 2.1mm at 800nm, and doubled with a standard frequency doubler to generate the 400nm wavelength with a FWHM diameter of 1.1mm. The beam is split into two arms by a beamsplitter (BS), and each arm is reflected back to the beam splitter using mirrors (M1, M2). The M2 arm, which we will consider to be the sample arm, contains a pair of 10X microscope objectives that illuminates a biological sample. In this configuration, both wavelengths on the sample arm will make a double pass through the sample. The M1 arm, the reference arm, has a compensator to match path lengths of the reference arm to the sample arm. This is particularly important because this experiment uses low coherent light sources, and fringes will not be visible if the two arms are off by more than the coherence length of the laser. The M1 mirror moves at a constant velocity of 1 mm/s to induce a Doppler frequency shift on the reference beam. This frequency shift is given by

$$\Delta f = \frac{2v}{\lambda},$$  (3.25)

so we expect heterodyne frequencies of 2.5KHz in the fundamental and 5KHz at the harmonic wavelength. The two arms of the Michelson are recombined at the beamsplitter, and then separated into their component wavelengths using a dichroic mirror, and measured with separate photodetectors, which convert the optical signal into an electrical signal that is then digitized using a 16-bit 100KHz analog-to-digital converter (ADC). Both digitized signals are bandpassed around the center heterodyne frequency and the Hilbert transform is used extract their respective phases. The phase difference is measured and then displayed on the computer. The time for acquisition and processing was about 3 seconds per pixel, and a 100x100 pixel sample took several hours to generate. [12, Chap 5]

### 3.3.2 Experimental Results

In Figure 3-4, a drop of water is placed on a slide alongside a drop of 1% DNA in
Figure 3-4: Images of a drop of water (left) and DNA solution (right). From C.H. Yang et al, Optics Letters (2000). Color in PDM (lower) image corresponds to phase difference between 400nm and 800nm wavelengths.

Figure 3-5: Images of white and gray brain matter interface. A PCM image, a PDM image, and am image of the same slide with H and E stain is shown.

water solution. The top image shows this sample analyzed through phase contrast; as we can easily see, PCM is unable to differentiate between the two drops because
the DNA solution does not effectively scatter light. The bottom image is generated by using the phase dispersion microscope (PDM), and shows that we can see that PDM has the ability to differentiate between the two drops. Figure 3-5 shows a slide of the white and gray brain matter interface analyzed through PCM, PDM, and the standard method of staining with H & E. Once again, PCM is unable to produce significant levels of contrast to distinguish the two tissue types. Although PDM is clearly a useful technique, its practical application is limited by the slow acquisition time, approximately 3 seconds per pixel. Imaging an area of 100 by 100 μm with μm resolution would require over eight hours of data collection.

3.4 Improvements

Although the proof of principle experiment showed the potential that phase dispersion microscope has for sampling biological samples, the difficulties in alignment and the slow speed of the instrument limited its effectiveness. Thus, a new instrument utilizing the phase dispersion technique is desired, with the following modifications:

1. *Simplified optical setup* that removes return beam paths, which can be difficult to align. This excludes the Michelson interferometer. A Mach-Zender interferometer does not require overlapping return beams, and is well-suited for the needs of the new instrument.

2. *Faster scanning of the sample* will allow for images to be acquired in a much shorter period of time. If scanning is made fast enough, it is also possible to use phase dispersion microscopy to study dynamic cell movements.

3. *Higher frequency heterodyne signals* will also aid in decreasing the time of acquisition, since several waves are required to reliably extract the phase difference. The proof of principle instrument had heterodyne frequencies of 2.5KHz and 5KHz, which made it sensitive to noise in that frequency range. The noise level decreases at increasing frequencies, so shifting the heterodyne signals to higher
frequencies will decrease the amount of noise the instrument will have. Unfortunately, the original instrument was limited by the speed by which it could move the mirror at constant velocity. A new method for frequency shifting will need to used for the new instrument.

4. Improved phase extraction methods will allow the instrument to measure phases of the higher-frequency heterodyne signals. Currently the electronic signals from the photodetectors are digitized and processed in a computer running LabVIEW. This setup would limit the heterodyne frequency because the ADC have a maximum sampling frequency of 100kHz. By the Nyquist criterion, this would limit our heterodyne signal to an ideal limit of 50kHz, which would only provide a marginal improvement in the speed of the instrument and in the system's signal-to-noise ratio. Furthermore, we would also be limited by how quickly the computer could extract the phase of the two wavelengths. By removing a portion of the phase extraction method, using analog circuitry or digital signal processing, the speed of the instrument will be greatly improved.

3.5 Summary

We have discussed the basic principles of interferometry, and of noise reduction using harmonically related light sources. We have introduced the phase dispersion microscope, a new method to achieve contrast in biological samples without staining. We will discuss the design and implementation of the new instrument, which we now name the Fast Phase Dispersion Microscope (FastPDM), in Chapters 4 and 5.
Chapter 4

Experimental Setup: Optics

The FastPDM instrument is based on the Mach-Zender interferometer. As described in the previous chapter, this instrument will utilize harmonic wavelengths for stabilization from noise, as well as to produce contrast for imaging biological samples.

This chapter overviews the design and implementation of the optics for the FastPDM. This includes the microscope objectives, the translation system, the laser source, and the interferometer itself. In addition, there is a bright-field microscope interfaced with the FastPDM that will be described.

4.1 Mach-Zender interferometer

The Mach-Zender interferometer operates on the same principle of the standard Michelson interferometer described in Chapter 3, in a slightly different configuration that avoids overlapping beam paths.

4.1.1 Optical Principle

The basic optical setup is shown in Figure 4-1. The light source produces a
beam that hits the first beam splitter (BS1 in Figure 4-1) and is split into two arms, the sample arm and the reference arm. The light on the two arms travel down separate optical paths, being aligned by mirrors 1 and 2 (M1, M2) respectively and then recombined at a second beamsplitter (BS2) into an interference arm, where the combined signal can be detected and measured. This interferometer is equivalent to the Michelson in Figure 3-2, with the exception that M1 and M2 reflect the beam at an angle and towards a second beam splitter, instead of the single beamsplitter in the Michelson interferometer. This has the advantage that M1 and M2 do not have to be aligned to ensure that the reflected beam precisely matches the optical path of the incident beam. In addition, if an element such as a lens or a sample are placed in either arm of the Mach-Zender, the light beam will only make a single pass through that element, whereas in a Michelson the beam will typically make a double pass.

![Optical principle setup for a Mach-Zender interferometer](image)

Figure 4-1: Optical principle setup for a Mach-Zender interferometer. See text for explanation

The alignment of a Mach-Zender interferometer can be more difficult in some situations compared to the Michelson. The angles and positions of BS1, BS2, M1 and M2 must be made such that the beams will overlap properly for interference to be observed. This is somewhat simplified in the Michelson because M1 and M2 must simply be perpendicular to the incident beam. In addition, it is harder to match the lengths of both arms. For the purposes of FastPDM, because the light sources are expected to have long coherence lengths, matching arm lengths was not found to be crucial.
4.1.2 Heterodyne Generation

In the Michelson interferometer, heterodyne generation was accomplished by means of a Doppler shift from mechanical motion in one of the mirrors. In principle, this could also be done in a Mach-Zender, by mechanical motion of either M1 or M2 in Figure 4-1. However, doing so in the Mach-Zender geometry would decrease the overlap of the sample and reference beams at BS2, which would decrease the amplitude of the heterodyne signal. Furthermore, the heterodyne frequency would again be limited by the maximum velocity of the mirror actuator.

An alternative means of heterodyne generation can be accomplished through the use of acousto-optic frequency shifters (AOFS), also known sometimes as acousto-optic modulators (AOM). An AOFS is a crystal in which an RF signal of constant frequency is applied, inducing acoustic waves within the crystal that modulates its index of refraction. This acts as an optical grating and causes Bragg diffraction within the crystal, up- or down-shifting the frequency of the incident light as well as producing an angle proportional to the order of the diffraction. The vibrational motion conferred upon the crystal by the acoustic wave is quantized, in the form of a phonon, and one photon can absorb multiple phonons as it passes through the crystal. [12, pp 89-90]

4.2 Harmonic wavelengths

4.2.1 Light Source

The light source for the FastPDM needs to produce light at a fundamental wavelength of \( \lambda \) and its second harmonic wavelength of \( \frac{\lambda}{2} \). Because depth resolution is not important for this instrument, the light sources do not have to be broadband and a monochromatic light source can be used. This simplifies the alignment because the need for matching path lengths for the two arms of the Mach-Zender interferometer
is not as prevalent as it would be for a low-coherent light source.

With these specifications, a frequency-doubled Nd:YAG laser was selected from Intelite, Inc. (Minden, NV), a custom order of a GIS-100.10 532nm/1064nm laser system. This laser is a diode pumped solid state laser with intracavity frequency doubling operating in CW mode, and produces light at a fundamental wavelength of 1064nm with 50mW of power, and the second harmonic at 532nm and 100mW of power. Although the power stability of the laser was advertised as ±10%, we found that this number was greater than ±25%, and in fact this noise level has caused many difficulties in constructing and operating the FastPDM. We believe that problems with this laser will ultimately require its repair or replacement. The laser noise may possibly be from the laser mode-hopping as a result of poor alignment of optics within the laser cavity, or malfunctioning control electronics. This noise, and our attempts to minimize its impact on the phase measurement, are characterized in Chapter 5.

![Figure 4-2: Images of 532nm/1064nm beams as they are moved towards the focal point of a 20X microscope objective. Notice the significant diffraction on the 1064nm beam](image)

In addition, the laser, which was claimed to have both orders of light completely overlapped and collimated, in fact was found to have a small angle between the two
beams. Furthermore, the beams were not clean Gaussian beams, and in particular
the infrared (1064nm) beam had significant abberation. This is evidenced in Figure
4-2, which imaged the beams for both colors as the beams moved toward the focus of
a 20X microscope objective. The significant diffraction in the 1064nm beam indicates
that the beam is not a clean Gaussian beam. This leads us to believe that there
may be a small misalignment of the frequency doubling crystal. To what extent this
affects the exact harmonic relationship between the two wavelengths is unclear.

The solution to the problem of aberrated laser beams was to separate the output
of the laser into its component 1064nm and 532nm wavelengths using a dichroic
mirror, image the beam into two separate, single mode fibers, and then recollimate
and recombine the two beams with a second dichroic mirror. The outputs of the
fibers are carefully aligned to insure that both wavelengths are completely overlapped.
Methods to confirm this overlap will be presented shortly. The single mode fibers will
only transmit one mode of the input laser beam, a Gaussian TEM00 beam will be
emitted. Although this method added many components to the system, it allowed us
to overcome two of the shortcomings of the laser.

4.2.2 Microscope Objectives

In order to probe biological samples, where the typical cell size can range from
tens of microns to half a millimeter, the spot size of the laser beam must be small,
on the order of two or three microns at worst.

Microscope objectives are compound lenses, consisting of several simple lenses
carefully aligned. Modern objectives are often chromatically corrected for a range of
wavelengths (usually in the visible range) as well as corrected for spherical aberrations.
Because we are dealing with two widely separated wavelengths, with the 1064nm
beam in the near-IR region and the 532nm in the green visible region, off-the-shelf
microscope objectives will have a chromatic aberration associated with these two
wavelengths. In addition, the amount of aberration will depend on the design of the
objective itself. Thus, a means of characterizing objectives must be applied.
Characterizing Objectives

Using the fundamental equation of Gaussian beam optics, the diameter (defined as the distance between the points where the intensity drops to $\frac{1}{e^2}$ from the peak) is

$$2w_o = \frac{4\lambda}{\pi} \cdot \frac{f}{D},$$

(4.1)

where $\lambda$ is the wavelength, $f$ is the focal length of the lens, $w_o$ is the waist beam radius at the focal plane of the objective, and $D$ is the beam diameter prior to entering the lens.

Although Equation 4.1 would be very helpful in the design of the optical system for the FastPDM, the focal length is dependent on the wavelength of light. Unfortunately, the focal lengths at specific wavelengths is generally not published by manufacturers of microscope objectives. Instead, a generic focal length is often published in catalogs that refers to the focal length at a single wavelength, such as 546.1nm (in the visible range).

In addition to Equation 4.1, another mathematical method of characterizing the optical system is required. One such method is ray matrices.[6, pp299-301] In this analysis, a laser beam is characterized by its component rays, with each ray having a specific position, $\rho$ and angle $\theta$. There are specific matrices which modify a specific ray’s position and angle, depending on what the ray propagates through. For instance, the matrix denoting the propagation of the ray across a distance $d$ is given by

$$\begin{bmatrix} 1 & d \\ 0 & 1 \end{bmatrix}$$

(4.2)

The matrix denoting the transformation of a ray by a simple lens is given by

$$\begin{bmatrix} 1 & 0 \\ -\frac{1}{f} & 1 \end{bmatrix},$$

(4.3)
where \( f \) is the focal length of the lens, and is dependent on the shape and the type of glass used to form the lens.

![Ray analysis of a simple lens](image)

**Figure 4-3: Ray analysis of a simple lens**

Using Equations 4.2 and 4.3, the position and angle through any number of lenses and mirrors can be calculated. For instance, consider the simple lens in Figure 4-3. A ray with position relative to the optical axis of \( \rho_1 \) and angle \( \theta_1 \) is transformed by a lens and propagates to the focal plane, located a distance \( f \) from the principle plane of the lens. At the focal plane, the position of the ray is \( \rho_2 \) and has an angle \( \theta_2 \). Equations 4.2 and 4.3 will give,

\[
\begin{bmatrix}
\rho_2 \\
\theta_2
\end{bmatrix} = \begin{bmatrix} 1 & f \\ 0 & 1 \end{bmatrix} \begin{bmatrix} 1 & 0 \\ -\frac{1}{f} & 1 \end{bmatrix} \begin{bmatrix} \rho_1 \\
\theta_1
\end{bmatrix}
\]  \hspace{1cm} (4.4)

Solving for \( \rho_2 \) and \( \theta_2 \), we find

\[
\rho_2 = f\theta_1 
\]  \hspace{1cm} (4.5)

and

\[
\theta_2 = -\frac{\rho_1}{f} + \theta_1 
\]  \hspace{1cm} (4.6)

As mentioned previously, this analysis is only useful in a theoretical sense, because we were unable to get actual figures for the focal lengths of the objectives used in
our system. For comparison, we were able to obtain empirical data from Carl Zeiss for a 20X fluor. The focal length at 532nm was given as 8.22498mm, and at 1064nm was 8.23194mm. The 6.5μm observed difference between the two wavelengths is a result of dispersion in the optics of the objective. This information, and the above equations, will become very important as we consider the scanning system necessary for creating images.

4.2.3 Acousto-Optic Frequency Shifters

The acousto-optic frequency shifters are necessary for generation of the heterodyne signal. The AOFS generally have a center RF frequency in the order of 100MHz and a 3dB bandwidth of approximately 30MHz.

We selected the Model TEF-110-30-.532 AOFS from Brimrose Corporation (Baltimore, MD), which has a center RF frequency of 110MHz with a 30MHz 3dB bandwidth and a high power broadband coating to improve transmission of the two wavelengths of light, because of the wide separation between the two wavelengths. The uncoated raw crystal is tellurium dioxide (TeO2), with maximum RF power of between 1 and 2 watts and a diffraction efficiency of greater than 85% for 532nm and 50% for 1064nm. The difference in diffraction efficiency between the two wavelengths is considered normal.

The diffraction angle is proportional to the wavelength of light. Although this would at first seem to be a problem in the setup of the FastPDM, because of the use of harmonic light sources, alignment issues can be overlooked if the first order diffraction of the fundamental wavelength is used along with the second order diffraction of the second harmonic wavelength. The resulting diffraction angle is the same (excluding the dispersion of the crystal, which is a small effect).

The two shifters were placed on the reference arm of the Mach-Zender interferometer. This was done so that any misalignment of the output of the AOFS due to dispersion will not affect the illumination of the sample. There is a loss in power associated with this alignment, although it was not significant.
4.2.4 Alignment

Alignment for the FastPDM was particularly difficult because the two wavelengths must be precisely overlapped, even though the fundamental wavelength is in the near-infrared region and is thus invisible to the human eye. Decidedly low-tech methods for aligning the two colors were performed using a long, empty stretch of optical table, as well as pinholes and power detectors.

In order for a good heterodyne signal to be detected, the sample and the reference arms for both wavelengths must be carefully aligned. They must meet at the same point on the second beamsplitter (BS2 in Figure 4-1) as well as on both the transmitted and reflected arms of BS2. This was done by careful adjustment of the position and angles of BS2, M1 and M2. A distant wall is used to insure that the beams are well aligned. This was done to get a general alignment of the two beams. Precise alignment of the beams requires reiterative testing of the alignment and adjustment of the beam splitter and mirrors.

Verifying the overlap of the two beams is crucial. In particular, both beams must pass through the same point on the specimen plane of the microscope objectives. The following section describes the methods used to verify the overlap of the 532nm and 1064nm beams.

Figure 4-4: Setup of knife edge experiment
4.2.5 Testing

At the specimen plane of the microscope objectives, the beam sizes are expected to be less than 10 \( \mu m \) in diameter. These beams are too small to discern by the naked eye, and even small displacements in between the 532nm and the 1064nm beam would be detrimental to the proper functioning of the FastPDM. Because of this, a technique using a scanning knife edge was implemented to quantify the alignment of the two colors.

Using a knife edge that is precisely positioned and scanned transversely across the beam path of a laser, as shown in Figure 4-4, the amount of power that is not blocked by the knife edge is measured by an optical power meter. As the knife edge is translated and blocks more of the beam, the amount of power measured at the optical power meter will decrease accordingly. If this experiment is repeated many times, with the knife edge moving a small amount relative to the size of the beam for each iteration of this experiment, then a plot of the power transmitted vs. the position of the knife can be graphed. This function will be the error function, which is the integral of the Gaussian. By fitting data in MATLAB to the error function, the center and the waist radius of the Gaussian laser beam can be determined. This method will be shown later in this chapter.

4.3 Scanning

The FastPDM can only measure the phase of a single point of the sample placed between the microscope objectives. Thus, some method of translation is required—either mechanical movement of the sample ("Sample Translation"), or movement of the laser beam itself ("Beam Translation"). Because beam translation allows faster scanning, this method was attempted as the scanning method for the FastPDM; however, due to difficulties in this method, the sample scanning approach was eventually adopted. The design and implementation of both methods is presented here.
4.3.1 Beam Translation

One way of forming images is by scanning the laser beam across the sample. This is done using a scanning galvanometer, which is a rapidly rotating mirror. The optical system can be manipulated such that the scanning galvanometer, which will change the angle that a beam makes with a plane, will translate the beam by known amounts.

Figure 4-5 is a diagram of a simple 4-f system. It is called a 4-f system because there are two lenses with identical focal lengths of \( f \), spaced with their principle planes a distance \( 2f \) apart, and we consider the position and angle of rays of the laser beam at a plane a distance \( f \) in front of the first lens (plane 1 in the figure), at the focus of the first lens (plane 2), and a distance \( 4f \) behind the second lens (plane 3). For each of the three planes being considered, the position of the beam relative to the plane \( (\rho_n, \text{ where } n \text{ is the plane being considered, 1, 2, or 3}), \) and the angle of the beam relative to the optical axis \( (\theta_n) \) is considered. Using Equations 4.3 and 4.2, we can calculate how modulating the angle of a ray at plane 1 will affect the position and angle of the same ray at planes 2 and 3.

Consider first the ray that passes through the front focal point of the first lens, at the intersection between plane 1 and the optical axis. This ray has \( \rho_1 = 0 \). For any angle \( \theta_1 \), this ray will always be parallel to the optical axis between the two lenses; in other words, \( \theta_2 = 0 \) regardless of what \( \theta_1 \) is. This is apparent from Equation 4.6.
Furthermore, from Equation 4.5, the position of the ray on the back focal plane of the first lens (the front focal plane of the second lens) will be simply \( f \theta_1 \). In fact, \( \rho_2 \) has no dependence on \( \rho_1 \) and is completely determined by \( \theta_1 \). In other words, a beam that has a center that intersects the front focal plane of the first lens in the system at the optical axis will come to a focus in the back focal plane of the first lens. The center of this beam between the two lenses will travel parallel to the optical axis.

If the two lenses are identical, then symmetry can be used to determine \( \rho_3 \) and \( \theta_3 \). Consider, instead of propagating past plane 2, a mirror were placed there instead. The beam would reflect back towards the first lens, and would return identically to its position and angle at plane 1. Plane 1 and plane 3 are exactly symmetric. This can also be proven algebraically.

This analysis shows that if \( \theta_1 \) is carefully modulated, the beam will scan across plane 2.

Notice that the 4-f system is highly dependent on the focal lengths of these lenses. The distances of the lenses must carefully be matched. However, as mentioned previously, due to dispersion between the two harmonic wavelengths in the FastPDM, the focal lengths of lenses will generally not be the same. This adds a complication to the system.

Figure 4-6: FastPDM optical setup using scanning galvanometers as the image-forming mechanism

Figure 4-6 shows the optical system and the galvos that are used. The laser source
produces a composite 100mW of 532nm light and 50mW of 1064nm, which is split into its component wavelengths with a dichroic mirror. The individual wavelengths are then transmitted through a fiber (not pictured) and recombined at a second dichroic mirror. They are reflected towards the interferometer, which begins at “BS1” in the figure. The beamsplitter splits the light into two arms, the sample arm (vertical reflection from BS1) and the reference arm (transmitted through BS1). The reference arm has the two AOFS devices, which upshift the frequency of the reference arm relative to the sample arm. The shift at the 532nm wavelength will be 20MHz, and 10MHz on the 1064nm arm. The sample arm has a more complicated optical setup. In order to scan an image in two dimensions, two scanning galvanometers are required. The angle shifts from the galvos also produce changes in position, which are compensated for both additional 4-f systems like the one depicted in Figure 4-5. The first 4-f system, between galvo 1 and galvo 2, has the first galvo on the front focal plane of the first lens, and places the two lenses a distance of 2-f apart. The second galvo is placed on the back focal plane of the second lens.

A second 4-f system is placed between galvo 2 and the first microscope objective. Galvo 2 is placed at the front focal plane of the first lens in this system, with the two lenses a distance of 2-f apart, and the back focal plane of the second lens corresponds with the front focal plane of the microscope objective. With the galvo mirrors and the lenses aligned properly in this fashion, the angle modulation generated by the set of galvanometers will not change the position of the center of the beam in the front focal plane of the first microscope objective.

The pair of microscope objectives is in itself a 4-f system. The sample of interest is placed in the focal plane between the two microscope objectives. The beam from the sample arm is the beam from the reference arm, at the second beamsplitter (“BS2”), producing an interference pattern for both wavelengths that can be detected if the composite beam is separated into its component wavelengths and detected using separate photodetectors.
4.3.2 Sample Translation

Imaging by carefully moving the sample is a simpler method than scanning the beam. However, scanning the sample is slower than scanning the laser beam. Instead of scanning the sample as described in Section 4.3.1, the beam is fixed at a single location and the sample is moved using motorized positioning stages. This can be either an actuator or an oscillator such as a piezo-electric transducer (PZT). Although the optics is much simpler for this type of setup, this method is limited because it will always be slower than scanning via beam translation.

4.3.3 Experimental Results: Beam Scanning

Because of the increased speed of the galvanometers, beam scanning was the original image-forming method chosen for the FastPDM. The optical system diagrammed in Figure 4-6 was implemented in the Spectroscopy Lab.

Two scanning galvanometers, HarmonicScan-15, were purchased from Nutfield Technology (Windham, NH), along with corresponding QuantumScan servo drivers. These drivers are electronic circuits that control the galvo movement, measure its positions, and correct any abnormalities. These galvanometers are specified with ±20° (optical) travel range, and 5 μrad repeatability. The QuantumScan servo drivers require an analog electronic signal to determine what the proper position of the galvo should be. The control signals for the galvos, which have to be coordinated, were generated from a special electronic circuit. The schematic and details of this circuit will be described in Chapter 5. The servo driver was custom tuned so that the mirror movement will be ±1° for ±1V of command voltage.

The optical setup using the scanning galvanometers was aligned by setting the galvos to its “zero” position and aligning the optics with the galvos powered. The entire optical setup was constructed in this fashion, from alignment of the two colors to the detection of the heterodyne signals. After, to adjust any small mismatch between
the 532nm beam and the 1064 nm beam, the knife scanning experiment depicted in Figure 4-4 was used. However, instead of scanning a stationary beam, one of the galvos scanned a small angle. As discussed in Section 4.3.1, the focal length of a lens is dependent on the wavelength of light in question. Equation 4.6 states that the position of the beam at the back focal plane of the lens is proportional to the focal length.

Figure 4-7 demonstrates a sample of the data collected in this experiment. The galvo scans $\pm 0.1^\circ$ optical, and the knife is stationary blocking a portion of the beam. The data from the 532nm and the 1064nm beams are collected simultaneously and recorded into a computer running LabView. The data is then fit to the Gaussian error function using MATLAB. The knife is then moved several microns and the

![Figure 4-7: Sample data from knife experiment with galvo scanning. Data fit to error function](image)

Figure 4-7: Sample data from knife experiment with galvo scanning. Data fit to error function.
Figure 4-8: Data from the knife experiment showing the center beam position experiment is repeated until the beam is completely blocked by the knife at all angles of the galvo mirror. The result of these experiments is shown in Figure 4-8 for the beam position and in Figure 4-9 for the waist radius. Each data point on the abscissa corresponds to a position of the knife, and the experiment is repeated each time the knife is moved. Data is fit for each experiment, and the resulting plots can tell us where the focus is for each wavelength (the minimum waist radius) as well as how well the two beams are overlapped.

Multiple iterations of this experiment must be performed, with minute adjustments made in the optical configuration. This is a time-consuming and frustrating process, but it is possible to get within a micron of overlap without great difficulty. In the beam scanning configuration, there will always be a mismatch of the two colors because of the chromatic aberration in the microscope objectives. The experimental data from Figure 4-8 showed that this error need not be more than two microns for
a 100 µm by 100 µm field size. However, in the design of the FastPDM with the scanning galvanometers, the effect of the angle shift was not taken into account as the beams interfered with each other at the photodetectors. Because the angle of the sample arm will constantly change, there will be fringing of the interference signal on the photodetector. The net effect is that this will greatly decrease the heterodyne efficiency of the system, and decrease the signal that can be detected. Because of this, the galvo scanning system as set up in Figure 4-6 will not work. With this problem stalling progress, we decided to convert the system to sample scanning.

4.3.4 Experimental Results: Sample Scanning

Converting the experiment from a beam scanning configuration to a sample scanning configuration was not very difficult. The knife experiment, which moved the knife edge through the focus of the laser beam between the two objectives, can also
be used to scan the sample. The speed of this process would make the design object of one frame per second impossible.

Speeding up the scanning process would require using a faster translation stage. Traditional stages, which use precise motors, are several orders of magnitude too slow for the purposes of the FastPDM. The alternative, a PZT, could be used to replace the translation stages. However, due to the cost of a PZT stage capable of quick movements needed for the one frame per second design goal, the FastPDM was implemented using the slow motorized stages. At some point, these will be replaced.

4.4 Final Design and Implementation

The final optical setup is shown in Figure 4-10. This includes the addition of a bright field microscope to provide an amplitude image to compare with the phase dispersion image. The laser in the upper left produces the composite 532nm/1064nm light into a dichroic mirror ("DCM1") which splits the light into its component wavelengths and which is then transmitted to two single-mode fibers. The light exits the fibers and are recombined at a second dichroic mirror ("DCM2"). The recombined light then enters the Mach-Zender interferometer, starting at the first beamsplitter ("BS1") which splits the light into two arms, the sample arm containing the microscope objectives and the sample, and the reference arm containing the acousto-optic frequency shifters. Imaging is accomplished by translation of the sample between the two microscope objectives ("O1" and "O2"), and the two arms are recombined at "BS2", after which they are split again into component wavelengths at "DCM3" and are detected by two separate photodetectors. In addition to the Mach-Zender interferometer, the FastPDM also has a bright field illumination, with a flashlight providing light behind the "BS1", which is transmitted through "O1" and "O2", and imaged by a long focal-length tube lens into a CCD camera. This bright field microscope is useful for verifying the overlap of the 532nm and 1064nm beams, as well as verifying that both the laser beams and the sample is at the focal plane of the two.
objectives.

Figure 4-10: Final optical setup with sample scanning and bright field microscope

4.4.1 Alternative Designs and Implementations

There are alternative implementations which may have allowed the use of a beam-scanning configuration. These designs are more complicated than even the setup in Figure 4-6. One possibility would be to put the galvanometers before the first beam splitter in the Mach-Zender. This would force angle shifts in both the sample and the reference arms. However, this means that the beam will have angle shifts through the AOFS; because we have two AOFS shifters on one arm, which might cause complications with alignment and efficiency through the shifters. More analysis would be needed before this design could be finalized and implemented, but if the
sample scanning method proves to be too slow, this sort of implementation might be possible.
Chapter 5

Experimental Setup: Electronics

In addition to the optical system of the FastPDM, there are numerous electronic systems that are necessary. This chapter describes the electronics of the FastPDM.

5.1 Heterodyne/Detection scheme

In the interferometer, the phase information is encoded in the electric field of the laser beams. In order for this phase information to be extracted, this data must be converted from electric field to electrons.

5.1.1 Photodetectors

In order to understand how photodetectors work, some basic solid state physics must be described. Semiconductors are crystalline solids, made of materials such as silicon (Si) or gallium arsenide (GaAs) that are covalently bonded to each other in a lattice configuration. Each atom in the lattice has a electrons associated with it; core electrons, which are tightly bound to the nucleus of each atom, and valence electrons, which occupy the highest energy atomic orbit and have a higher energy then the core
electrons. In fact, in a crystal structure, valence electrons cannot be associated with a specific atom, but belong to the crystal, shared by all of the component atoms. These electrons can occupy different energy states, but in general these energy states are clustered into distinct bands, and separated by bandgaps of energy states that electrons cannot exist in. At 0K, there is no excess energy in the crystal lattice, and electrons will occupy the lowest available energy states. The highest occupied energy band at 0K is known as the valence band. The energy difference between the valence band and the next available energy band, the conduction band, is known as the fundamental bandgap. [4, pp 23-25]

Photodetectors require a process of band-to-band optical generation, or optical absorption, in which a photon of energy $h\nu$ supplies the energy required for a covalent bond to be broken, creating an electron-hole pair. This is depicted in an energy diagram as an electron jumping from the valence band to the conduction band (Figure 5-1). This photon-generated electrical current requires that the energy of the photon is greater than the difference between the energy of the conduction band and the energy of the valence band, $E_g$. [4, pp 88-92] A photodetection system will also include a transimpedance amplifier, which will convert the photon-generated current into a voltage difference that can be captured and analyzed using electronics.
<table>
<thead>
<tr>
<th></th>
<th>1801FS-AC</th>
<th>1811FS-AC</th>
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<tr>
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</tr>
<tr>
<td>Responsivity at 1064nm (apprx)</td>
<td>0.1 A/W</td>
<td>0.55 A/W</td>
</tr>
</tbody>
</table>

Table 5.1: Specifications for photodetectors

Two photodetectors from New Focus (San Jose, CA) were chosen because of their speed. The New Focus 1801FS-AC has a responsivity to light in the range of 400nm wavelength to 1100nm wavelength, and is used for the 532nm beam in the FastPDM. The New Focus 1811FS-AC has a responsivity to light in the wavelength range of 800nm-1800nm and is used for the 1064nm beam. A list of the relevant specifications for the two photodetectors is shown in Table 5.1.

5.1.2 Electronic Filters

Due to the laser intensity noise described in Chapter 4, the heterodyne signal detected by the photodetectors was not a simple sinusoidal waveform, but instead had significant amplitude modulation. This can be seen in Figures 5-2 and 5-3, which show the interference signals generated from the photodetector. The top graph of each figure shows the Unfiltered heterodyne signal, which, rather than a sinusoid, has a sinusoid on top of large pulses. The noise was not steady state but instead varied in frequency from tens to hundreds of kilohertz.

It is evident from Figures 5-2 and 5-3 that there is a very low signal-to-noise ratio in the detected signal. In order to obtain a good phase measurement, the noise in this
signal must be reduced. This is accomplished using electronic filters, which selectively reduce the amount of signal at specific wavelengths. If the fundamental frequency of the noise is several orders of magnitude in frequency away from the frequency of our heterodyne signal, then simple, single-order filters can be used.

Thus, the noise in the laser has limited the possibilities for heterodyne frequency and the electronic setup. We choose to drive the AOFS at 110MHz and 120MHz, for an offset frequency of 10MHz. This means that the first order of the fundamental (1064nm) wavelength will have a center frequency of 10MHz, while the second order of the second harmonic wavelength will have a frequency of 20MHz. High frequency passive elliptic response bandpass filters from Mini-Circuits (Brooklyn, NY) were used.

Figure 5-2: Unfiltered (top) and filtered (bottom) electronic signals from the 532nm photodetector. The top graph shows the low signal-to-noise ratio as a result of amplitude fluctuations in the laser. The bottom graph shows that electronic filters can partially recover a clean signal.
Figure 5-3: Unfiltered (top) and filtered (bottom) electronic signals from the 1064nm photodetector. The top graph shows the low signal-to-noise ratio as a result of amplitude fluctuations in the laser. The bottom graph shows that electronic filters can partially recover a clean signal to increase the signal-to-noise ratio. BBP-10.7 has a 10.7MHz center frequency with a 9.5MHz-11.5MHz passband (1.5dB maximum attenuation), and was ideal for the 1064nm wavelength signal; BBP-21.4 has a 21.4MHz center frequency and a passband of 19.2MHz-23.6MHz, and was used for the 532nm wavelength signal. The results of the filtering are seen in the bottom half of Figures 5-2 and 5-3.

5.1.3 Amplification and Frequency Doubling

As discussed in Chapter 3, one of the primary advantages of using the FastPDM is the noise correction provided by the harmonic wavelength technique. In order to get
a noise-correct phase difference, Equation 3.24 stated that we needed to double the frequency of the fundamental wavelength. This is done using an electronic frequency doubler, which is essentially an RF mixer. A Mini-Circuits FD-2 frequency doubler has appropriate specifications for our purposes. The FD-2 has an input frequency range of 5MHz-500MHz, with a typical insertion loss of 13dB for a 10MHz signal input signal. The FD-2 also requires a minimum of 1dB of input signal.

![Conversion graph of power (dBm) versus peak-to-peak voltage](image)

**Figure 5-4**: Conversion graph of power (dBM) versus peak-to-peak voltage

The specifications for the photodetectors given in Table 5.1 will give the output current in response to a photoexcitation. This is converted into a voltage by a transimpedance amplifier. Most high frequency components are specified in terms of power, in particular to decibels of power centered at 1mW. This is known as 1dBm. A conversion graph of voltage (peak-to-peak) into a 50Ω impedance is given in Figure

76
Using the equation
\[
dBM = 10 \log_{10} \left( \frac{V_{pp}^2}{50} \cdot 1000 \right)
\]  
(5.1)

Thus, from Equation 5.1, for a 1dBm input RF power, we would need a single with a peak-to-peak voltage of greater than 700mV. From Table 5.1, we can easily calculate that the amount of light of the 1064 nm wavelength needed to achieve this peak-to-peak voltage is 42 μW. However, in the actual FastPDM electronics setup, the signals from the photodetectors need to be filtered, which has a conversion loss associated with each filter, and then split several times. In addition, because of power losses through the optical system, we cannot get a 100% heterodyne efficiency. Therefore, we need to amplify the 1064nm signal. This was accomplished using an RF amplifier, model ZFL-500LN, from Mini-Circuits. This provided a minimum gain of 24dB for a frequency range of 0.1MHz to 500MHz.

After doubling the 1064nm heterodyne signal from 10MHz to 20MHz, the 20MHz signal was bandpassed using the BBP-21.4 band-pass filter, to remove any harmonics from the frequency doubling process.

### 5.2 Phase detection

There are numerous methods for measuring the phase difference between two signals of the same frequency. They can be generalized into two categories, those converting the heterodyne signal first into a digital signal, or those using analog techniques to measure phase, and then converting the resulting phase measurement into a digital signal that can be interpreted by a computer.

Originally, the FastPDM was to have a digital phase detection scheme, using a phase lookup table. However, due to the complexity of such a system, as well as the availability of cheap and accurate analog phase detectors, the analog scheme was eventually chosen.
5.2.1 Digital Design

In a digital design scheme, the analog heterodyne signal generated by the photodetectors is converted into a digital signal using analog-to-digital converters (ADC). This digital data can then be processed using digital signal processing techniques.

There are two options for using a digital scheme. The first would be to use a digital signal processor (DSP), a specialized integrated circuit that is optimized for signal processing.

![Functional block and CPU (DSP core) diagram](image)

Figure 5-5: Block diagram of Texas Instruments TMS320C6711 digital signal processor core [11, p7]

The block diagram of the Texas Instruments’ TMS320C6711 digital signal processor is shown in Figure 5-5. A peripheral bus can be accessed through the chip’s external memory interface (EMIF), which is a 32-bit bus that interfaces with I/O devices and memory. Timing between the optical system, the scanning control system,
and the DSP chip can occur by triggering interrupt signals that will inform the chip that data should be processed. Building a circuit board for this application would be extremely difficult, because the chip only comes in a 256-pin plastic ball grid array (BGA), which requires specialized wave-soldering apparatus.

Tests were undertaken using an evaluation board and TI’s own Code Composer Studio, which uses a variant of the C programming language tailored for writing DSP algorithms. Unfortunately, it soon became clear that the amount of time required to perform the calculations to extract the phase would be far too slow to allow a 10,000 pixel rendered phase image. We suspect that this is probably because the DSP chip is instructed to perform the inverse tangent function, which is probably calculated using a Taylor series. Although the DSP chip is designed for just such an operation, it cannot be performed quickly enough for the purposes of the FastPDM.

![Figure 5-6: EPROM-based phase extraction system. Phase data is stored on two EPROMs (IC5, IC6)](image)

An alternative digital implementation is to calculate the phase given two input signals. This minimizes the amount of calculation that needs to be done in real-time, because all of the time-consuming trigonometric calculations can be done off-line using MATLAB and programmed onto programmable logic. One such implementation uses electronically programmable read only memories (EPROM). This circuit is diagrammed in Figure 5-7, and the parts list is given in Table 5.2.

The EPROM implementation takes the two analog heterodyne signals from the photodetectors, 1532 and 11064, and digitizes them using analog-to-digital converters (IC1 and IC2). The EPROM phase extraction circuit also requires control signals that are generated from a clock generator circuit (Figure 5-6) and the control circuitry for the scanning galvanometers (Figure 5-10). In particular, the clock generator and the
Figure 5-7: EPROM-based phase extraction system. Phase data is stored on two EPROMs (IC5, IC6)

<table>
<thead>
<tr>
<th>Schematic Label</th>
<th>Part Number</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>IC1,2</td>
<td>-</td>
<td>8-bit analog-to-digital converter</td>
</tr>
<tr>
<td>IC3,4,7,8,10,12,13</td>
<td>74F175</td>
<td>D-type flip-flop</td>
</tr>
<tr>
<td>IC5,6</td>
<td>AT27C512R</td>
<td>EPROM</td>
</tr>
<tr>
<td>IC9</td>
<td>-</td>
<td>8-bit adder/subtraction circuit</td>
</tr>
<tr>
<td>IC11</td>
<td>ATV2500B</td>
<td>Complex Programmable Logic Device</td>
</tr>
<tr>
<td>IC14</td>
<td>SX28AC</td>
<td>8-bit 50MHz microcontroller</td>
</tr>
</tbody>
</table>

Table 5.2: Parts list for EPROM-based phase extraction circuit

galvo control circuits generate two signals to the phase extraction circuit, ADCCLK and PCLK. These two clock signals are square waves at the same frequency, but delayed in time to meet the timing parameters of the ADC chips (IC1 and IC2) and the D-Q flip-flops (IC3, IC4). ADCCLK is a special clock that signals the ADC chips to sample the analog signals I532 and I1064. The signal rate is exactly four times the 532nm heterodyne frequency and the 1064nm doubled heterodyne frequency. This matching is done in the clock generator circuit through the use of an RF mixer that mixes the driving signals of the AOFS. The output of the mixer will have the sum and difference frequencies of the AOFS driver. The difference frequency, filtered, will also be the heterodyne frequency of the second order, second harmonic beam from
The EPR(M-based phase extraction circuit has several pipeline stages to increase the throughput of the device. The first stage is the ADC. The second stage, between IC4, IC13 and IC7, IC8, has the EPROMs (IC5 and IC6). The third stage of the pipeline has a subtraction circuit (IC9) which can be any CMOS or TTL digital adder/subtractor. The fourth stage is a multi-bit adder circuit, which will add the outputs of the third stage and will average several phase measurements, before generating an output circuit that will be transmitted to a computer through the PC parallel port. This circuit, IC11 in Figure 5-7, was implemented on an ATV2500B complex programmable logic device (CPLD) from Atmel, Inc. (San Jose, CA)

This circuit would require a minimum of 14 integrated circuits (although this number could be minimized by utilizing quad- or octal-D-Q-flip flop chips). The timing requirements necessitated by the ADC and the EPROMs would limit the choice of heterodyne frequencies. For this reason, although the design presented here would be suitable for the phase extraction system, this design was eventually abandoned due to its restrictions and complexity. Another, simpler approach was needed, and an analog design method was chosen.

5.2.2 Analog Design

Phase measurements using analog integrated circuits is a simple task. RF mixers multiply two high frequency signals, and output the sum frequency, the difference frequency, and a phase term. Consider two signals, one at frequency $\omega_1$ and another at frequency $\omega_2$. Using the analog terminology for an RF mixer, there are two input signals, the RF signal and the local oscillator (LO) signal. The output signal is the interference frequency, or IF. Consider a circuit with an RF signal with a frequency of $\omega_1$ and an LO signal with a frequency of $\omega_2$. Then, the multiplication of these two
signal would result in

\[ IF = RF \cdot LO \]  
\[ = |RF| e^{i(\omega_1 + \phi_1)} \cdot |LO| e^{i(\omega_2 + \phi_2)} \]  
\[ = |RF||LO| e^{i((\omega_1 + \phi_1) \pm (\omega_2 + \phi_2))}, \]

where \(|RF|\) and \(|LO|\) are the magnitudes of the RF and LO signals, respectively.

Assuming, for the sake of discussion, that \(|RF|\) and \(|LO|\) are constant, known quantities. If \(\omega_1 = \omega_2\), and the output is low-pass filtered, then Equation 5.4 becomes

\[ IF = e^{i(\phi_1 - \phi_2)}. \]

By taking this output and repeating the experiment with one of the heterodyne signals shifted by 90°, known as the quadrature component, the phase difference between the

---

Figure 5-8: Internal system block diagram for the Analog Devices AD8302 phase detector chip

---

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two signals can be extracted. The full notation would then be

\[
\phi_{\text{diff}} = \arg\left( \frac{I_{F_{\text{in-phase}}}}{I_{F_{\text{quadrature}}}} \right)
\]  

(5.6)

Mini-Circuits has mixers that are specifically designed to be used as phase detectors. These are the ZRPD-1, which has a frequency range of 1MHz to 100MHz, but requires a input power of 7dBm. From Figure 5-4, this corresponds to roughly 1.7V peak-to-peak. Thus, we would need RF amplifiers on both the 532nm and 1064nm heterodyne signals.

An alternative is an analog integrated circuit, such as the one shown in Figure 5-8, the AD8302 from Analog Devices (Norwood, MA). This IC is a gain and phase detector for frequencies up to 2.7GHz. The heart of this device is a mixer like the one described above, but it also includes logarithmic amplifiers that attempts to eliminate the amplitude dependence of the phase measurement, as well as eliminates the need to amplify the input signals. The input range of this chip is -60dBm to 0dBm.

Obviously, the AD8302 has many advantages compared to the ZRPD-1 or using a generic analog RF mixer. However, the small scale of the AD8302 makes it difficult to prototype. The AD8302 is available only in a 14-lead thin shrink small outline package (TSSOP), which requires a printed circuit board to be fabricated and the chip attached using surface mount technology. Although this is better in the long run because it is a smaller package, and will be less sensitive to electronic noise, as well as more compact, a significant effort would have to be expended to prototype the system. In order to minimize the amount of time required to get a working prototype of the FastPDM, we decided to use an evaluation board.

5.2.3 Final Design

The final design of the phase extraction circuit, based on the evaluation board for the AD8302 phase detector, is shown in Figure 5-9. The parts list is given in Table 5.3. The analog signal from the photodetectors is generated by PD1 for the fundamental
wavelength, and PD2 for the second harmonic. The heterodyne frequency at the detectors is 10MHz and 20MHz, respectively. These electronic signals are then band-passed at their respective center frequencies with U10 and U6. The fundamental signal is then amplified with U9, frequency doubled using U8, and then bandpassed at the 20MHz center frequency with U7. Both signals are then split using U3 and U4, and sent to the AD8302 phase detectors, U1 and U2. One of the signals split from U3 is phase delayed by 90° using X1, a delay line. The outputs of the phase detectors are then sent to a computer (PC) which analyzes the data and displays it for the operator.

Eventually, this entire circuit will be placed on a printed circuit board. This will reduce the amount of space and free wires that the current circuit takes up, which will eliminate the chance of accidentally tripping on a cable and destroying the circuit. In addition, it should have better noise tolerance.
### Table 5.3: Parts list for AD8302 based phase detection circuit

<table>
<thead>
<tr>
<th>Schematic Label</th>
<th>Part Number</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>PD1</td>
<td>1811FS-AC</td>
<td>InGaAs photodetector (infrared region)</td>
</tr>
<tr>
<td>PD2</td>
<td>1801FS-AC</td>
<td>Silicon photodetector (visible region)</td>
</tr>
<tr>
<td>U1,U2</td>
<td>AD8302</td>
<td>Phase detector</td>
</tr>
<tr>
<td>U3,U4</td>
<td>ZSC-2-4</td>
<td>Power splitter</td>
</tr>
<tr>
<td>U6,U7</td>
<td>BBP-21.7</td>
<td>20Mhz bandpass filter</td>
</tr>
<tr>
<td>U8</td>
<td>FD-2</td>
<td>Frequency doubler</td>
</tr>
<tr>
<td>U9</td>
<td>ZFL-500LN</td>
<td>RF power amplifier</td>
</tr>
<tr>
<td>U10</td>
<td>BBP-10.4</td>
<td>10MHz bandpass filter</td>
</tr>
<tr>
<td>X1</td>
<td></td>
<td>Variable delay line</td>
</tr>
<tr>
<td>PC</td>
<td></td>
<td>Windows Computer with data acquisition board</td>
</tr>
</tbody>
</table>

#### 5.3 Control Systems

In addition to measuring the phase difference, there must be electronics to control the scanning system. As discussed in Chapter 4, there were two options for the scanning system, beam translation or sample translation. Although the sample translation method was the technique implemented for the FastPDM, both control mechanisms will be discussed here.

![Figure 5-10: Scanning galvanometer control circuit](image-url)
5.3.1 Beam Scanning Control

As discussed in Chapter 4, the scanning galvanometers that was to have been used in the beam scanning setup are driven using QuantumScan servo drivers. These servo drivers accept an analog input, which it translates into a position that the mirror on the scanning galvanometer should be. The servo has the feedback and control mechanisms to ensure that the mirror is properly positioned. Thus, if we put the appropriate voltage across the control input pins of the servo driver, we can assume that this corresponds exactly to the position of the mirror on the galvo.

Because there are two galvanometers in the beam scanning experiment, we will therefore require two analog inputs. These inputs must be carefully coordinates such that the laser beam, after being reflected by both galvo mirrors, will uniformly scan the entire sample in the two axes of concern. This is done using the circuit described in Figure 5-10.

The critical component in the galvanometer control circuit is the microcontroller (U1 in Figure 5-11). The microcontroller assembly language code used for the galvo control is in Figure 5-12. The resulting printed circuit board layout is shown in the Appendix, as well as the schematic errata. The circuit operates as follows: the microcontroller is essentially a counter. An 8-bit value representing the position of the fast axis galvanometer is stored in register “XPOS” and output to port B. “XPOS” is incremented, resulting in an increase in the value of port B. The microcontroller then sleeps for 40,000 clock cycles, representing 10ms. If “XPOS” is less than 100,
Figure 5-12: GALVOCONTROL.SRC: Microcontroller assembly language code for galvanometer control
This process is repeated until "XPOS" equals 100. Otherwise, the loop is broken, "XPOS" is cleared, and the register "YPOS," which represents the position of the slow axis galvanometer, is incremented. The value of "YPOS" is output to port C. If "YPOS" is less than 100, the process begins at the very beginning, with "XPOS" cleared and incremented, until "YPOS" equals 100. When this occurs, control lines are asserted high to signal to the FastPDM instrument that a new frame has begun, and the values of "XPOS" and "YPOS" are cleared to zero, and the sequence begins again.

This circuit worked efficiently and was effective in synchronizing the movements of both galvanometers. However, for reasons due to the optics and difficulty in getting high heterodyne efficiency as the beam was translated, as outlined in Chapter 4, the beam scanning scheme was abandoned. However, in the future, if it is deemed appropriate to use galvanometers for the FastPDM or similar instrument, this circuit will operate well.

### 5.3.2 Sample Scanning Control

The sample scanning method for forming images uses three Newport 433 translation stages. These are moved using Newport 850G ball bearing linear stages with sub-micron repeatability and accuracy. There are controlled through a computer running LabVIEW and connected via serial cable to a Newport ESP300 universal motion controller/driver. The LabVIEW code for motion control is intertwined with the data acquisition and display.

### 5.4 Data Acquisition and Display

The data acquisition and display system has the following responsibilities:

1. Control the scanning system, and know exactly where the laser beam is at any
given point in the scan

2. Acquire data from the phase detection system, and process the data to give a full $-\pi$ radian to $+\pi$ radian range of data

3. Display the data on a computer screen

4. Save the data for later analysis

The data acquisition and display system used a computer running Windows 98 and the National Instruments LabVIEW software. The computer had a data acquisition board that takes analog inputs, digitizes and transfers the data so that LabVIEW can acquire samples.

LabVIEW is a software package produced by National Instruments (Austin, TX) that is designed to provide a graphical development environment for automation and data acquisition applications. Rather than lines of iterative code, LabVIEW applications are programmed using the graphical “G” programming language, which represents data flow using wires and data manipulations using “sub-VIs”.

The FastPDM has several VIs, depending on what measurement is being performed. They all operate on the two analog electronic signals sent from the AD8302 phase detectors, in a circuit described in Figure 5-9. “Input-Quadrature.vi” measures phase with time, so that the operator of the FastPDM can ascertain whether the instrument has reached a steady-state and that the laser noise, which is always present, is not adversely affecting the phase measurement. “2D-Scan-Quadrature.vi” performs the actual image scanning. The full documentation of the LabVIEW VI’s for the data acquisition and scanning is in Appendix B.

The quadrature measurement in LabVIEW, which takes the signals from the two phase detectors and produces a phase measurement with a full range of $-\pi$ to $\pi$, is done by inputting the in-phase and quadrature signals as the real and imaginary parts of the same signal into a sub-VI that converts this into polar coordinates. The argument of this conversion is then taken, which gives the modulus and the angle.
Chapter 6

Results

This chapter describes the preliminary results of the FastPDM instrument, using the implementation discussed in Chapters 4 and 5. We will discuss the calibration experiments to verify that the FastPDM is capable of correctly measuring the dispersion of simple, non-biological structures. The first phase dispersion images from the FastPDM using biological samples will be shown.

It is relevant to note that these are preliminary images that have not been fully analyzed. Furthermore, as discussed previously, there are still significant issues with the FastPDM that have made it difficult to fully interpret the phase dispersion data. For every image shown here, the experiment was conducted many times and many experiments were thrown out due to noise issues with the FastPDM. In addition, the long periods of time required to produce a single FastPDM image is problematic, as it increases the sensitivity of the instrument to slow phase drifts. The poor resolution of the FastPDM makes it very difficult to study subcellular structures, or to interpret specific tissue structures. However, the data that has been taken and is being presented here shows that the FastPDM has promise as a vital tool for cell biology.
6.1 Proof of principle

Before we could study complicated biological structures, we needed to verify that the FastPDM was working properly. Although the noise cancellation using the second harmonic was not perfect, for unknown reasons, the phase measurement was at times reasonably stable.

6.1.1 Calibration Experiment: Microscope Slide

A microscope slide is merely a thin (approximately 1mm thick) piece of glass. By varying the angle of incidence that the microscope slide makes with the laser beam, the beam will have to travel through varying thicknesses of glass. This experiment is complicated by the fact that dispersion, which will give us a phase change, will also physically separate the two colors. Therefore, extremely large angles are not possible using this experiment. However, for small angles, this experiment can be used to calibrate the experiment and verify that the phase measurements made by the FastPDM are accurate.

A standard microscope slide, made of soda-lime glass, was placed in the sample arm of the interferometer, in front of the microscope objectives. It was attached to a rotation stage with demarcation every two degrees, and phase measurements were taken every two degrees and plotted in Figure 6-1. This figure shows two experiments, one taking data after every $2^\circ$ of rotation (green line with blue circles), and a separate one taking data every $1^\circ$ of rotation (red line with blue X). The dotted blue line was an attempt to fit this data to what was expected. Unfortunately, this was difficult for a number of reasons. First is that this data was taken using only one phase detector. Therefore the range of phases was limited to $0$ to $\pi$, with nonlinearities towards $0$ and $\pi$ radians. In addition, for the type of glass used in the microscope slide, a soda-lime glass, the dispersion between the 532nm and the 1064nm wavelength is not known. Therefore it was difficult to predict what would be expected. However,
these experiments show that although there is an unknown dispersion at our two wavelengths, the shape of the data follows what would be expected, and that the experiments are reasonably repeatable.

6.1.2 Phase Grating

A better calibration experiment would involve a sample with numerically known values for the dispersion at the 532nm and 1064nm wavelengths. The next experiment concerned a phase grating, which is a piece of glass of known material (such as BK7) where the heights vary as a square function. In other words, the glass has varying thickness; the thinner regions have a thickness of approximately 1mm, and the thicker
regions have that height plus an increase of approximately 1.1\( \mu \text{m} \). These regions are equispaced across the top of the glass. We will refer to the thicker regions, which has a width of 75\( \mu \text{m} \), as the plateau region, and thinner region as the valley region, which also has a width of 75\( \mu \text{m} \). Figure 6-2 has the basic setup of this grating. The laser passes through the grating and is phase delayed at different amounts depending on whether it is traveling through the plateau region or the valley region.

![Figure 6-2: Pictorial of phase grating](image)

Figure 6-3 shows the phase dispersion image taken from the grating with plateau-to-valley height of approximately 1.1 \( \mu \text{m} \). This height variation was measured using a profilometer. The width of each plateau and each valley was measured using a standard light microscope. A horizontal cross-section taken at 15\( \mu \text{m} \) is shown in Figure 6-4.

From Chapter 3, we know that

\[
\phi_{diff} = \frac{2\pi}{\lambda_2} \Delta n L,
\]  

(6.1)
where $\lambda_2$ is the wavelength of the second harmonic light, $\Delta n$ is the difference in refractive index between the two wavelengths, and $L$ is the thickness of the material. The characteristics of BK7 are well known. The index of refraction at the 532nm wavelength is $n_{532\text{nm}} = 1.51947$, and at 1064nm is $n_{1064\text{nm}} = 1.50663$. Therefore $\Delta n = 0.013$. Plugging into Equation 6.1, we find that the expected phase difference between the plateau and the valley of the phase grating is approximately 0.17 radians, if we ignore the dispersion between 532nm and 1064nm of air. This assumption is valid because the amount of dispersion in air will be much less than the dispersion of the BK7 glass. The cross-section of the phase dispersion image of the phase grating in Figure 6-4 shows that the phase difference between the plateau and valley regions is roughly 0.2 radians. Therefore, we are reasonably satisfied that the FastPDM is
Examining the cross-section in Figure 6-4, it is obvious that the area between the plateau and the valley has a large phase shift compared to the phase shift between the plateau and the valley (such as at 40um, 110um, 190um on the abscissa). This is somewhat unexpected, and there are a number of theories about why this is occurring. The first theory is that there is a slight displacement between the two beams (on the order of one micron), which produces a Nomarski-like effect that measures the gradient of phase of one wavelength versus another. However, if this were the case, we would expect that the concavity of the phase jumps would stagger from one edge to another, and this is not the case. In other words, if this effect were due to a separation of the beams, the phase would shift up when the edge was where a plateau
turned into a valley, and would shift the other direction when the edge was a valley into a plateau. Therefore, even though there may be a slight spatial displacement of the two beams, this is not a likely explanation for the large phase shifts.

An alternative explanation is that the beam sizes are different, and the phase measurement integrates the field of the beam as it travels through the grating. Using a CCD camera, we determined that the two beams are at most displaced by 1μm in either axis, but have beam sizes of 4μm for the 532nm beam and 13μm for the 1064nm beam. Furthermore, if the beams weren't focused, there might be unpredictable measurements such as the ones we have been seeing.

Regardless of the cause, it is apparent that the FastPDM measures substantial phase shifts at boundaries. Although this effect is not necessarily detrimental to the operation of the FastPDM, the cause of this phenomenon will be studied, and hopefully eliminated.

There is also a slow phase drift that is evident in Figure 6-3. The scan started at the lower left-hand corner (the origin is at 0 microns) and then scanned horizontally. Once this scan was completed, the sample was moved back to 0 microns and moved five microns horizontally. This was completed until the entire field was scanned. There are obvious areas in this figure where the area between the edges of the phase grating that have higher or lower phase than the corresponding region in a previous horizontal scan. This slow drift can have a variety of sources. One source might be thermal drift in the interferometer, although this is not likely because the system was on for many hours before this scan was taken. By that point, we would expect that the system would reach a thermal steady state, if there are no external factors affecting the system. Vibrational motion that is not completely canceled out by the second harmonic wavelength is also a possibility. However, the most likely candidate is simply noise in the laser. Frequency fluctuations in the laser would be particularly deleterious to a clean phase difference measurement if the path lengths of the arms of the interferometer.

In addition to the slow drift in the interferometer, a higher frequency noise is evident in Figure 6-4. If the noise cancellation was perfect in the FastPDM, instead
of oscillations, there would be straight lines in the areas of the plateaus and the valleys. Again, this is probably due to a number of factors, including noise in the laser and possibly non-linearities in the movement of the scanning motors.

Although there are some noise issues to contend with in analyzing the phase grating data, in general these results are promising, and shows that small changes in the relative phase can be measured using this implementation of the FastPDM.

6.2 Biological Samples

The purpose of the FastPDM is to image biological samples without the need for external contrast agents. We examined several samples that have structures that are identifiable under conventional microscopy, in order to ensure that the phase dispersion image correlates spatially and structurally with what is seen with a conventional microscope. With evidence that the FastPDM is working reasonably well, we decided to study several different types of biological samples.

6.2.1 Onion Skin

A common, easily obtainable sample is the skin of an onion. The onion skin is thin, fairly transparent with well defined structure at cell walls. The size of a typical onion cell is 50-75 \( \mu \text{m} \) width and longer length of several hundred microns. A conventional microscopy image of onion skin, with magnification of 400X and stained with non-specific red stain is shown in Figure 6-5.

Figure 6-6 is a phase dispersion image of several cells from a similar onion skin. The field size of the image is 600 \( \mu \text{m} \) by 300 \( \mu \text{m} \), which is enough to see portions of several cells. The cells run lengthwise from lower left to upper right. The only obvious structures visible are the cell walls, indicated on the figure. In the cytoplasm, there also appears to be some structure that has no definable shape. The areas where the phase difference jumps from dark blue to dark red, for instance, is indicative of
Figure 6-5: A stained conventional microscopy image of onion skin, 400X. Stained with red stain. [1]

Figure 6-6: 300μm by 600μm phase dispersion image of onion skin. The cells are several hundred microns in length and about 75 microns in width.
a $2\pi$ phase wrap. This does not appear to be related to the noise in the system, because they repeat over several scans. There are also regions in the cytoplasm, several microns away from the cell walls, that show a brief phase shift of several radians (such as the green spots in several locations in Figure 6-6). These could correspond to real changes in dispersion in the cell. However, there are no discernible patterns that would indicate that these changes are due to specific structures.

A closeup of the lower left-hand corner (inside black box) of Figure 6-6 is shown in Figure 6-8, with higher resolution. In addition, the same portion of the onion was viewed using the bright-field microscope capabilities of the FastPDM in Figure 6-7, to compare the two structures. In particular, the cell wall is clearly visible in both the amplitude image and the phase dispersion image as a sideways "T" structure. The laser beam (bright green spot) is also evident in the bright-field image.

The experiments with the onion skin cells shows that biological samples can be viewed. We would like to see more interesting structures, however, since onion cells have very little internal structure other than the cell walls.

### 6.2.2 Carotid Artery

For the first pathological sample viewed with the FastPDM, we used a 10μm thick slide of diseased human carotid artery. As with the breast tissue, there are few cells in the carotid artery. The majority of the structure in the carotid artery are collagen fibers and extracellular matrix.

Figure 6-9 shows a 150μm by 150μm phase dispersion image of the 10μm thick carotid artery, with a bright field image of the same region. The lower region of each image shows where the tissue stops and there is just blank microscope slide. This corresponds to the light blue region in the phase dispersion image at around 5μm above the bottom of the image. Within the tissue, there are ripple-like structures which might correspond to collagen fibers or extracellular matrix. Although the structures do not seem to match up identically with the bright field image on the upper right, this is more likely due to the poor quality of the bright field image than
Figure 6-7: Brightfield image of a closeup of the lower left-hand corner of Figure 6-6.

Figure 6-8: Phase dispersion image of a closeup of the lower left-hand corner of Figure 6-6. This is the same region shown in the brightfield image in Figure 6-7.
any issue with the phase dispersion microscope.

A larger field (1mm by 1mm) of the same carotid artery was taken and shown in Figure 6-10. The entire cross section of the carotid artery can be seen. The lower portion of the image is the inside of the artery, where the blood cells flow. There are three layers in the carotid artery: the intima, the layer closest to the inside of the artery; the medial layer; and the adventitia. The adventitia generally has denser structure than the medial layer. In diseased tissue, plaque forms that causes a thickening of the intima from one cell layer thick to tens or hundreds of microns. Although not entirely evident from this image, there seems to be regions of changing density from the upper 100 μm of tissue. We believe that this may be the distinction between the adventitia and the medial layer. In addition, in the lower portion of the image there appears to be some structures that might be collagen fibers. There are also regions of the cell where the tissue appears to be empty. Although we have not been able to perform a complete analysis of this image, we feel confident that there is interesting structure evident in using the FastPDM with this type of structure, and

Figure 6-9: Phase dispersion image and bright-field image of diseased carotid artery
Figure 6-10: A larger field of view of the same carotid artery warrants further investigation.
Figure 6-11: A single 40\(\mu\)m polystyrene sphere in glycerol.

6.2.3 Polystyrene Beads

In order to understand the phase wrapping from the carotid artery, we examined 40 \(\mu\)m polystyrene beads. Because these beads are spherical, there are no abrupt boundaries such as the ones seen with the phase grating. Instead, we would expect to see gradual changes in the phase as the laser beam is scanned across a sphere.

The beads were prepared from Cat No 4240A 40 \(\mu\)m polymer microspheres in water from Duke Scientific Corporation (Palo Alto, CA). A drop of this solution was placed on a glass microscope coverslip and immersed in glycerol. A second coverslip was placed on top of this mixture, and the two slides were taped together. Figure 6-11 is a phase dispersion image of a polystyrene bead. It is clear that there are large phase changes across the sphere. However, this change is more gradual, as expected.
A cross section across this sphere is shown in Figure 6-12.

The wrapped image of the bead was unwrapped in two dimensions using MATLAB and manually. The result is shown in Figure 6-13. The result is a spherical cone which matches what we would expect the phase dispersion profile of the sphere to be. This result shows that it is possible to unwrap the wrapped images such as the carotid artery. Unfortunately, we have yet to discover an automatic method for doing this. Because the dispersion profile of the artery is not known, we cannot unwrap the image by hand. New methods of automatically unwrapping images with large phase changes must be discovered.
Figure 6-13: A single 40µm polystyrene sphere in glycerol, unwrapped, of Figure 6-11.

6.2.4 Summary

We have shown using a microscope slide on a rotation stage, and a phase grating, then the FastPDM can measure dispersion in non-biological samples. We have demonstrated that this instrument can also identify more complex structures in the carotid artery. Due to the issues with the boundary effect, phase noise from unknown sources, and slow phase drifts in the interferometer, there is some ambiguity about how well dispersion can be measured in more complex structures. However, we are confident that with minor adjustments and troubleshooting on the current FastPDM implementation, that this instrument can be a valuable tool for cellular microscopy.
6.3 Potential for pathology

There are several techniques through which a pathologist may be able to image cells without the need for chemical staining, as described in Chapter 2. Because of numerous limitations, however, they do not have the same potential that is available for the FastPDM.

As an example, let us consider the 10 μm slice of carotid artery in Figure 6-10 again. As described previously in this chapter, there should be three distinct strata of tissue. Under phase contrast, the three layers may be discernible to a trained physician or technician, but most of this would be achieved through guesswork and conjecture. Furthermore, since images taken under phase contrast are not quantitative, it would not be possible to analyze this data computationally. However, under phase dispersion, we may be able to distinguish the different densities of tissue structures. In the case of the carotid artery examined under the FastPDM, mathematically methods such as the spatial Fourier transform, or simple analysis using variances might be performed on data similar to the one shown in this image. Because diseased carotid artery has plaque formation, the intima is many times thicker in the diseased tissue than in a healthy one. Using this data, it might be possible for a computer to automatically diagnose a pathology sample as diseased or normal.

Of course, because of the current limitations of the FastPDM, such an analysis would not be practical. As we will discuss in Chapter 7, we plan on making improvements to the current implementation of the FastPDM in order to decrease the amount of time it takes to scan a sample, as well as improve the resolution. If the boundary effect that we’ve shown can be solved, the type of analysis described above might take merely a second or two of time. There are many practical applications that having a quantitative means of analyzing phase might provide.
Chapter 7

Conclusions and Discussion

In this thesis, we have overviewed several standard methods of amplitude-based and phase-based microscopy; we have introduced the concepts of optical dispersion and its application and potential to the field of cellular microscopy; and we have introduced the prototype of a new instrument that has been built using this technique, the FastPDM. We have demonstrated that the FastPDM can detect small phase changes as a result of dispersion in glass, and we have shown that the FastPDM can be used to image thin biological samples without staining, such as onion cells and sections of the human carotid artery.

This FastPDM prototype has several shortcomings that must be improved before being ready for practical use. In particular, the resolution and the speed of the FastPDM needs to be increased. In addition, Chapter 6 described the preliminary results, and it is apparent that there is some sort of boundary effect where there are changes in refractive index. This results in a phase change larger than expected. Given that most cells we are interested in will have a size on the order of ten to fifteen microns, and that we would be interested in imaging subcellular structures, in order to do so we must improve the resolution to be less than two microns. In addition, the FastPDM is intended to be an instrument that can be used as a tool by biologists, and will be an instrument in the Spectroscopy Lab for routine study of biological tissue samples. Very few, if any, cell biologists would be willing to wait 45 minutes
Table 7.1: Comparison between the original, Michelson-based PDM described in Chapter 3, the current FastPDM, and future improvements to the current implementation for a 100μm by 100μm phase dispersion image.

Table 7.1 lists the major differences between the original phase dispersion microscope, described in Chapter 3, and the FastPDM instrument as it currently exists. The third column describes additional work that will be performed on the current FastPDM to improve its performance and make it suitable for practical use. In particular, the speed of the scanning must be improved by several orders of magnitude, and the resolution should be improved by at least a factor of two in order to discern subcellular structures.

The proposed improvements to the FastPDM will include replacing the current motorized actuators used in to form images with a PZT stage. In addition to replacing the translation stages currently in place, the control system will have to be updated to accommodate the PZT stage. This should reduce the scanning time to the desired one to two second range for a 100μm by 100μm image.

In addition, the resolution can be improved by expanding the beam prior to the microscope objectives, or by using higher numerical aperture objectives. The 532nm and the 1064nm beam should also be the same size; this can be accomplished by expanding the 1064nm beam after the single mode fiber, and before the second dichroic mirror in the system. Alternative means of improving the resolution, such as using an imaging lens and a pinhole between the microscope objectives and the photodetectors, will also be considered.
The phase detection system currently consists of two AD8302 evaluation boards, in addition to several coaxial power splitters, power amplifiers, and filters. This configuration not only takes up a lot of space, it also requires numerous BNC connections that increase the amount of electrical noise in the system. We plan to re-implement the entire phase detection electronics onto one printed circuit board. We hope the lower the noise in the phase measurement, such that phase differences of less than $5^\circ$ can be accurately measured. This will probably also require the replacement or repair of the laser.

The results from Chapter 6 have shown that the FastPDM can image small changes in dispersion in biological samples. It has also shown that there is a phase wrapping problem at boundaries of different structures, as well as when there are large changes in phase. In order to quantitatively analyze biological samples, the phase wrapping issue must be understood and dealt with. In addition, we must be able to interpret phase measurements in terms of the biological structures they represent.

We believe that with these refinements, the FastPDM will be an instrument that will provide biologists with a quantitative imaging method, and will allow pathologists to quickly examine biological samples without extensive preparation. Furthermore, continued research of biological samples using phase dispersion will give new insights into the structure of biological and cellular components.
Appendix A

Beam Scanning

A.1 waist-scan.vi

A.1.1 Connector Pane

A.1.2 Front Panel

A.1.3 Controls and Indicators
Block Diagram

[Diagram of a block diagram showing Power Motors, Define Home, Initialize Serial Port, and Simple Error Handler.]
Appendix B

Single Point Quadrature Phase Measurement

B.1 Input-Quadrature

B.1.1 Connector Pane

B.1.2 Front Panel
B.1.3 Controls and Indicators

\[ \text{Phase} \quad \text{avg}[\text{phase}+90] \quad \text{Phase+90} \quad \text{avg}[\text{phase}] \]
\[ \text{avg}[\text{phase}] - \text{avg}[\text{phase}+90] \quad \text{Theta} \quad \text{DBL} \quad \text{Phase} \]

B.1.4 Block Diagrams
Appendix C

Image Scanning and Phase Measurement

C.1 2D-Phase-Scan-Quad-Repeat

C.1.1 Connector Pane

C.1.2 Front Panel

C.1.3 Controls and Indicators

FA Motor #   FA Step Size   FA Step #   StartMotor
SA Motor #   SA Step Size   SA Step #   Delay After FA Scan (s)
C.1.4 Block Diagram
Initialize Serial Port

MotorReady

StartMotor

Simple Error Handler
Bibliography


