QUANTITATIVE PHASE IMAGING FOR CELLULAR BIOLOGY

BY

MUSTAFA AIZED HASAN MIR

DISSERTATION

Submitted in partial fulfillment of the requirements
for the degree of Doctor of Philosophy in Electrical and Computer Engineering
in the Graduate College of the
University of Illinois at Urbana-Champaign, 2013

Urbana, Illinois

Doctoral Committee:

Assistant Professor Gabriel Popescu, Chair
Assistant Professor Supriya G. Prasanth
Professor Stephen Allen Boppart
Professor Rashid Bashir
ABSTRACT

Measuring cellular level phenomena is challenging because of the transparent nature of cells and tissues, the multiple temporal and spatial scales involved, and the need for both high sensitivity (to single cell density, morphology, motility, etc.) and the ability to measure a large number of cells. Quantitative phase imaging (QPI) is an emerging field that addresses this need. New quantitative phase imaging modalities have emerged that provide highly sensitive information on cellular growth, motility, dynamics and spatial organization. These parameters can be measured from the sub-micron to millimeter scales and timescales ranging from milliseconds to days. In this thesis I discuss the development and use of QPI tools and analysis methods to explore several applications in both clinical and research settings. Through these applications I demonstrate that the quantitative information provided by QPI methods allows for analyzing biological systems in an unprecedented manner, creating opportunities to answer longstanding questions in biological sciences, and also enabling the study of phenomena that were previously inaccessible. Here I show results on blood cell analysis, single cell growth, cellular proliferation assays and neural network formation. These results prove that QPI provides unique and important insight into the behavior of biological systems and can be utilized to help address important needs in clinical settings as well as answer fundamental biological questions.
ACKNOWLEDGMENTS

As I finish my time here at Illinois, the realization that this is just the beginning of my career is beginning to set in and I am excited to take what I have learned here out into the world. The successful completion of the work presented in this dissertation would not be possible without the incredible support system and resources that I have been lucky enough to have available to me. I know that I will continue to carry the personal connections I have made here and the experiences and knowledge that I have gained throughout my career. Most importantly, what I have learned from the incredible people I have been exposed to, is the importance of striving to be an excellent educator, communicator, and scientist so that I can inspire the next generation of researchers just as I have been inspired. I have learned that science is truly about standing on the shoulders of giants and it’s the outstanding contributions of others that have enabled my successes.

First of all, I would like to thank my advisor, Prof. Gabriel (Gabi) Popescu for all of his guidance, support and inspiration. I joined Gabi’s group just a few months after its creation and have witnessed its transformation from a small group with big ideas to a big group with a world renowned reputation and even bigger ideas. This transformation would not be possible without Gabi’s ingenuity, tireless work ethic, and determination to be a trail blazer. I am truly fortunate to have been a part of this group and will carry what I learned from Gabi throughout my life.

I have also had the incredible fortune of working with a very talented team of colleagues each with their own expertise and unique insights. Dr. Zhuo Wang was responsible for bringing me in to work with Gabi. Zhuo is one of the most skilled and knowledgeable students I have had the privilege of knowing and also a good friend. My other fellow grad students: Dr. Ru Wang, Shamira Sridharan, Hoa Pham, Taewoo Kim, Renjie Zhuo, Chris Edwards and Tan Nguyen have
all provided me with important insights, support and inspiration when I have needed it most, and
I am truly lucky to have had them as my colleagues and friends. Prof. Lynford Goddard, a close
collaborator of our group and co-advisor of some of the students, has also provided me with
important insight and inspiration. I would also like to acknowledge the undergraduate students
who have contributed to my work, often doing very laborious tasks: Ryan Tapping, Mike Xiang
and Anwen Jiang.

None of the work I have done would have been possible without my collaborators who
have really shaped my research and allowed me to make contributions beyond my area of
expertise. First on this list is Dr. Krishnarao Tangella whom I collaborated with on my work on
blood screening. Working with Dr. Tangella was truly inspiring as he defines the meaning of the
word tireless. I would also like to thank Prof. Supriya Prasanth and her student Shen Zhen for
their support and advice on my work on U2OS cell growth; Prof. Ido Golding and his student
Michael Bednarz for my work with _E. coli_, Prof. Steve Stice and his post-doc Dr. Anirban
Majumder for work on human neurons and stem cells, Prof. Martha Gillette and her student
Chris Liu on work with rat neurons, Prof. Benita Katzenellenbogen and her post-doc Dr. Anna
Bergamaschi for the work on breast cancer cells, and Dr. Derin Babacan for work on 3D de-
convolution. I have also been lucky to be a part of an NSF-STC (EBICS) which has exposed me
to many incredible scientists who are too many to name individually. I would also like to thank
Prof. Scott Carney who has been a mentor and friend throughout my graduate career. Scott cares
about his students and the educational experience more than any other professor I have
encountered and my discussions with him have always left me motivated to do my best.
I am also grateful to my committee members, Profs. Rashid Bashir, Steve Boppart, and Supriya Prasanth, who have provided me with excellent advice and suggestions to improve this dissertation.

This acknowledgment would be incomplete without recognizing the hard work of all the faculty and staff at the ECE department and the Beckman institute. In particular, I would like to thank Julie McCartney for always being there for help with ordering supplies, organizing meetings and navigating bureaucratic channels.

My time at Illinois was not only an incredible educational and professional experience, it also allowed me to form many friendships that I will always value. I would like to thank them all for all their support and for making my time in the corn fields enjoyable.

Going to graduate school here also allowed me to meet my wonderful and loving wife Dr. Lydia Majure. She is both an amazing person and an accomplished scientist who always inspires me to do my best and be the best person I can. I cannot begin to describe what her love and support mean to me.

Finally, and most importantly, I am thankful to my parents and family. My earliest memories are of my parents teaching me to learn and be curious about the world. They tirelessly pushed me to excel academically and personally, despite all my stubbornness. I would not be here without their love and support and I am eternally grateful for it.
## CONTENTS

CHAPTER 1. INTRODUCTION .............................................................................................................. 1

CHAPTER 2. QUANTITATIVE PHASE IMAGING ............................................................................. 4
  2.1 Principles of Full-Field QPI....................................................................................................... 5
  2.2 Diffraction Phase Microscopy ............................................................................................... 10
  2.3 Spatial Light Interference Microscopy .................................................................................. 13
  2.4 Discussion ............................................................................................................................ 22

CHAPTER 3. ANALYSIS OF QUANTITATIVE PHASE DATA .......................................................... 24
  3.1 Topography and Refractometry ............................................................................................ 25
  3.2 Dry Mass Measurements ....................................................................................................... 28
  3.3 Fourier Transform Light Scattering (FTLS) ......................................................................... 30
  3.4 Dispersion Analysis: Membrane Fluctuations and Mass Transport .................................... 31
  3.5 Spatial Light Interference Tomography (SLIT) ..................................................................... 35
  3.6 Visualizing Subcellular Structure using Deconvolved Spatial Light Interference Tomography (dSLIT) .............................................................................................................. 38
  3.7 Discussion ............................................................................................................................ 53

CHAPTER 4. RED BLOOD CELL CYTOMETRY ............................................................................ 55
  4.1 Diffraction Phase Cytometry ............................................................................................... 56
  4.2 Simultaneous Measurement of Morphology and Hemoglobin ............................................ 64
  4.3 Discussion ............................................................................................................................ 74

CHAPTER 5. CELL GROWTH ......................................................................................................... 75
  5.1 *E. coli* Growth ...................................................................................................................... 77
  5.2 Cell Cycle Dependency .......................................................................................................... 80
  5.3 Cell Growth and Motility ..................................................................................................... 86
  5.4 Discussion ............................................................................................................................ 88

CHAPTER 6. BREAST CANCER GROWTH KINETICS ................................................................. 90
  6.1 Effects of Estrogen on MCF-7 Growth Kinetics................................................................. 93
  6.2 Discussion ............................................................................................................................ 100

CHAPTER 7. NEURAL NETWORK FORMATION ........................................................................ 103
  7.1 Measuring a Forming Network ............................................................................................ 106
  7.2 Discussion ............................................................................................................................ 114

CHAPTER 8. SUMMARY AND OUTLOOK .................................................................................. 116
CHAPTER 1. INTRODUCTION

“It is very easy to answer many of these fundamental biological questions; you just look at the thing!” - Richard Feynman, There is plenty of room at the bottom, 1959.

The fields of cell biology and microscopy emerged simultaneously in the 17th century, when van Leeuwenhoek first used a light microscope to observe microscopic objects such as bacteria and human cells. Since their inception, these two fields have contended with two major issues: poor optical contrast, because of the thin and optically transparent nature of cells, and diffraction limited resolution. The lower limit on the spatial resolution of images is approximately half the wavelength of the illumination light, as first calculated by Abbe in 1873 [1]. Several approaches have been developed to circumvent this resolution limit, but all have practical limitations and severe tradeoffs between resolution and throughput. Efforts to improve optical contrast include engineering exogenous contrast agents and exploiting the optics of the light-specimen interaction to better reveal the endogenous contrast provided by naturally occurring structures [2].

Currently, the most commonly used exogenous contrast technique in cell biology is fluorescence microscopy, which allows specific structures to be labeled and provides optimal contrast [3]. A key development in fluorescence microscopy was to genetically engineer the cell to express fluorescent proteins [4], essentially combining the intrinsic and exogenous contrast imaging fields. The advent of this technology made it possible to genetically modify a cell to naturally express GFP and bind the cell to prescribed cellular structures. Despite its ubiquitous use in biology, fluorescence imaging has several well characterized disadvantages, such as phototoxic effects, photo-bleaching and the need for expensive optical sources and filters.

* Although in this quote Feynman is referring to building more powerful electron microscopes, the sentiment of how important visualization is to biological sciences resonates with the topic of this thesis.
For *endogenous* contrast imaging, there are currently two widely used methods, differential interference contrast (DIC or Nomarski) and phase contrast [3]. Both of these techniques rely on the realization by Abbe that image formation, and thus contrast generation, is caused by interference between scattered and unscattered light fields [1]. This concept allowed Zernike to develop phase contrast microscopy [5]. Phase contrast improves the contrast of an image by introducing a quarter-wavelength shift between the light scattered by the specimen and the unscattered light. Phase contrast has enabled many exciting live cell imaging studies and earned Zernike the 1953 Nobel Prize in Physics; however, the information provided by this method is qualitative and the spatial resolution is diffraction limited.

It has become increasingly clear that an elucidation of cellular function requires the measurement of cellular activity with high resolution, in three dimensions, across a wide range of spatial and temporal scales, and in a minimally invasive manner. This multi-scale capability is essential, since the emergent cellular behavior is the result of integrating information from intracellular molecular reactions, inter-cellular interactions, and myriad environmental stimuli. The response to these cues also has different implications at various scales. At the cellular level, changes may be observed in the growth rate, in the cell cycle, morphology, or motility or in various inter-cellular processes, such as the production of a certain protein. At the level of the cellular culture or population, changes may be observed in the overall proliferation rate, shifts in homeostasis points, spatial architecture, etc. Thus, to truly understand cellular behavior, there is a need for a measurement method that can quantify all these parameters simultaneously, across all relevant spatial and temporal scales.

Quantitative phase imaging (QPI) techniques have shown the potential to address this need [2, 6]. In QPI, both image contrast and resolution are improved by measuring the phase
shift of light travelling through a biological specimen [6]. Additionally, measuring the phase provides quantitative data on several fundamental optical properties of living cells and tissues [2]. The QPI field has been growing rapidly over the past decade and a variety of methods have been developed [2, 7-11]. Recently we have developed a new QPI modality known as Spatial Light Interference Microscopy (SLIM) [12]. SLIM is a broadband (white light) illumination technique that provides phase sensitive measurements of thin transparent structures with unprecedented sensitivity [13, 14], femtogram sensitivity to changes in dry mass, and excellent depth sectioning capabilities which enable tomographic imaging [15]. Furthermore, SLIM is designed as an add-on module to a commercial phase contrast microscope providing the capability to simultaneously use existing modalities such as fluorescence imaging and to leverage existing peripheral technologies such as environmental controls for extended live cell imaging.

In this thesis I demonstrate the potential of QPI technology through several applications. The thesis is organized as follows: In Chapter 2 the principles behind QPI are discussed and diffraction phase microscopy (DPM) and spatial light interference microscopy (SLIM) are described. In Chapter 3 I discuss various analytical tools for extracting biologically relevant information from quantitative phase data. In Chapter 4 I show how QPI can be used as a highly sensitive point of care blood screening instrument. In Chapter 5 abilities for single cell growth measurements, including cell cycle dependent trends, are demonstrated. In Chapter 6 I show how the capabilities of measuring cell growth can be extended for use as a proliferation assay. In Chapter 7 the growth measurement capabilities are combined with analysis of spatial structure and mass transport to investigate neural network development. Finally, in Chapter 8 the results are reviewed and the future outlook of the technology is discussed.
CHAPTER 2. QUANTITATIVE PHASE IMAGING*

Quantitative phase imaging (QPI) is an emerging field aimed at studying weakly scattering and absorbing specimens [16]. The main challenge in generating intrinsic contrast from optically thin specimens including live cells is that, generally, they do not absorb or scatter light significantly, i.e. they are transparent, or phase objects. In his theory, Abbe described image formation as an interference phenomenon [1], opening the door for formulating the problem of contrast precisely like in interferometry. Based on this idea, in the 1930s Zernike developed phase contrast microscopy (PCM), in which the contrast of the interferogram generated by the scattered and unscattered light, i.e., the image contrast, is enhanced by shifting their relative phase by a quarter wavelength and further matching their relative power [17, 18]. PCM represents a major advance in intrinsic contrast imaging, as it reveals inner details of transparent structures without staining or tagging. However, the resulting phase contrast image is an intensity distribution, in which the phase information is coupled nonlinearly and cannot be retrieved quantitatively.

Gabor understood the significance of the phase information and, in the 1940s, proposed holography as an approach to exploit it for imaging purposes [19]. It became clear that knowing both the amplitude and phase of the field allows imaging to be treated as transmission of information, akin to radio communication [20].

In essence, QPI combines the pioneering ideas of Abbe, Zernike, and Gabor. The measured image in QPI is a map of pathlength shifts associated with the specimen. This image contains quantitative information about both the local thickness and refractive index of the

---

* This chapter is reproduced from (with some modifications and additional material) a review article: M. Mir, B. Bhaduri, R. Wang, R. Zhu and G. Popescu, “Quantitative Phase Imaging,” Progress in Optics, Chennai, B.V.: (2012), pp. 133-217. This material is reproduced with the permission of the publisher and is available using the ISBN: 978-0-444-59422-8.
structure. As discussed below QPI provides a powerful means to study dynamics associated with both thickness and refractive index fluctuations.

In this chapter I review two full-field QPI methods that have proven successful in biological investigations. Section 2.1 provides a basic introduction to the principles of QPI and the main approaches: off-axis, phase-shifting, common path, white light, and their figures of merit. In Section 2.2 I discuss an off-axis method known as diffraction phase microscopy (DPM) and in Section 2.3 spatial light interference microscopy (SLIM) is discussed. Analysis of the measured quantitative phase information is discussed in Chapter 3.

2.1 Principles of Full-Field QPI

Quantitative phase imaging (QPI) deals with measuring the phase shift produced by a specimen at each point within the field of view. Full-field phase measurement techniques provide simultaneous information from the whole image field on the sample, which has the benefit of providing information on both the temporal and the spatial behavior of the specimen under investigation. Typically, an imaging system gives a magnified image of the specimen and the image field can be expressed in space-time as

\[
U_i(x, y; t) = U_0(x, y)h(x, y) = U_0(x, y)e^{i\phi(x, y)}.
\]

Clearly, if the image is recorded by the detector as is, only the modulus squared of the field, \( |U_i(x, y)|^2 \), is obtained, and thus, the phase information is lost. However, if the image field is mixed (i.e. interfered) with another (reference) field, \( U_r \), the resulting intensity retains information about the phase,
\[ I(x, y) = \left( |U_i(x, y)| + |U_R|^2 \right)^2 \]

\[ = |U_i(x, y)|^2 + |U_R|^2 + 2|U_R||U_i(x, y)| \cdot \cos \left[ \left( \langle \omega \rangle (t - t_R) - \left( \langle k \rangle - k_R \right) \cdot r + \phi(x, y) \right) \right] . \tag{2.2} \]

In Eq. 2.2, \( \langle \omega \rangle \) is the mean frequency, \( \langle k \rangle \) the mean wavevector, and \( \phi \) is the phase shift of interest. For an arbitrary optical field, the frequency spread around \( \langle \omega \rangle \) defines temporal coherence and the wavevector spread around \( \langle k \rangle \) characterizes the spatial coherence of the field, as described earlier. We assume that the reference field can have both a delay, \( t_R \), and a different direction of propagation along \( k_R \). It can be seen that measurements at different delays \( t_R \) or at different points across the image plane, \( r \), can both provide enough information to extract \( \phi \).

Modulating the time delay is typically referred to as the phase shifting where three or more intensity patterns are recorded to extract \( \phi \). Using a tilted reference beam is commonly called the off-axis (or shear) method, from which phase information can be extracted from a single recorded intensity pattern. In some interferometric systems, the object and reference beams travel the same optical path; these are known as common path methods; furthermore, some systems use broadband white light as an illumination source and are known as white light methods. In practice, the phase shifting and off-axis methods are not normally used simultaneously; however, they are often implemented in a common path geometry or with white light illumination for better performance. Furthermore, phase information can also be retrieved through non-interferometric methods, for example, by recording a stack of defocused intensity images for solving the transport of intensity equation (TIE).

Like all instruments, QPI systems are characterized by certain parameters that quantify their performance. The main figures of merit are: acquisition rate, transverse resolution and phase sensitivity, both temporally and spatially.
**Temporal sampling: Acquisition Rate**

 Acquisition rate establishes the fastest phenomena that can be studied by a QPI method. According to the Nyquist sampling theorem (or Nyquist-Shannon theorem), the sampling frequency has to be at least twice the frequency of the signal of interest \[ [21, 22]. \] In QPI, the required acquisition rates vary broadly with the application, from 100s of Hz in the case of membrane fluctuations to 1/1000 Hz when studying the cell cycle. The acquisition rate of QPI systems depends on the modality used for phase retrieval. Off-axis interferometry gives the phase map from a single camera exposure and is thus the fastest. On the other hand, phase-shifting techniques are slower as they require at least 3 intensity images for each phase image and hence the overall acquisition rate is at best 3 times lower than that of the camera.

**Spatial sampling: Transverse Resolution**

 As in all imaging methods, it is desirable to preserve the diffraction limited resolution provided by the microscope \[ [23]. \] Defining a proper measure of transverse resolution in QPI is nontrivial and perhaps worth pursuing by theoretical researchers. Of course, such a definition must take into account that the coherent imaging system is not linear in phase (or in intensity), but in the complex field.

 Phase-shifting methods are more likely than off-axis methods to preserve the diffraction limited resolution of the instrument. In off-axis geometries, the issue is complicated by the additional length scale introduced by the spatial modulation frequency (i.e. the fringe period). Following the Nyquist sampling theorem, this frequency must be high enough to recover the maximum frequency allowed by the numerical aperture of the objective. Furthermore, the spatial filtering involving Fourier transformations back and forth has the detrimental effect of adding
noise to the reconstructed image. By contrast, in phase shifting, the phase image recovery involves only simple operations of summation and subtraction, which is overall less noisy.

**Temporal Stability: Temporal Phase Sensitivity**

Temporal stability is perhaps the most challenging feature to achieve in QPI. In studying dynamic phenomena by QPI, the question that often arises is: what is the smallest phase change that can be detected at a given point in the field of view? For instance, studying red blood cell membrane fluctuations requires a path length displacement sensitivity on the order of 1 nm, which translates roughly to a temporal phase sensitivity of 5-10 mrad, depending on the wavelength. In time-resolved interferometric experiments uncorrelated noise between the two fields of the interferometer always limits the temporal phase sensitivity; i.e. the resulting interference signal contains a random phase in the cross-term,

$$I(t) = |U_1|^2 + |U_2|^2 + 2|U_1||U_2| \cos[\phi(t) + \delta\phi(t)],$$

where $\phi$ is the phase under investigation and $\delta\phi(t)$ is the temporal phase noise. If $\delta\phi$ fluctuates randomly over the entire interval $(-\pi, \pi]$ during the time scales relevant to the measurement, the information about the quantity of interest, $\phi$, is completely lost, i.e. the last term in Eq. 2.3 averages to zero. Sources of phase noise include air fluctuations, mechanical vibrations of optical components, vibrations in the optical table, etc. In order to improve the stability of QPI systems, there are several approaches typically pursued:

i) **Passive stabilization** includes damping mechanical oscillations from the system (e.g. from the optical table), placing the interferometer in vacuum sealed enclosures, etc. To some extent, most QPI systems incorporate some degree of passive stabilization; floating the optical table is one such example. Unfortunately, these procedures are often insufficient to ensure sensitive phase measurements of biological relevance.
ii) **Active stabilization** involves the continuous cancellation of noise via a feedback loop and an active element (e.g. a piezoelectric transducer) that tunes the pathlength difference in the interferometer. This principle has been implemented in various geometries in the past with some success. Of course, such active stabilization drastically complicates the measurement by adding dedicated electronics and optical components.

iii) **Differential measurements** can also be used effectively to increase QPI sensitivity. The main idea is to perform two noisy measurements whereby the noise in the two signals is correlated and, thus, can be subtracted.

iv) **Common path interferometry** refers to QPI geometries where the two fields travel along paths that are physically very close. In this case, the noise in both fields is very similar and hence automatically cancels in the interference (cross) term.

**Spatial Uniformity: Spatial Phase Sensitivity**

Analog to the “frame-to-frame” phase noise discussed in the previous section, there is a “point-to-point” (spatial) phase noise that affects the QPI measurement. This spatial phase sensitivity limits the smallest topographic change that the QPI system can detect.

Unlike with temporal noise, there are no clear cut solutions to improve spatial sensitivity besides keeping the optics pristine and decreasing the coherence length of the illumination light. The spatial non-uniformities in the phase background are mainly due to the random interference pattern (i.e. speckle) produced by fields scattered from impurities on optics, specular reflections from the various surfaces in the system, etc. This spatial noise is worst in highly coherent sources, i.e. lasers. Using white light as illumination drastically reduces the effects of speckle while preserving the requirement of a coherence area that is at least as large as the field of view. In post-processing, sometimes subtracting the constant phase background (no sample QPI) helps.
The above discussion makes it apparent that there is no perfect QPI method, i.e. there is no technique that performs optimally with respect to all figures of merit identified in the last section. The off-axis methods are fast as they are single shot, phase-shifting preserves the diffraction-limited transverse resolution without special measures, common-path methods are stable and white light illumination suffers less from speckle and, thus, is more spatially uniform. However, as we will see in the following sections, by combining these four approaches, the respective individual benefits may be added together.

2.2 Diffraction Phase Microscopy

Diffraction phase microscopy (DPM) is a full-field common path interferometry technique introduced by Popescu et al, in 2006 [8]. Figure 2.1 shows a typical DPM setup. The illumination source may be selected depending on the application at hand, and any source is sufficient as long as it is spatially coherent. Since DPM is usually implemented as an add-on to an inverted microscope, the only additional components that are necessary are a relay lens, a diffraction grating, and a 4-f spatial filtering setup. The image plane from the microscope is projected onto the diffraction grating via the relay lens. This generates several diffraction orders in the Fourier plane, which is projected onto the spatial filter via lens L1. Lens L2 is the inverse Fourier lens in the 4-f system and projects the interferogram onto a CCD.
Figure 2.1 DPM schematic (a) FC, fiber collimator; M, mirror; S, sample; O, objective lens; TL, tube lens; G, grating; SF, spatial filter; L1 and L2, lenses. (b) Close-up of a DPM image of 4 RBCs.

The spatial filter allows passing the entire frequency content of the 1st diffraction order beam and blocks all the other orders. The 1st order is thus the imaging field and the 0th order plays the role of the reference field. The two beams traverse the same optical components, i.e. they propagate along a common optical path, thus significantly reducing the longitudinal phase noise. If the direction of the spatial modulation is chosen at an angle of 45° with respect to the x and y axes of the CCD, the total field at the CCD plane has the form

$$U(x, y) = |U_0| e^{i[\phi_0 + \beta(x, y)]} + |U_1(x, y)| e^{i\phi(x, y)}. \quad (2.4)$$

In Eq. 2.4, $|U_{0,1}|$ and $\phi_{0,1}$ are the amplitudes and the phase, respectively, of the orders of diffraction 0, 1, while $\beta$ represents the spatial frequency shift induced by the grating to the 0th order (i.e. the spatial frequency of the grating itself). Note that, as a consequence of the central ordinate theorem, the reference field is proportional to the spatial average of the microscope image field,

$$|U_0| e^{i\phi_0} \propto \frac{1}{A} \int |U(x, y)| e^{i\phi(x, y)} \, dx \, dy, \quad (2.5)$$
where $A$ is the total image area. The spatial average of an image field has been successfully used before as a stable reference for extracting spatially resolved phase information [9].

The CCD recording may be written as

$$I(x, y) = |U_1(x, y)|^2 + |U_2|^2 + 2|U_1(x, y)||U_2| \cos[qx + \phi(x, y)],$$

(2.6)

where $q$ is the spatial frequency shift from the diffraction grating. The interferogram is then spatially high-pass filtered to isolate the sinusoidal cross term. For phase objects, $U_1(x, y)$ is expected to have weak spatial dependence and thus the isolated term can be interpreted as the real part of a spatial complex analytic signal. The corresponding imaginary part can then be obtained via a Hilbert transform:

$$\sin[qx + \phi(x, y)] = P \cos\left[\frac{qx^2 + \phi(x', y)}{x - x'}\right],$$

(2.7)

where $P$ is the principle value integral. The phase may then be recovered as

$$\phi(x, y) + qx = \arg\left[\cos(qx + \phi), \sin(qx + \phi)\right].$$

(2.8)

The diffraction limited resolution of the system can be preserved through proper selection of the spatial modulation, $q$. As with any other signal, this must meet the requirements of the Nyquist theorem, that it is twice the highest resolvable frequency. Since this value $q$ is known from the period of the diffraction grating, it may simply be subtracted to retrieve $\phi(x, y)$. However since $qx$ can be much higher than $2\pi$, $\phi(x, y) + qx$, can be highly wrapped. An unwrapping algorithm is thus applied prior to subtracting $qx$. Such algorithms search for $2\pi$ jumps in the phase and correct them. In this manner the phase can be recovered from a single CCD recording.

Recently, DPM has been combined with epi-fluorescence microscopy in diffraction phase and fluorescence microscopy (DPF) to simultaneously image both the nanoscale structure and dynamics, and the specific functional information in live cells [24]. Further, confocal diffraction
phase microscopy (cDPM), has also been presented which provides quantitative phase measurements from localized sites on a sample with high sensitivity [25]. The ability of DPM to study live cells has been demonstrated on various systems such as whole blood measurements [26], imaging kidney cells in culture [24], Fresnel particle tracking [27], red blood cell mechanics [28], imaging of malaria-infected RBCs [29] etc. In Chapter 4 I describe the use of a DPM platform for quantitative cytometry of red blood cells.

2.3 Spatial Light Interference Microscopy

As discussed above, a large number of experimental setups have been developed for QPI however, the contrast in QPI images has always been limited by speckles resulting from the practice of using highly coherent light sources such as lasers. The spatial non-uniformity caused by speckles is due to random interference phenomena caused by the coherent superposition of various fields from the specimen and those scattered from optical surfaces, imperfections or dirt [30]. Since this superposition of fields is coherent only if the path length difference between the fields is less than the coherence length ($l_c$) of the light, it follows that if broadband light with a shorter coherence length is used, the speckle will be reduced. Due to this, the image quality of laser based QPI methods has never reached the level of white light techniques ($l_c \sim 1 \mu m$) such as phase contrast or DIC as discussed below.

To address this issue I participated in the development of a new QPI method called Spatial Light Interference Microscopy (SLIM) [31, 32]. SLIM combines two classical ideas in optics and microscopy: Zernike’s phase contrast method [5] for using the intrinsic contrast of transparent samples and Gabor’s holography to quantitatively retrieve the phase information [33]. SLIM thus provides the spatial uniformity associated with white light methods and the stability associated with common path interferometry. In fact, as described in greater detail
below, the spatial and temporal sensitivities of SLIM to optical path length changes have been measured to be 0.3 nm and 0.03 nm respectively. In addition, due to the short coherence length of the illumination, SLIM also provides excellent optical sectioning, enabling three-dimensional tomography [15].

In the laser based methods the physical definition of the phase shifts that are measured is relatively straightforward since the light source is highly monochromatic. However, for broadband illumination the meaning of the phase that is measured must be considered carefully. It was recently shown by Wolf [34] that if a broadband field is spatially coherent, the phase information that is measured is that of a monochromatic field which oscillates at the average frequency of the broadband spectrum. This concept is the key to interpreting the phase measured by SLIM. In this section I will first discuss the physical principles behind broadband phase measurements using SLIM, then the experimental implementation and finally various applications.

The idea that any arbitrary image may be described as an interference phenomenon was first proposed more than a century ago by Abbe in the context of microscopy: “The microscope image is the interference effect of a diffraction phenomenon” [1]. This idea served as the basis for both Zernike’s phase contrast [5] and is also the principle behind SLIM. The underlying concept here is that under spatially coherent illumination, the light passing through a sample may be decomposed into its spatial average (unscattered component) and its spatially varying (scattered component) as

\[ U(\mathbf{r}; \omega) = U_0(\omega) + U_1(\mathbf{r}; \omega) = |U_0(\omega)| e^{i \phi_0(\omega)} + |U_1(\mathbf{r}; \omega)| e^{i \phi_1(\mathbf{r}; \omega)}, \]

where \( \mathbf{r} = (x, y) \). In the Fourier plane (back focal plane) of the objective lens these two components are spatially separated, with the unscattered light being focused on-axis as shown
Fig. 2.2. In the spatial Fourier transform of the field $U$, $\vec{U}(q;\omega)$, it is apparent that average field $U_0$ is proportional to the DC component $\vec{U}(0;\omega)$. This is equivalent to saying that if the coherence area of the illuminating field is larger than the field of view of the image, the average field may be written as

$$U_0 = \langle U(x,y) \rangle = \frac{1}{A} \int U(x,y) dx dy.$$  

Thus the final image may be regarded as the interference between this DC component and the spatially varying component. Thus the final intensity that is measured may be written as:

$$I(x,y) = |U_0|^2 + |U_1(x,y)|^2 + 2|U_0||U_1(x,y)| \cos[\Delta \phi(x,y)],$$  

where $\Delta \phi$ is the phase difference between the two components. Since for thin transparent samples this phase difference is extremely small and since the Taylor expansion of the cosine term around 0 is quadratic, i.e. $\cos(\Delta \phi) \approx 1 - \frac{\Delta \phi^2}{2}$, the intensity distribution does not reveal much detail. Zernike realized that the spatial decomposition of the field in the Fourier plane allows one to modulate the phase and amplitude of the scattered and un-scattered components relative to each other. Thus he inserted a phase-shifting material in the back focal plane that adds a $\pi/2$ shift ($k=1$ in Fig. 2.2) to the un-scattered light relative to the scattered light, essentially converting the cosine to a sine which is rapidly varying around 0 ($\sin(\Delta \phi) \approx \Delta \phi$). Thus Zernike coupled the phase information into the intensity distribution and invented phase contrast microscopy. Phase contrast (PC) has revolutionized live cell microscopy and is widely used today; however, the quantitative phase information is still lost in the final intensity measurement. SLIM extends Zernike’s idea to provide this quantitative information.
As in PC microscopy, SLIM relies on the spatial decomposition of the image field into its scattered and un-scattered components and the concept of image formation as the interference between these two components. Thus in the space-frequency domain we may express the cross spectral density as [35, 36]:

\[
W_{01}(r; \omega) = \langle U_0(\omega) \cdot U_1^*(r; \omega) \rangle
\]  

(2.12)

where the * denotes complex conjugation and the angular brackets indicate an ensemble average. If the power spectrum \( S(\omega) = \langle |U_0(\omega)|^2 \rangle \) has a mean frequency \( \omega_0 \), we may factorize the cross spectral density as

\[
W_{01}(r; \omega - \omega_0) = |W_{01}(r; \omega - \omega_0)| e^{i[\Delta \phi(r; \omega - \omega_0)]}.
\]  

(2.13)

From the Wiener–Kintchen theorem [36], the temporal cross correlation function is related to the cross spectral density through a Fourier transform and can be expressed as

\[
\Gamma_{01}(r; \tau) = |\Gamma_{01}(r; \tau)| e^{i[\omega_0 \tau + \Delta \phi(r; \tau)]},
\]  

(2.14)

where \( \Delta \phi(r) = \phi_0 - \phi_1(r) \) is the spatially varying phase difference. It is evident from Eq. 2.14 that the phase may be retrieved by measuring the intensity at various time delays, \( \tau \). The retrieved phase is equivalent to that of monochromatic light at frequency \( \omega_0 \). This can be understood by calculating the auto-correlation function from the spectrum of the white-light source being used (Fig. 2.2 c-d). It can be seen in the plot of the autocorrelation function in Fig. 2.2 d that the white light does indeed behave as a monochromatic field oscillating at a mean frequency of \( \omega_0 \). Evidently, the coherence length is less than 2μm, which as expected is significantly shorter compared to quasi-monochromatic light sources such as lasers and LEDs. However, as can be
seen, within this coherence length there are several full cycle modulations, and in addition, the envelope is still flat near the central peak.

**Figure 2.2. Imaging as an interference effect.** (a) A simple schematic of a microscope is shown where L1 is the objective lens which generates a Fourier transform of the image field at its back focal plane. The un-scattered component of the field is focused on-axis and may be modulated by the phase modulator (PM). The tube lens L2 performs an inverse Fourier transform, projecting the image plane onto a CCD for measurement. (b) Spectrum of the white light emitted by a halogen lamp source, with center wavelength of 531.9 nm. (c) Resampled spectrum with respect to frequency. (d) Autocorrelation function (solid line) and its envelope (dotted line). The four circles correspond to the phase shifts that are produced by the PM in SLIM.

When the delay between $U_0$ and $U_1$ is varied, the interference is obtained simultaneously at each pixel of the CCD; thus, the CCD may be considered as an array of interferometers. The average field, $U_0$, is constant over the field of view and serves as a common reference for each pixel. It is also important to note that $U_0$ and $U_1$ share a common optical path, thus minimizing any noise in the phase measurement due to vibrations. The intensity at the image plane may be expressed as a function of the time delay as:
\[ I(\mathbf{r}; \tau) = I_0 + I_1(\mathbf{r}) + 2|\Gamma_{01}(\mathbf{r}; \tau)|\cos[\omega \tau + \Delta \phi(\mathbf{r})], \quad (2.15) \]

In SLIM, to quantitatively retrieve the phase the time delay is varied to get phase delays of \(-\pi, \pi/2, 0\) and \(\pi/2\) (\(\omega \tau_k = k\pi / 2\), \(k = 0, 1, 2, 3\)) as illustrated in Fig. 2.2d. An intensity map is recorded at each delay and may be combined as:

\[ I(\mathbf{r}; 0) - I(\mathbf{r}; -\pi) = 2\left[\hat{\Gamma}(0) + \hat{\Gamma}(-\pi)\right]\cos[\Delta \phi(\mathbf{r})] \quad (2.16) \]

\[ I(\mathbf{r}; \frac{\pi}{2}) - I(\mathbf{r}; -\frac{\pi}{2}) = 2\left[\hat{\Gamma}\left(-\frac{\pi}{2}\right) + \hat{\Gamma}\left(\frac{\pi}{2}\right)\right]\sin[\Delta \phi(\mathbf{r})]. \quad (2.17) \]

For time delays around 0 that are comparable to the optical period, \(|\Gamma|\) can be assumed to vary slowly at each point as shown in Fig. 2.2d. Thus for cases where the relationship \(\hat{\Gamma}(0) + \hat{\Gamma}(-\pi) = \hat{\Gamma}\left(-\frac{\pi}{2}\right) + \hat{\Gamma}\left(\frac{\pi}{2}\right)\) holds true the spatially varying phase component may be expressed as:

\[ \Delta \phi(\mathbf{r}) = \arg\left[\frac{I(\mathbf{r}; -\pi / 2) - I(\mathbf{r}; \pi / 2)}{I(\mathbf{r}; 0) - I(\mathbf{r}; -\pi)}\right]. \quad (2.18) \]

Letting \(\beta(\mathbf{r}) = |U_1(\mathbf{r})|/|U_0(\mathbf{r})|\), the phase associated with the image field is determined as:

\[ \phi(\mathbf{r}) = \arg\left[\frac{\beta(\mathbf{r})\sin(\Delta \phi(\mathbf{r}))}{1 + \beta(\mathbf{r})\cos(\Delta \phi(\mathbf{r}))}\right]. \quad (2.19) \]

Thus by measuring 4 intensity maps the quantitative phase map may be uniquely determined.

Next we will discuss the experimental implementation of SLIM and its performance.
A schematic of the SLIM setup is shown in Fig. 2.3a. SLIM is designed as an add-on module to a commercial phase contrast microscope (more details about the SLIM design and peripheral accessories can be found in Appendix B). In order to match the illumination ring with the aperture of the spatial light modulator (SLM), the intermediate image is relayed by a 4f system (L1 and L2). The polarizer P ensures the SLM is operating in a phase modulation only mode. The lenses L3 and L4 form another 4f system. The SLM is placed in the Fourier plane of this system which is conjugate to the back focal plane of the objective which contains the phase contrast ring. The active pattern on the SLM is modulated to precisely match the size and position of the phase contrast ring such that the phase delay between the scattered and unscattered components may be controlled as discussed above.

To determine the relationship between the 8-bit VGA signal that is sent to the SLM and the imparted phase delay, it is first necessary to calibrate the liquid crystal array as follows. The SLM is first placed between two polarizers which are adjusted to be 45° to the SLM axis such that it operates in amplitude modulation mode. Once in this configuration the 8-bit grayscale signal sent to the SLM is modulated from a value of 0 to 127 (the response from 128 to 255 is symmetric). The intensity reflected by the SLM is then plotted vs. the grayscale value as shown in Fig. 2.3b. The phase response is calculated from the amplitude response via a Hilbert transform (Fig. 2.3c). From this phase response the 3 phase shifts necessary for quantitative phase reconstruction may be obtained as shown in Fig. 2.3d. Finally a quantitative phase map image may be determined as described above.

Figure 2.3e shows a quantitative phase measurement of a cultured hippocampal neuron; the color indicates the optical path in nanometers at each pixel. The measured phase can be approximated as
\[
\phi(x, y) = k_0 \int_0^{h(x,y)} \left[ n(x, y, z) - n_0 \right] dz
= k_0 \Delta n(x, y) h(x, y),
\]

(2.20)

where \( k_0 = 2\pi/\lambda \), \( n(x, y, z) - n_0 \) is the local refractive index contrast between the cell and the surrounding culture medium, \( \Delta n(x, y) = \frac{1}{h(x, y)} \int_0^{h(x,y)} \left[ n(x, y, z) - n_0 \right] dz \), the axially-averaged refractive index contrast, \( h(x,y) \) the local thickness of the cell, and \( \lambda \) the mean wavelength of the illumination light. The typical irradiance at the sample plane is \( \sim 1 \) nW/\( \mu m^2 \). The exposure time is typically 1-50 ms, which is 6-7 orders of magnitude less than that of confocal microscopy [37], and thus there is very limited damage due to phototoxic effects. In the original SLIM system the phase modulator has a maximum refresh rate of 60 Hz and the camera has a maximum acquisition rate of 11 Hz; due to this the maximum rate for SLIM imaging was 2.7 Hz. Of course this is only a practical limitation as both faster phase modulators and cameras are available commercially.
Figure 2.3 Experimental setup. (a) The SLIM module is attached to a commercial phase contrast microscope (AxioObserver Z1, Zeiss). The first 4-f system (lenses L1 and L2) expands the field of view to maintain the resolution of the microscope. The polarizer, P, is used to align the polarization of the field with the slow axis of the Spatial Light Modulator (SLM). Lens L3 projects the back focal plane of the objective, containing the phase ring onto the SLM which is used to impart phase shifts of 0, π/2, π and 3π/2 to the un-scattered light relative to the scattered light as shown in the inset. Lens L4 then projects the image plane onto the CCD for measurement. (b) Intensity modulation obtained by displaying different grayscale values on the SLM. (c) Phase modulation vs. grayscale value obtained by a Hilbert transform on the data in b. (d) The 4 phase rings and their corresponding images recorded by a CCD. (e) Reconstructed quantitative phase image of a hippocampal neuron, the color bar indicates the optical path length in nanometers.

To quantify the spatiotemporal sensitivity of SLIM a series of 256 images with a field of view of 10 x 10 µm² were acquired with no sample in place. Figure 2.4a shows the spatial and temporal histograms associated with this data. The spatial and temporal sensitivities were
measured to be 0.28 nm and 0.029 nm respectively. Figure 2.4b-c compares SLIM images with those acquired using a diffraction phase microscope (DPM) [8] that was interfaced with the same commercial microscope. The advantages provided by the broadband illumination are clear as the SLIM background image has no structure or speckle as compared to those acquired by DPM.

![Figure 2.4 SLIM sensitivity](image)

**Figure 2.4. SLIM sensitivity.** (a) Spatial and temporal optical path length noise level, solid lines indicate Gaussian fits. (b) Topographic noise in SLIM. (c) Topographic noise in DPM a laser based method. The color bar is in nanometers.

### 2.4 Discussion

I anticipate that QPI technologies will become a dominant field in biomedical optics in the years to come. Clearly, the methods have come a long way and have exciting potential for enabling new biological studies with the required resolution, repeatability, and compatibility with existing techniques. QPI provides sensitivity to spatial and temporal pathlength changes down to the *nanoscale*. This has been exploited, for example, in studies of red blood cell fluctuations and topography of nanostructures. However this *sensitivity* should not be referred to
as axial *resolution*. Nanometer resolution or resolving power would describe the ability of QPI to resolve two objects separated axially by 1 nm. Of course, this is impossible, due to the uncertainty principle.

As discussed in Chapter 3, the phase information allows us to interpret the data as an image or scattering map, depending on whether we are interested in keeping the spatial information, and average the angular scattering information or vice versa. Most importantly, the quantitative phase image represents a density map, whose behavior in space and time can be analyzed and understood quantitatively using physical models. Whether a morphological feature can report on tissue cancer or a dynamic behavior teaches us about cell transport, QPI is a new powerful approach to biomedicine. In Chapter 3 I discuss how QPI data can be analyzed and interpreted to provide such biological insights. In the years to come, I believe that QPI can become a significant tool in the current transition of biology from empirical to quantitative science.
CHAPTER 3. ANALYSIS OF QUANTITATIVE PHASE DATA *

As in all QPI techniques the phase information measured by SLIM and DPM is proportional to the refractive index times the thickness of the sample (see Eq. 2.20). Due to the coupling of these two variables the natural choices for applying a QPI instrument are in situations where either the refractive index (topography) or the thickness (refractometry) is known [14]. When these parameters are measured dynamically, they can be used to measure membrane or density fluctuations, providing mechanical information on cellular structures [38]. Moreover, it was realized soon after the conception of quantitative phase microscopy, that the integrated phase shift through a cell is proportional to its dry mass (non-aqueous content) [39, 40], which enables studying cell mass growth [13, 41] and mass transport [38, 42] in living cells. Furthermore, when the low-coherence illumination is combined with a high numerical aperture, objective SLIM provides excellent depth sectioning. When this capability is combined with a linear forward model of the instrument, it can be used to perform three-dimensional tomography on living cells [15] with sub-micron resolution. Also, QPI data may be used to generated highly sensitive scattering measurements through a simple Fourier transform [43-47]. Thus the current major applications of SLIM may be broken down into five basic categories: refractometry, topography, dry mass measurement, tomography and scattering. In addition to basic science applications, SLIM has also been applied to clinical applications such as blood screening [48] and cancer diagnosis [49].

Since SLIM is coupled to a commercial phase contrast microscope that is equipped with complete environmental control (heating, CO₂, humidity), it is possible to perform long term live cell imaging. In fact with SLIM measurements of up to a week have been performed [13]. Due to

* Portions of this chapter are reproduced from a review article: M. Mir, B. Bhaduri, R. Wang, R. Zhu and G. Popescu, “Quantitative Phase Imaging”, Progress in Optics, Chennai, B.V.: (2012), pp. 133-217. This material is reproduced with the permission of the publisher and is available using the ISBN: 978-0-444-59422-8.
the coupling with the commercial microscope, it is also possible to utilize all other commonly used modalities such as fluorescence simultaneously. Fluorescence imaging can be used to add specificity to SLIM measurements such as for identifying the stage of a cell cycle or the identity of an observed structure. Furthermore, since it is possible to resolve sub-cellular structure with high resolution, the inter- and intra- cellular transport of dry mass may also be quantified. Using mosaic style imaging, it is also possible to image entire slides with sub-micron resolution imaging by tiling and stitching adjacent fields of view. Thus SLIM may be used to study phenomena on time scales ranging from milliseconds to days and spatial scales ranging from sub-micron to millimeters. In my work I have leveraged all the capabilities discussed above. In the chapter I will describe in greater detail the analysis methods that I have used to interpret QPI data and provide physical meaning.

3.1 Topography and Refractometry

To assess the accuracy of topographic SLIM measurements, an amorphous carbon film (of known refractive index) was imaged using both SLIM and an atomic force microscope as shown in Fig 3.1. It can be seen that the two measurements agree within a fraction of a nanometer (Fig. 3.1a). It is important to note that both SLIM and AFM are characterized by smaller errors than indicated by the widths of the histogram modes, which reflect irregularities in surface profile due to errors in the fabrication process. Unlike AFM, SLIM is non-contact and parallel and more than 3 orders of magnitude faster. AFM can measure a 10 x 10 \( \mu m^2 \) field of view in 21 minutes whereas SLIM can optically measure a 75 x 100 \( \mu m^2 \) area in 0.5 seconds. Of course, unlike AFM, SLIM provides nanoscale accuracy in topographic measurements but still has the diffraction limited transverse resolution associated with the optical microscope.
Figure 3.1. Comparison between SLIM and AFM. (a) Topographical histograms for SLIM and AFM. (b) SLIM image of an amorphous carbon film. (c) AFM image of the same sample.

Having established the nano-scale sensitivity and accuracy of SLIM, its topographic capabilities were further tested through measurements on graphene flakes [14] where it is necessary to resolve single atomic layers. Graphene is a two-dimensional lattice of hexagonally arranged and $sp^2$-bonded carbon atoms. The graphene sample was obtained by mechanically exfoliating a natural graphite crystal using adhesive which was then deposited on a glass slide. This process results in both single-layer and multi-layer flakes being deposited on the slide with later dimensions on the order of tens of microns. Figure 3.2a shows the SLIM image of such a graphene flake. It can be qualitatively deduced from this image that the background noise is below the signal from the sample. To perform topographic measurements, the height at each pixel is calculated using Eq. 2.20 and inputting the known refractive index of graphite, $n=2.6$. Figure 3.2c shows the histograms of the height information. It can be seen in the overall histogram that there are local maxima in the distribution at heights of 0 nm (background), 0.55 nm, 1.1 nm and 1.6 nm, indicating that the sample has a staircase profile in increments of 0.55 nm. These values are comparable to the reported thickness of individual atomic layers of graphene measured using AFM in air (~ 1nm) or with a scanning tunneling microscope (STM,
0.4 nm) in ultra-high vacuum. The difference between the AFM and STM measurements is likely due to the presence of ambient species (nitrogen, oxygen, water, organic molecules) on the graphene sheet. From these results it can be concluded that SLIM is capable of measuring single atomic layers, with topographic accuracy comparable to AFM with a much faster acquisition time and in a non-contact manner.

Figure 3.2. Topography and Refractometry (a) SLIM image of a graphene flake. (b) Topographic histograms of the regions indicated in (a). (c) Tube structure with refractive index and thickness of layers shown. (d) Histogram of the refractive index contrast, n− 1, of the selected area in the inset. Inset, distribution of refractive index contrast, n− 1.

The refractometry capabilities of SLIM were demonstrated through measurements on semi-conductor nanotubes (SNT) [14]. SNTs are an emerging nanotechnology building block that are formed by the self-rolling of residually strained thin films that are grown epitaxially and defined lithographically. Since the nanotubes have a known cylindrical geometry, it is possible to deduce the thickness of the tubes from the projected width which is directly measurable in the image. Assuming that the thickness and the width are equal, it is possible to extract the average
refractive index of the tube using Eq. 2.20. The expected value of the refracted index was calculated by averaging the refractive indices of the layered structure shown in Fig. 3.2c. The measured values shown in Fig. 3.2c agree very well with the expected values ($\Delta n_{\text{measured}}=0.093$, $\Delta n_{\text{expected}}=0.087$). The fluctuations observed in the refractive index are most likely due to physical inhomogeneities in the tube itself. Thus SLIM provides a way to do high throughput refractometry on nanofabricated structures. A similar procedure was also demonstrated for measuring the refractive index of neural processes which are also cylindrical [14].

3.2 Dry Mass Measurements

Along with differentiation and morphogenesis, cell growth is one of the fundamental processes of developmental biology [50]. Due to its fundamental importance and the practical difficulties involved measuring cell growth, the question of how cells regulate and coordinate their growth has been described as “one of the last big unsolved problems in cell biology”[51]. The reason that this measurement has been elusive despite decades of effort is simply that cells are small, weighing in on the order of pictograms, and they only double their size during their lifecycle. For this reason, the accuracy required to answer basic questions such as whether the growth is exponential or linear is on the order of femtograms [52].

The traditional approach for measuring cell growth is to use a Coulter counter to measure the volume distribution of a large population of cells and perform careful statistical analysis to deduce the behavior of single cells [52]. This type of analysis does not provide single cell information and does not permit cell cycle studies without synchronizing the population using techniques that may alter the behavior. For cells with regular shapes such as *E. coli* and other relatively simple cells, traditional microscopy techniques have also been used to study size parameters such as projected area and length in great detail [53]. However, this approach
assumes that the cell density remains constant, such that the size is analogous to the mass which is not always true as the size may change disproportionately to mass due to osmotic responses [41]. More recently several novel microelectromechanical (MEMS) devices have been developed to essentially weigh single cells by measuring the shift in the resonant frequency of micro-scale structures as cells interact with them [54-56]. Although, these devices are impressive in terms of throughput, they are limited to either measuring a large number of cells without the ability for single cell analysis or measuring only one cell at a time. It is well recognized that the ideal approach should have the capability to measure single cells and their progeny, be non-invasive and provide information at both the cell and population level with the required sensitivity.

Quantitative phase measurements are thus a natural choice to study cell growth. In fact it was realized in the 1950s, soon after the invention of phase contrast microscopy, that the integrated phase shift through a cell is linearly proportional to its dry mass [39, 40]. This may be understood by expressing the refractive index of a cell as:

\[ n_c(x, y) = n_0 + \beta C(x, y) \]  \hspace{1cm} (3.1)

where \( \beta \) (ml/g) is known as the refractive increment, which relates the change in concentration of protein, \( C \) (g/ml), to the change in refractive index. Here \( n_0 \) is the refractive index of surrounding cytoplasm. According to intuition an uncertainty arises in determining the refractive increment method when considering the heterogeneous and complex intracellular environment. However, measurements indicate that this value varies less than 5% across a wide range of common biological molecules [39, 40]. It was also recently shown using Fourier phase microscopy [9] that the surface integral of the phase map is invariant to small osmotic changes, which establishes the validity of using QPI techniques for cell dry mass measurements. Using Eq. 3.1 the dry mass surface density at each pixel of a quantitative phase image is calculated as:
This method of measuring cellular dry mass has been used by several groups over the past half century [57-59]; however, until the development of SLIM, QPI instruments have generally been limited in their sensitivity and stability as described in detail earlier. Specifically, SLIM’s path length sensitivities of 0.3 nm spatially and 0.03 nm temporally translate to temporal dry mass sensitivities of 1.5 fg/µm² and 0.15 fg/µm² respectively. Thus SLIM finally enabled the optical measurement of cell growth with the required sensitivity. I recently demonstrated these capabilities through measurements on both *Escherichia coli* (*E. coli*) cells and a mammalian human osteosarcoma cell line [13] as discussed in Chapter 4. Furthermore this idea was leveraged to create a cancer cell proliferation assay as described in Chapter 5 and to study neural network formation as described in Chapter 6.

### 3.3 Fourier Transform Light Scattering (FTLS)

Perhaps one of the most striking features of QPI is that it can generate *light scattering* data with extreme sensitivity. This happens because full knowledge of the complex (i.e., amplitude and phase) field at a given plane (the image plane) allows us to infer the field distribution at any other plane, including in the far zone. In other words, the image and scattering fields are simply Fourier transforms of each other; this relationship does not hold in intensity. This approach, called Fourier transform light scattering (FTLS) [43-47] is much more sensitive than common, goniometer-based angular scattering because the measurement takes place at the image plane, where the optical field is most uniform. As a result, FTLS can render with ease scattering properties of minute subcellular structures, which is an unprecedented capability.

When measuring mostly transparent, optically thin samples, we can assume that the amplitude of the optical field is left unperturbed and that only the phase measured by SLIM is
altered by the sample. In this case, SLIM provides a measure of the complex optical field, \( U(\mathbf{r}, t) = |U(\mathbf{r}, t)| e^{i\phi(\mathbf{r}, t)} \), at the sample plane. This field may then be numerically propagated to the far-field or scattering plane by simply calculating its spatial Fourier transform as 
\[
\hat{U}(\mathbf{q}) = \int U(\mathbf{r}) e^{-i\mathbf{q} \cdot \mathbf{r}} d^2 \mathbf{r},
\]
where \( \mathbf{q} \) is the scattering wave vector. The modulus square of this function, \( P(\mathbf{q}) = |\hat{U}(\mathbf{q})|^2 \), is related to the spatial auto-correlation of the measured complex field through a Fourier transform, 
\[
\int P(\mathbf{q}) e^{i\mathbf{q} \cdot \mathbf{r}} d^2 \mathbf{q} = \int U(\mathbf{r}') U(\mathbf{r} - \mathbf{r}') d^2 \mathbf{r}',
\]
and thus describes the spatial correlation of the scattering particles in the sample. Since the signal is measured and reconstructed in the image plane, rather than in the far field as in traditional scattering experiments, all the scattering angles that are allowed by the numerical aperture of the microscope objective are measured simultaneously. This greatly enhances the sensitivity to scattering compared to the traditional approach of goniometric measurements.

Fourier transform light scattering has already been used for the purposes of studying scattering from entire organs, cancer diagnosis and prognosis and scattering from single cells [43-47]. In Chapter 7, I show how FTLS can be used to measure spatial organization in developing neural networks.

### 3.4 Dispersion Analysis: Membrane Fluctuations and Mass Transport

In addition to simply growing, single cells must also organize and transport mass in forms ranging from single molecules to large complexes in order to achieve their functions. Cells rely on both passive (diffusive) and active (directed) transport to accomplish this task. Active transport, typically over long spatial, scales is accomplished using molecular motors, which have been tracked and measured previously using single molecule fluorescence techniques (e.g., see [60]). Establishing a more complete view of the spatial and temporal distribution of mass
transport in living cells remains a challenging problem; addressing this problem requires measuring the microscopic spatiotemporal heterogeneity inside the cells. This has been addressed in the past by both active and passive particle tracking [61, 62]. Recently it was shown that this may also be accomplished using QPI techniques by measurements taken on living cells using SLIM [38, 42].

If measured over time the changes in path-length that are measured by SLIM can be expressed to the first order as:

\[
\Delta s(r,t) = s(r,t) - \langle s(r,t) \rangle_{r,t}
\]

\[
= \left[ \langle h(r,t) \rangle_{r,t} + \Delta h(r,t) \right] \left[ \langle n(r,t) \rangle_{r,t} + \Delta n(r,t) \right] - \langle h(r,t) \rangle_{r,t} \langle n(r,t) \rangle_{r,t}
\]

\[
\approx \langle n(r,t) \rangle_{r,t} \Delta h(r,t) + \langle h(r,t) \rangle_{r,t} \Delta n(r,t),
\]

where \(s(r,t)\) is the optical path length, \(r=(x,y)\), \(h\) is the local thickness and \(n\) is the local refractive index contrast. As can be seen in Eq. 2.15 the fluctuations in the path length contain information about out-of-plane fluctuations in the thickness and in plane fluctuations in the refractive index. The out-of-plane fluctuations have previously been extensively measured in the context of red blood cell membrane fluctuations using QPI [28, 63], which typically occur at fast temporal frequencies. The in-plane fluctuations correspond to intracellular mass transport. Separating the membrane fluctuations and mass transport components from \(\Delta s\) can be performed by ensuring that the image acquisition rate is lower than the decay rates associated with the bending and tension modes of membrane fluctuations.

As discussed in the section on growth above, the SLIM image may be regarded as a 2D dry mass density map and thus the changes in this map satisfy an advection-diffusion equation that includes contributions from both directed and diffusive transport [64]:

\[
D \nabla^2 \rho(r,t) - \mathbf{v} \cdot \nabla \rho(r,t) - \frac{\partial}{\partial t} \rho(r,t) = 0.
\]
where \( D \) is the diffusion coefficient, \( v \) is the advection velocity and \( \rho \) is the dry mass density.

The spatiotemporal autocorrelation function of the density can be calculated as:

\[
g(\mathbf{r}', \tau) = \langle \rho(\mathbf{r}, t) \rho(\mathbf{r} + \mathbf{r}', t + \tau) \rangle_{t, t'} .
\]

(3.5)

Taking a spatial Fourier transform of Eq. 3.5 the temporal autocorrelation may be expressed for each spatial mode, \( q \), as:

\[
g(q, \tau) = e^{iq \cdot v \cdot \tau - Dq^2 \tau}
\]

(3.6)

thus relating the measuring temporal autocorrelation function to the diffusion coefficient and velocity of matter. This is the same autocorrelation function that can be measured in dynamic light scattering at a fixed angle. In SLIM the entire forward scattering half space is measured simultaneously, limited only by the numerical aperture of the objective. Thus SLIM essentially functions as a highly sensitive light scattering measurement instrument.

The measured data is averaged over a range of advection velocities so Eq. 3.6 must be averaged as:

\[
g(q, \tau) = \left( e^{iq \cdot v \cdot \tau - Dq^2 \tau} \right) \left( e^{-Dq^2 \tau} \int P(|v - v_0|) e^{iq \cdot v \cdot \tau} d^2v \right) .
\]

(3.7)

Since the maximum speeds of molecular motors are approximately 0.8 \( \mu \text{m/s} \) and since there is transport over a large range of directions, the average velocity that is measured must be significantly lower than this value. Hence, it was proposed that the probability distribution, \( P \), of local advection velocities is a Lorentzian of width \( \Delta v \) and that the mean advection velocity averaged over the scattering value is much smaller, \( v_0 \ll \Delta v \). Thus, Eq. 3.7 may be evaluated as

\[
g(q, \tau) = e^{iq \cdot v_0 \cdot \tau} e^{-q \cdot \Delta v \cdot \tau - Dq^2 \tau}
\]

(3.8)
The mean velocity produces a frequency modulation \( \omega(q) = \mathbf{v}_0 \cdot \mathbf{q} \) to the temporal autocorrelation, which decays exponentially at a rate

\[
\Gamma(q) = \Delta v q + D q^2.
\]  
(3.9)

Equation 3.9 is the dispersion relationship which gives the technique its name of Dispersion Phase Spectroscopy (DPS). Thus from a 3D \((x,y,t)\) SLIM dataset, the dispersion relationship \(\Gamma(q_x, q_y)\) may be calculated by first performing a spatial Fourier transform of each frame and then by calculating the temporal bandwidth at each spatial frequency by performing a temporal Fourier transform. The radial function, \(\Gamma(q)\), where \(q = \sqrt{q_x^2 + q_y^2}\), is obtained by an azimuthal average of the data.

To verify this approach SLIM was used to image the Brownian motion of 1 µm polystyrene sphere in a 99% glycerol solution (Fig. 3.3 a). Images were acquired for 10 minutes at a rate of 1 Hz. The diffusion coefficient was first determined by conventional particle tracking (Fig. 3.3 b) and then using DPS (Fig. 3.3 c-d) with excellent agreement. The DPS approach is significantly faster as it does not require tracking individual particles and also applies to particles which are smaller than the diffraction spot of the microscopy. In addition, in the case of living cells where there are usually no intrinsic particles available for tracking, DPS provides a simpler alternative than adding extrinsic particles to the cells. Using DPS several cell types have been measured including neurons, and glial and microglial cells [42, 64]. Figure 3.3 e-f shows such a measurement on a microglial cell. The dispersion curve shown in Fig. 3.3 f is associated with a narrow strip whose long axis is oriented radially with respect to the cell’s nucleus (white box). It can be seen that the transport is diffusive below spatial scales of 2 µm and directed above. The findings suggest that both diffusion and the advection velocities are inhomogeneous and anisotropic.
DPS thus provides the ability to quantify mass transport in continuous and transparent systems in a label-free manner. Experiments on live cells using this method have shown that the transport is diffusive at scales below a micron and deterministic at larger scales as expected from current knowledge about biology. Since DPS uses SLIM to acquire the phase maps, the total dry mass of the cell and other information such as fluorescence may be acquired simultaneously.

**Figure 3.3 Dispersion Phase Spectroscopy** (a) Quantitative phase image of 1μm polystyrene beads in glycerol. Colorbar indicates pathlength in nm. (b) Mean squared displacements (MSD) obtained by tracking individual beads in a. The inset illustrates the trajectory of a single bead. (c) Decay rate vs. spatial mode, Γ(q), associated with the beads in a. The dash ring indicates the maximum q values allowed by the resolution limit of the microscope. (d) Azimuthal average of data in (c) to yield Γ(q). The fit with the quadratic function yields the value of the diffusion coefficient as indicated. (e) SLIM image of a microglial cell. (f) Dispersion curves, Γ(q), associated with the white box regions in (e). The corresponding fits and resulting D and Δv values are indicated. The green and red lines indicate directed motion and diffusion, respectively, with the results of the fit as indicated in the legend.

### 3.5 Spatial Light Interference Tomography (SLIT)

In addition to rendering high resolution 2D quantitative phase maps, SLIM has the ability to provide optical sectioning, providing a pathway to 3D tomographic measurements [15]. This sectioning capability is inherent in SLIM due to main factors. First, there is coherence gating due
to the short coherence length (~ 1.2 µm) of the white-light illumination. If the coherence length is shorter that the optical path difference between two scattering particles, the interference term between the scattered and unscattered light disappears, thus providing sectioning. Second, using a high numerical aperture objective in conjunction with SLIM provides depth-of-focus gating. Since in SLIM the two interfering fields are inherently overlapped, so are the two optical gates.

Recently it was shown that it is possible to render three-dimensional refractive index maps from SLIM 3D images using a linear forward model based on the first order Born approximation. This technique has appropriately been dubbed Spatial Light Interference Tomography (SLIT) [15]. The scattering model was formulated by first considering a plane wave incident on a specimen which becomes a source for a secondary field. That is, the fields that are scattered by every point in the sample propagate as spherical waves and interfere with the un-scattered plane wave. The microscope objective may simply be considered as a band-pass filter in the wave vector (k) space. Thus at each of the optical frequencies the 3D field distribution may be measured by SLIM via depth scanning; the measured field may considered as a convolution between the susceptibility of the specimen $\chi$ and the point spread function, $P$, of the microscope

$$U(\mathbf{r}) = \iiint \chi(\mathbf{r}') P(\mathbf{r} - \mathbf{r}') d^3 \mathbf{r},$$

(3.10)

where $\chi(\mathbf{r}) = n^2(\mathbf{r}) - 1$, the spatial component of the susceptibility is the quantity of interest. In the frequency domain Eq. 3.10 may be written as

$$U(\mathbf{q}) = \chi(\mathbf{q}) P(\mathbf{q}).$$

(3.11)

Thus in the frequency domain, the susceptibility may be simply obtained as the ratio of the measured field and the PSF. For SLIT the point spread function was determined experimentally by imaging microspheres with diameters less than a third of the diffraction spot. Using the
measured PSF it is then possible to perform 3D tomography on various transparent samples, rendering inhomogeneous refractive index maps. Figure 3.4 (from [15]) provides an example of SLIT measurements on a living neuron which show excellent agreement with previous results. Figure 3.4 c-d provides a comparison of the 3D rendering provided by SLIM and a fluorescence confocal microscope. It can be seen that qualitatively the morphology of the two images is very close. The higher resolution provided by the confocal microscope is due to the fact that a higher NA objective was used for those measurements; furthermore, for confocal microscopy the neuron had to be stained and fixed. In contrast, SLIT is label-free and can non-invasively image living cells. In the next section I will discuss how super-resolution 3D tomography can be accomplished using sparse deconvolution algorithms with SLIT.

Figure 3.4. Tomography capability. (a)-(b) Refractive index distribution through a live neuron at position $z = 0.4 \, \mu m$ (a) and $6.0 \, \mu m$ (b). The soma and nucleolus (arrow) are clearly visible. Scale bars, $10 \, \mu m$. (c) 3D rendering of the same cell. The field of view is $100 \, \mu m \times 75 \, \mu m \times 14 \, \mu m$ and NA = 0.75. (d) confocal microscopy of a stained neuron with same field of view and NA = 1.2. Neurons were labeled with anti-polysialic acid IgG #735. The 3D rendering in (c) and (d) was done by ImageJ 3D viewer.
3.6 Visualizing Subcellular Structure using Deconvolved Spatial Light Interference Tomography (dSLIT)

Over the past two decades, fluorescence microscopy has enabled a number of super-resolution technologies, including Stimulated Emission Depletion microscopy (STED) [65], Stochastic Optical Reconstruction Microscopy (STORM) [66], (Fluorescence) Photo-Activated Localization Microscopy, (f)PALM [67, 68], Spatially Structured Illumination Microscopy (SSIM) [69], etc., collectively referred to as far-field nanoscopy techniques (for a review, see Ref. [70]). Impressively, these methods provide a transverse resolution of 20-30 nm. Abbe’s resolution limit is overcome by taking advantage of the nonlinear properties (e.g., saturation, switching) of the engineered fluorophores. Since these methods require scanning or many frames to reconstruct the final image, they are often limited by severe tradeoffs between acquisition time and field of view. More importantly, the nonlinear light-specimen interactions require a high level of illumination intensity, which in turn adds limitations due to photodamage and photobleaching.

In recent years it has become increasingly clear that to truly understand cellular function, it is necessary to image with high resolution in three dimensions; many of the fluorescence techniques mentioned above have also been adapted to work in 3D. Confocal microscopy is the most commonly used technique for 3D imaging and provides an axial resolution of approximately 500 nm [37]. 4Pi microscopy yields an axial resolution of 90 nm [71], while, more recently, 3D-STORM provides 50 – 60 nm resolution [72]. Another approach for 3D reconstruction is deconvolution microscopy, in which the blurring of the fluorescence image due to

* This section appeared in its entirety (with some modifications) in M. Mir, S.D. Babacan, M. Bednarz, M. N. Do, I. Golding and G. Popescu, Visualizing Escherichia coli Sub-Cellular Structure using Sparse Deconvolution Spatial Light Interference Tomography, PLoS One, 7, (6), e38916 (2012). This material is reproduced with the permission of the publisher.
diffraction is treated as a linear problem and reversed numerically. Note that, of course, only the amplitude (intensity) of the field is measured in all these methods.

As discussed in Section 3.5, if instead of just measuring intensity, the complex field (i.e., phase and amplitude) is measured, the 3D reconstruction of the specimen structure can be obtained without the need for exogenous contrast agents. In addition to SLIT [15], advances in QPI methods have enabled three-dimensional optical tomography of transparent biological samples using Radon transform based algorithms that were originally developed for X-ray computed imaging [73-75]. QPI based projection tomography has also been demonstrated on live cells with several approaches demonstrating high resolution [76-81].

Despite the advantages provided by QPI methods, their resolution is still diffraction limited [82]. Such degradations are common to all optical instruments and may be reduced to an extent through post-processing methods such as deconvolution. Deconvolution works by inverting the optical transfer function of the instrument and has been widely used in intensity based techniques [83, 84]. However, such methods have not been investigated thoroughly on complex fields measured by QPI instruments. Previous work [85] suggests that the noise-amplification that is commonly encountered when applying deconvolution to intensity images is not significant when they are applied to complex field measurements. The high SNR phase measurements obtained by SLIM provide a far more accurate modeling of the convolution with the PSF of the optical system in the complex fields, compared to the approximate convolution model typically used for intensity based methods. So far two novel deconvolution methods have been developed for SLIM. First, a non-linear method [86] was developed that estimates the unknown amplitude and phase through a combination of variable projection and quadratic regularization on the phase. The second method, called dSLIM [87], is based on modeling the
image using sparsity principles. This type of modeling is very effective in capturing the fine-scale structural information that is lost due to the instruments optical transfer function. It was shown that dSLIM provides a resolution increase by a factor of 2.3, enabling super-resolution imaging with SLIM. Here I present sparse deconvolution spatial light interference tomography (dSLIT). This new method provides super-resolution in 3D and allows us to study the fine scale sub-cellular structure present in *E. coli*.

The idea that the sub-cellular environment of *E. coli* cells is simply an amorphous mix have been proven to be incorrect, mostly due to the availability of high-resolution fluorescence methods. Numerous structures and distinct localizations of proteins have been studied such as the MinCD complex [88, 89], FtsZ [90-92] and MreB [89]. Furthermore, localization and structure has also been observed in the deposition of Lipopolysaccharides [93]. Interestingly many of these proteins have been found to lie in a helical or coil formation. The nature of these helices and coils is still under active investigation and many important questions remain to be answered. However, studying these sub-cellular structures using fluorescence requires specialized strains and probes, which inhibit the observation of these structures in wild-type strains in a non-invasive manner. In this section, I show that dSLIT can be used to render high-resolution images of the three-dimensional subcellular structure in *E. coli* cells. I found that dSLIT can characterize the behavior and interactions of these structures without using fluorescent labels.

The following notation is used in this section: Bold letters \( \mathbf{h} \) and \( \mathbf{H} \) denote vectors and matrices, respectively, with transposes \( \mathbf{h}^T \) and \( \mathbf{H}^T \). The spatial coordinates within an image are denoted by \((x, y, z)\), operator * denotes convolution, and \( i \) is equal to \( \sqrt{-1} \). Finally, \( \{ \} \) is used to denote a set created with its argument.
As described in reference [32], under the first order Born approximation, the 3D complex field distribution measured by SLIM, \( U(r) = |U(r)|\exp[i\Phi(r)] \), can be expressed as a convolution between the susceptibility of the object, \( \chi(r) = n^2(r) - 1 \) (where \( n \) is the refractive index), and the point spread function of the microscope, \( h(r) \). Essentially, the imaging system acts as a bandpass filter in the spatial frequency domain. Thus the measured field can be written as

\[
\tilde{U}(r) = \int \int \int \chi(r)h(r - r')d^3r',
\]

where \( \tilde{U} \) is the complex analytic signal associated with the real field. The PSF, \( h \), can be determined within the Born approximation by considering the contribution of all the optical components in the system. However, this calculation only provides the response of an idealized system. Thus, I measured the PSF experimentally by imaging microspheres with diameters less than the size of the diffraction spot such that they essentially behave as point scatterers. Therefore, for the purposes of the deconvolution procedure presented here we may model the measured field, \( \tilde{U} \), as

\[
\tilde{U}(r) = \chi(r) * h(r) + \xi(r),
\]

(3.12)

where \( \xi(r) \) is the additive signal-independent noise. Generally, both the magnitude and the phase of the image function are affected by the PSF and the noise. However, the degradation in the magnitude field is much smaller compared to the degradation in the phase \([85]\). Moreover, most of the useful biological information is contained in the phase image, and the magnitude image is not of much interest. Therefore, it is reasonable to assume that the magnitude of the image is constant and passes through the instrument without degradation, i.e.,

\[
|U(r)| = |\tilde{U}(r)| \approx const.
\]

This assumption allows us to remove the magnitude component, and write the deconvolution problem solely in terms of the phase \( \Phi(r) \) as
\[ \Phi(\mathbf{r}) = \arg\min_{\Phi(\mathbf{r})} \frac{1}{2\sigma^2} \left\| \exp[i\Phi(\mathbf{r})] - h(\mathbf{r})^* \exp[i\Phi(\mathbf{r})] \right\|^2 + \beta R(\Phi(\mathbf{r})), \] (3.13)

where \( \sigma^2 \) is the noise variance, and \( R(\cdot) \) is the regularization functional used to enforce certain image properties during deconvolution. Let us now denote by \( g(\mathbf{r}) \) the field \( \exp[i\Phi(\mathbf{r})] \) acquired by the microscope, and by \( f(\mathbf{r}) \) the unknown field \( \exp[i\Phi(\mathbf{r})] \) we are trying to recover. These fields can be represented as vectors \( \mathbf{g} \) and \( \mathbf{f} \), respectively, by stacking the images as single columns with \( N \) pixels. Using this representation, the image formation in Eq. 3.10 can be written in matrix-vector form as

\[ \mathbf{g} = \mathbf{H}\mathbf{f} + \xi. \] (3.14)

where \( \mathbf{H} \) is the convolution matrix corresponding to the PSF \( h(\mathbf{r}) \). Similarly, the deconvolution problem in Eq. 3.12 can be expressed as

\[ \hat{\mathbf{f}} = \arg\min_{\mathbf{f}} \frac{1}{2\sigma^2} \left\| \mathbf{g} - \mathbf{H}\mathbf{f} \right\|^2 + \beta R(\mathbf{f}) . \] (3.15)

The formulation in Eq. 3.14 can be expressed within the Bayesian framework by defining an observation model \( p(\mathbf{g}|\mathbf{f}, \sigma^2) \) and an image prior \( p(\mathbf{f}) \) as follows:

\[ p(\mathbf{g}|\mathbf{f}, \sigma^2) \propto \exp \left( -\frac{1}{2\sigma^2} \left\| \mathbf{g} - \mathbf{H}\mathbf{f} \right\|_2^2 \right), \] (3.16)

\[ p(\mathbf{f}) \propto \exp \left( -\beta R(\mathbf{f}) \right). \] (3.17)

The optimization problem in Eq. 3.15 then corresponds to finding the maximum (the mode) of the joint distribution \( p(\mathbf{g}|\mathbf{f}, \sigma^2) = p(\mathbf{g}|\mathbf{f}, \sigma^2) p(\mathbf{f}) \), corresponding to a maximum \textit{a posteriori} (MAP) estimation. In the following the Bayesian modeling formulation is followed.
Notice that in Eq. 3.15, the signal-independent noise $n$ is modeled as zero-mean, independent white Gaussian noise with variance $\sigma^2$. The Gaussian modeling accurately describes the noise characteristics in SLIM, since the signal-to-noise ratio (SNR) is very high (as in fluorescence microscopy [83]). In addition, the noise variance $\sigma^2$ can be estimated experimentally from a uniform area in the acquired image.

As mentioned earlier, the functional $R(\cdot)$ (and thus the image prior $p(f)$) is used to regularize the solution by enforcing certain image characteristics. The inverse filter solution can be obtained when no regularization is used ($R = 0$), but this approach generally does not produce good results due to excessive amplification of noise. The role of regularization is to impose desired characteristics on the image estimates, and to suppress the noise and ringing artifacts. The regularization parameter $\beta$ controls the trade-off between the data-fidelity and the strength of the regularization on the estimates.

I now present an image model suitable for characterizing both the specimen and the image instrument. Phase contrast imaging provides high sensitivity at the sharp object boundaries, but it is relatively insensitive to slow-variations in the background region. Thus, phase images generally exhibit high contrast around edges corresponding to, e.g., cell boundaries, which in turn provides accurate morphological information. In addition, in live cell imaging, the specimen contains a fine structure and small-scale dynamics.

Based on these characteristics, I propose to use the sparse representation/reconstruction framework [94] that is suitable for modeling phase images. Sparse representation and reconstruction have recently been used in a number of imaging problems with great success (e.g., [95, 96]). It has also been shown [95, 97] that sparsity-based deconvolution is generally superior to classical methods based on Wiener filtering and Tikhonov regularization.
As demonstrated below, phase images can be accurately represented sparsely in some transform domain; that is, when an appropriate transform is applied to the images, most of the transform coefficients become very small while only a few contain most of the signal energy. This transform sparsity allows us to capture the characteristics of spatial variations within the image. In this work, I use a collection of \( L \) linear transforms \( \mathbf{D}_k \) with \( k = 1, \ldots, L \), which are chosen as difference operators that capture spatial variation at varying scales and orientations. Specifically, I use the first and second order directional difference operators
\[
\begin{bmatrix}
-1 & 1
\end{bmatrix}, \begin{bmatrix}
-1 & 2 & -1
\end{bmatrix}
\]
and \( 45^\circ \) and \(-45^\circ \) first-order derivative filters
\[
\begin{bmatrix}
-1 & 0 \\
0 & 1
\end{bmatrix}, \begin{bmatrix}
0 & -1 \\
1 & 0
\end{bmatrix}
\]
These 2D transforms are applied on all three planes in the image, that is, on \( x-y \), \( y-z \) and \( x-z \) planes, which in total give 12 transforms to capture the local spatial variations within the 3D structure. More complicated transforms can also be incorporated in the proposed framework in a straightforward manner. As an illustration of the sparsity property of phase images, a SLIM phase image and the output of applying difference operators (in \( x \)-, \( y \)- and \( z \)- directions) are shown in Fig. 3.5, along with the corresponding log-histograms. It is evident that most of the structural information is accurately captured by the filtered images. In addition, the cell structure concealed in the acquired phase image is revealed in the filtered images (especially in the \( z \)-direction). Notice also that compared to the SLIM image, the sparsity level is significantly increased and the decrease in resolution due to the PSF can clearly be observed in the filtered images.
Figure 3.5. Sparsity property of phase images. Images show the original phase image, and the output images obtained by applying first order directional derivatives in the x, y, and z directions, as labeled, scale bar is 1 µm. The plot shows the corresponding log-histograms, the increase in sparsity is clearly visible.

Using these transforms, the image model can be constructed to exploit the sparsity in the transform coefficients. In this work, I employ separate Gaussian priors on each transform coefficient as

\[
p(f | \{A_k\}) \propto \exp \left( -\frac{1}{2} \sum_{k=1}^{K} \sum_{i=1}^{N} \alpha_{ki} \|D_k f\|_2^2 \right),
\]

(3.20)

where \(\alpha_{ki}\) are the weighting coefficients. The prior in Eq. 3.19 can be expressed in a more compact matrix-vector form as

\[
p(f | \{A_k\}) \propto \exp \left( -\frac{1}{2} \sum_{k=1}^{K} (D_k f)^T A_k (D_k f) \right),
\]

(3.21)
where $A_k$ are diagonal matrices with $\alpha_{ki}, i = 1, \ldots, N$ in the diagonal. The prior in Eq. 3.19 constitutes a sparse image prior, since the transform coefficients $(D_kf)_i$ at pixel $i$ are suppressed when the corresponding weight $\alpha_{ki}$ assumes very large values.

The weights $\alpha_{ki}$ also represent the local spatial activity at each location, and hence they are a measure of spatial variation in the corresponding filters direction. Since I do not know a priori which transform coefficients should be suppressed, they are estimated simultaneously with the image. For their estimation, I assign uniform priors as

$$p(\alpha_{ki}) = \text{const}, \forall k, i. \quad (3.22)$$

It should be noted that this image modeling based on the sparsity principle is used solely as an image prior, and it does not necessarily result in image estimates that are sparse in the transform domains. This is because the image estimate is still constrained with the acquired image $g$ via the data constraints (the first term in Eq. 3.14). Real images are generally only approximately sparse, with a few transform coefficients containing the majority of the image energy while the remaining majority of the coefficients have very small values. These small values may carry information about the subtle image features. The modeling employed in this work allows for adaptively estimating both the large and small coefficients by estimating the parameters $\alpha_{ki}$ simultaneously with the image.

Using the models for the noise in Eq. 3.15, the image in Eq. 3.21, and the parameters in Eq. 3.22, the problem of estimating the unknown complex image $f$ and the weights $\alpha_{ki}$ is formulated within the MAP framework as

$$\hat{f}, \hat{\alpha}_{ki} = \arg\min_{f, \alpha_{ki}} -\log \left[ p(g|f, \sigma^2)p(f|\{A_k\}) \prod_{k=1}^{L} \prod_{i=1}^{N} p(\alpha_{ki}) \right], \quad (3.23)$$
\[
= \arg\min_{\ell, \alpha_{\ell}} \frac{1}{\sigma^2} \| \mathbf{g} - \mathbf{Hf} \|^2_2 + \sum_{k=1}^{K} (D_k \mathbf{f})^T \mathbf{A}_k (D_k \mathbf{f}). \tag{3.24}
\]

I solve this problem using an alternating iterative minimization scheme where each unknown is estimated by keeping other variables fixed. Notice that this problem is convex in \( \mathbf{f} \) and \( \alpha_{\ell} \), but it is not jointly convex. For such problems, alternating minimization is shown to be an effective strategy, and it converges to a local minimum of the objective function (see [98, 99] for related discussions).

The estimate of the complex image \( \mathbf{f} \) is found by taking the derivative of Eq. 3.24 and setting it equal to zero, which yields

\[
\hat{\mathbf{f}} = \left( \mathbf{H}^T \mathbf{H} + \sigma^2 \sum_{k=1}^{K} \mathbf{D}_k^T \mathbf{A}_k \mathbf{D}_k \right)^{-1} \mathbf{H}^T \mathbf{g}. \tag{3.25}
\]

The parameters \( \alpha_{\ell} \) are estimated in a similar fashion by minimizing Eq. 3.24, which gives the update

\[
\hat{\alpha}_{\ell} = \frac{1}{\left\| (D_k \hat{\mathbf{f}})^T \right\|^2 + \varepsilon}, \tag{3.26}
\]

where \( \varepsilon \) is a small number (e.g., \( 10^{-6} \)) used to avoid the trivial solution (\( \hat{\alpha}_{\ell}^{-1} = 0 \)) for numerical stability. It is evident from (14) that the parameters \( \alpha_{\ell} \) are functions of the \( k^{th} \) filter response at pixel \( i \) of the image estimate \( \hat{\mathbf{f}} \). Thus, the strength of the enforced sparsity is varied spatially within the image, and it is adaptively estimated with each new image estimate. Through the use of the transforms, this can also be seen as controlling the amount of spatially-varying smoothness applied on the image estimate: When a parameter \( \alpha_{\ell} \) assumes a large value, a higher amount of
smoothness is applied at pixel $i$ (and vice versa). Low values of $\alpha_{ki}$ will therefore be obtained in the areas with more edge structure, preserving the image features.

Figure 3.6 Three-dimensional point spread function. (a) Comparison of raw and deconvolved PSF in the x-y plane; the deconvolution process reduces the FWHM from 397 nm to 153 nm. (b) Comparison of raw and deconvolved PSF in the x-z plane; the deconvolution process reduces the FWHM from 1218 nm to 357 nm. The dashed lines show the data and the circular markers indicate the Gaussian fit used to determine the FWHM.

In summary, the dSLIT deconvolution method estimates the complex image $f$ using Eq. 3.24 and the parameters $\alpha_{ki}$ using Eq. 3.25 in an alternating fashion. Estimation of the image in
Eq. 3.25 is performed using the conjugate gradient (CG) method. The operations involving the products with matrices $H$ and $D_k$ are done via multiplications in the Fourier domain. As mentioned earlier, the noise variance $\sigma^2$ is estimated from an approximately uniform background region in the image. The alternating minimization method employed here can be shown to belong to the family of half-quadratic minimization methods, for which certain theoretical convergence guarantees exist [98, 99]. The method is initialized with the acquired phase image $g$ without any pre-processing. Since the noise level in SLIM is very low, this image is a good estimate of the sharp image. In addition, the experimentally obtained point spread function (PSF) $h$ accurately represents the true PSF (see next section). Empirically I found that the proposed deconvolution algorithm is very robust and generally converges very rapidly within a few iterations. Finally, note that since the deconvolution is applied directly in the complex image domain, dSLIT does not alter the quantitative imaging property of SLIM. In contrast, traditional deconvolution methods applied on intensity images cannot preserve the quantitative information.

To quantify the increase in resolution I applied dSLIT to the experimentally measured point spread function (PSF). The experimental PSF was acquired by using SLIM to measure a sub-resolution (150nm) diameter polystyrene bead, while scanning the focus in $z$ in increments of 200 nm. Figure 3.6 shows the results of the deconvolution, the FWHM were calculated by fitting the experimental results with a Gaussian. In the x-y plane (Fig. 3.6a) an increase in resolution of 2.5 times is achieved as the FWHM is decreased from 397 nm to 153 nm. In the axial (z) direction the FWHM is reduced from 1218 nm to 357 nm, corresponding to an increase in resolution of 3.4 times that of SLIM (Fig. 3.6b). Next the method was evaluated on a biological specimen.
*E. coli* cells were prepared and imaged as described in Appendix A. Figure 3.7a shows a comparison of the SLIM and dSLIT images from two cells at different $z$-positions. It is clear that the deconvolution process reveals subcellular structure that is not visible in the SLIM images. Figure 3.7b shows the center slice of the 3D Fourier transform of the SLIM and dSLIT data. As discussed above SLIM measures the complex field at each $z$-position; thus the intensity distribution in far field or the scattering plane may be determined by calculating its Fourier transform, a technique known as Fourier transform light scattering (FTLS) [100]. Comparing the scattering maps obtained from the SLIM and deconvolved data, it is clear that there is more information available at higher scattering angles or spatial frequencies corresponding to smaller structures. Thus combining dSLIT with FTLS provides scattering information at the sub-cellular level for single *E. coli*. To our knowledge the scattering from single *E. coli* and their sub-cellular structures has not been studied, probably due to the practical difficulties involved in performing such measurements.
Figure 3.7. Comparison of raw and deconvolved data from two cells. (a) SLIM and dSLIT images at a variety of z-positions, with clearly visible coiled structures. (b) Scattering maps corresponding to the images shown in A. The increase in resolution is clearly visible from the extra information at higher angles in the dSLIT maps.

The dSLIT data reveal two sets of subcellular coil-like structures that are visible in most of the cells that were analyzed; Fig. 3.8 summarizes the measurements made on these structures. In the x-y plane a coiled structure is observed with an average period of 430 nm. Although the clarity and completeness of the structure vary from cell to cell, the period of the structure was measured to be invariant with the length of the cell. In the x-z plane another coil-like structure is apparent, which was measured to have a period of approximately half the length of the cell. This structure is not readily visible in smaller or freshly divided cells. The differences observed in x-y and x-z plane are likely due to the difference in resolution of the method in the axial and lateral
planes. Such coil-like structures have been observed in several contexts in *E. coli* cells including the MreB cytoskeletal element, MinCDE coiled arrays, outer membrane proteins and lipopolysaccharide [89, 93]. Fluorescence measurements of these structures indicate that they are most likely functionally distinct though little is known about their temporal behavior. Although dSLIT reveals these structures, there is no way to truly determine from the current data what the structures truly are. For this, it is necessary to conduct a study in which different subcellular structures are fluorescently labeled. Once the identity of the structure is determined, it will then be possible to study it in a label-free manner using dSLIT. This will enable practical experiments of the behavioral dynamics of these sub-cellular structures without the need for specialized strains or probes.

**Figure 3.8.** Measurement of prominent structures found in 26 cells. In the x-y plane a coil structure is visible that has a period of approximately 0.43 µm and does not vary with the length of the cell. In the x-z plane another structure is visible that has a period of half the cell-length.

In summary, dSLIT is a novel deconvolution microscopy method that retrieves sub-diffraction limited resolution information from the complex fields measured by SLIM. dSLIT operates on three key observations. First, the degradation of the image by a microscopy PSF can be modeled as a linear process. Second, due to the high SNR characteristic of SLIM, this PSF
may be measured experimentally. Third, the quantitative phase measurements of thin biological specimen, like *E. coli* cells, can be accurately modeled using sparsity principles. These properties of the measurement system allow for a very effective deconvolution process with a 2.5x resolution increase in the longitudinal resolution and a 3.4x increase in axial resolution, as shown in Fig. 3.6. This increase in resolution allowed us to measure sub-cellular structure in *E. coli* that was previously not visible in the SLIM data. Using dSLIT we found two consistent coil-like subcellular structures in *E. coli*, one that retains a constant period as the cell grows and one with a period of approximately half the length of the cell. Although several such structures have been previously identified, little is known about their function and behavior due to the practical difficulties involved in imaging them. The results presented here indicate that dSLIT can be used to characterize and study such sub-cellular structure in a practical and non-invasive manner, opening the door for a more in depth understanding of the biology.

### 3.7 Discussion

In summary, QPI and especially SLIM has expanded the capabilities and potential applications of quantitative phase imaging by providing high resolution speckle-free measurements. Due to this, SLIM has provided information with unprecedented detail on the structure and dynamics of living systems and has enabled many exciting biological applications. The major advances provided by SLIM and the analysis tools discussed here are:

1) Speckle-free images providing 0.3 nm spatial sensitivity to changes in optical path length
2) Temporal stability of 0.03 nm due to common path setup
3) 3D tomography due to inherent depth sectioning
4) Multiplexing with other microscopy modalities
5) Femtogram sensitivity to dry mass changes
6) Label-free measurements of mass transport

7) Single shot scattering measurements

In the remainder of this thesis I will show how these capabilities can be leveraged to construct novel clinical tools and address fundamental questions in cell biology.
CHAPTER 4. RED BLOOD CELL CYTOMETRY*

Existing clinical technologies used to characterize patient blood such as impedance counters and flow cytometers, though very effective in terms of throughput, offer limited information, are expensive, bulky, and costly to maintain, and often require careful calibration. Though there have been reports of using high throughput cytometers to characterize red cell morphology [101], this approach is limited as it only provides a general description of shape (e.g., ellipsoid vs. spherical) and is unable to provide the resolution required for aiding in differential diagnosis. Automated counters are thus designed to produce accurate measurements of normal blood and to alert the technician with “flags” when numerical abnormalities exist so that a smear may then be prepared and examined [102]. Even though automated blood analyzers have reduced the number of samples that require smears to 15%, the examination of a smear is still an indispensable tool in providing differential diagnosis (commonly for anemias and thrombocytopenia), recommending further tests, speedy diagnosis of certain infections and the identification of leukemia and lymphoma [103]. Despite the ability of the automated instruments to measure volume and hemoglobin concentrations, they are unable to accurately measure morphologic abnormalities and variations in shape, at the single cell level, and thus a pathologist is required to manually examine a smear. Other modern methods that can be used for accurately assessing red cell morphology, such as confocal microscopy, suffer from complicated procedures and the need for using specialized exogenous contrast agents.

The use of the optical phase shift through a sample as an endogenous contrast agent has served as a powerful technique in microscopy since the advent of techniques such as phase contrast (PC) and differential interference contrast (DIC) microscopy [104]. Even though these

---

* Portions of this chapter have been reproduced here from my master’s thesis (M. Mir, 2009) and the below referenced material in order to introduce the material in a complete manner. This material is reproduced with the permission of the publisher.
modalities greatly enhance the ability to observe details within transparent objects such as living cells, it was not until the development of quantitative phase imaging (QPI) techniques that the phase shift through a sample was able to provide quantitative information. Furthermore, with the development of common path interferometry and methods such as diffraction phase microscopy (DPM) [8] and SLIM [12] (see Chapter 2), we can now obtain quantitative phase images that are sensitive to sub-nanometer changes in optical path lengths over broad temporal scales.

4.1 Diffraction Phase Cytometry*

In this section I described Diffraction Phase Cytometry (DPC) which is based on DPM, and produces accurate measurements of normal blood and in addition is capable of characterizing specific morphological abnormalities in diseased blood. I previously demonstrated the simplicity and versatility of the DPM technique by combining it with CD-ROM technology for characterizing red blood cells (RBCs) [105]. By giving access to detailed 2D and 3D morphological parameters such as volume, surface area, sphericity, diameter, etc., DPC provides new information that is currently unavailable from commercial instruments. It is known that the distributions of these parameters and correlations between them reveal physiologically important information about a given blood sample [106]. For example, the minimum cylindrical diameter (MCD) can be used to predict the minimum capillary diameter that a given cell can squeeze through [107].

We present a comparison between the abilities of the DPM system and a state-of-the-art clinical impedance counter to measure and characterize RBCs. It is shown that after taking the mean cell hemoglobin concentration (MCHC) into account the DPM data correlates very well with the impedance counter. The advantages of using the DPM are also illustrated by an analysis

of the volume and sphericity distributions obtained from two patients. DPM also has the advantage that it can be easily implemented as an add-on modality to a microscope without adding any additional preparatory steps to the lab workflow. The results shown here are from measurements on whole blood samples, further illustrating the flexibility of the technology, as it can be applied both to peripheral blood smears and to samples stored according to clinical protocols. Considering the agreement with current techniques and the detailed morphological information provided by the DPM, it could prove to be both a powerful diagnostic tool and a way to improve blood testing efficiency by reducing the number of cases that require a manual smear analysis.

Whole blood was drawn from patients at a local community hospital via venepuncture by a certified phlebotomist and is stored in EDTA coated containers at room temperature. A complete blood count (CBC) analysis was then performed on each sample with the Coulter LH50 (Beckman-Coulter) impedance counter used for routine analysis in the hematology lab at the hospital. Each sample was marked with a unique identifier, and all unique personal patient information (name, id#, etc.) was removed in compliance with HIPPA regulations and the University of Illinois Institutional Review Board to maintain patient confidentiality.

The whole blood was then diluted with the same Coulter LH series diluent (Beckman Coulter) used by the impedance counter for a final concentration of 0.2% whole blood in solution. This concentration was chosen as it provides an adequate cell count for comparison with the CBC while being low enough to provide sufficient distribution of the cells, which is necessary for proper analysis given the large variations in patient hematocrit. Following dilution, the sample is pipetted into a 200 µm tall chamber, which was made in house by punching a hole in double-sided Scotch tape (3M) and sticking one side of the tape to a cover slip. After the
sample was introduced to the chamber, it was sealed using another cover slip. This sealed chamber technique reduces the mechanical stress imposed on the cells during sample preparation, offers precise control over the sample volume, prevents drying, and reduces cell translation. The samples were measured 5 minutes after being sealed to allow them to settle to the bottom of the chamber and to reach a steady state in the solution.

The DPC setup utilized in this experiment uses the diffraction phase microscope \([8, 100]\) as its core platform. In short, the DPC setup is a common path interferometer, in which a diffraction grating located at the image plane of a microscope is used to generate diffraction orders, each containing full spatial and phase information of the sample. The \(0^{th}\) order or undeviated beam is then spatially low-pass filtered using a pinhole in the Fourier plane so that it can be used as a reference beam; the +1 diffraction order is used as the sample beam and all the other orders are blocked. A second lens is then introduced to project the interferogram onto the CCD plane for recording. The phase map of the sample is then retrieved from a single CCD exposure by applying a spatial Hilbert transform \([7]\).

For each sample, 1600, 32 \(\mu\)m x 32 \(\mu\)m interferograms were recorded, which cover a total area of 1.64 mm\(^2\); a total of 5.3 minutes is required to scan this area at a rate of 5 frames/s. The analysis of the phase images is carried out in MATLAB (The Mathworks 2008) using a cell detection and analysis software developed in house. To find the cells in each image a standard particle detection algorithm was used \([108]\). Once the pixels occupied by individual cells are identified we can proceed to quantify the 2D and 3D morphological parameters of each cell. The 2D parameters such as diameter, projected area and circularity are easily obtained using region property descriptors available in MATLAB.
In order to obtain physiologically relevant and accurate 3D parameters from the retrieved phase map it was translated to a height map using an index of refraction calculated based on the mean cell hemoglobin concentration of each sample, as measured by the impedance analyzer. Due to the linear dependence on the protein concentration [41], the refractive index can be calculated as \( n_c = n_0 + \beta \times \text{MCHC} \), where \( \beta \) is the refractive increment of hemoglobin (0.002 dL/g) and MCHC is the concentration of dry protein expressed in g/dL. The phase map \( \phi(x,y) \) is then translated to a height map \( h(x,y) \) using the contrast in refractive index between the cells and surrounding media, \( \Delta n: h(x, y) = \frac{\lambda}{2\pi\Delta n}\phi(x, y) \), where \( \lambda = 532 \) nm is the wavelength of the illumination and \( \Delta n = n_c - n_0 \). Once the height information is retrieved, the volume of each cell is calculated by integrating the height map over the projected area as \( V = \iint h(x, y)dx\,dy \). The surface area of individual cells is determined using Monge parameterization [109], where the area of each pixel element, dA, is calculated as \( dA = dx\,dy\sqrt{1 + h_x^2 + h_y^2} \), where dx and dy are the width and height of each pixel and \( h_x \) and \( h_y \) are the gradients along the x and y directions respectively. The surface area of each cell is then the sum of all the area elements and the projected area, assuming the cell is sitting flat on the cover slip. Knowing the surface area and volume, we can calculate parameters such as sphericity (\( \psi \)) and minimum cylindrical diameter (MCD). The sphericity, \( \psi \), of RBCs was first determined as an important parameter by Canham and Burton [106]. It is defined as the ratio between the surface area (SA) of a sphere with the same volume as the cell, to the actual surface area of the cell, with values ranging from 0 (for a laminar disk) to 1 for a perfect sphere, and is calculated as \( \psi = 4.84 \frac{V^{2/3}}{SA} \). The MCD, also introduced by Canham and Burton, is a theoretical parameter that predicts the smallest capillary...
diameter that a given RBC can squeeze through. The MCD is obtained by solving the following polynomial equation that defines the cell volume: 

\[ V = SA \cdot MCD - \frac{\pi MCD^3}{12}. \]

Overall, for each cell imaged we obtain the following 17 parameters: perimeter, projected area, circular diameter, surface area, volume, sphericity, eccentricity, minimum, maximum and mean height, minimum cylindrical diameter, circularity, integrated density, kurtosis, skewness and variance. Thus, it is possible to identify and characterize abnormal cells that would otherwise be difficult or impossible to detect manually in a smear. This type of analysis could be utilized for early detection of diseases, infections and abnormalities such as poikliocytosis [102] and malarial infection [110], or of reactions to treatments such as chemotherapy and bone marrow transplants [111]. If manual analysis of the abnormality is still necessary to confirm the diagnosis, a physician may simply examine the cell images that have been labeled as abnormal by the DPC system, rather than manually scanning a smear in search of abnormalities. Given the wealth of information available about each cell, it is possible to study the distributions of and correlations between parameters in order to establish the parameters expected from a normal sample and to characterize various abnormalities.

In this study, samples from 32 patients were analyzed using both a clinical Coulter impedance counter and the DPC system; with the DPC system we analyzed an average of 828 cells per sample. We show that there is high correlation between the CBC and DPC data and provide examples of the advantages associated with our interferometric, image-based cytometry technique.
Figure 4.1 MCV values measured by DPC vs. impedance counter (complete blood count, CBC). The DPC data is shown before the correction for the refractive index (Raw) and after refractive index correction (Corrected). Pearson correlation coefficients for both data sets are shown in the legend. The straight line, included for comparison, represents the CBC MCV values.

In order to evaluate the consistency of the DPC analysis in comparison to that of the Coulter counter, we compared the mean corpuscular volumes (MCV) obtained by both methods (Fig. 4.1). Initially the data was analyzed assuming a constant refractive index contrast for all samples, which resulted in a weak correlation (Pearson correlation coefficient, ρ=0.52) between the DPC and CBC volume data (circular symbols in Figure 4.1). However, once the MCHC values are taken into account and the refractive index contrast is corrected, the correlation improves to ρ=0.84 (triangular symbols in Figure 4.1).

The MCHC is currently used by pathologists to help diagnose abnormalities such as anisochromasia (large variation in MCHC) and spherocytosis (high MCHC) [103]. However, with current automated counters a pathologist has to manually examine a smear to confirm diagnosis of spherocytosis or any other morphological abnormalities which would result in an
abnormal MCHC distribution. With the current DPC system it is possible to provide this diagnosis directly using the sphericity index. Figure 4.2 is an example of a sphericity distribution obtained from a 97-year-old female patient exhibiting anisocytosis (diagnosed by a large variation in MCV). By examining cell images along the sphericity distribution, the capabilities of the DPC to differentiate between flat and spherical cells is made clear. If a larger spherocytic subpopulation were to exist in this patient, it would appear as a secondary maximum in the distribution or could be identified by a positive shift in the samples’ mean sphericity value.

![Sphericity Distribution](image)

**Figure 4.2** Comparison of cells across the sphericity distribution for a 97 y/o female patient exhibiting anisocytosis. Examples of cells at the sphericity values as follows: (i) 0.50 (ii) 0.54 (iii) 0.57 (iv) 0.61 (v) 0.65 (vi) 0.72.

An important advantage of DPC as an emerging technology is that it recovers all metrics that are familiar and intuitive to pathologists, such as the MCV. One disorder that is fairly common and easy to diagnose using the MCV is anisocytosis, which is characterized by large variations in the cell volumes and quantified by the red cell distribution width (RDW). Figure 4.3
shows volume distributions from two patients, one normal and one exhibiting anisocytosis. Again we show images of cells across the distribution to illustrate the information available about each cell.

**Figure 4.3.** Comparison of volume distributions of patient exhibiting anisocytosis vs. a normal patient. The DPC measures red cell distribution width (RDW) values of 12.65 and 16.28 for the normal and abnormal patient respectively. More subpopulations are apparent in the patient with anisocytosis. Examples of cells at the different volume peaks as follows: (i) 64 fL (ii) 72 fL (iii) 84 fL (iv) 93 fL (v) 102 fL (vi) 117 fL.

This type of analysis enables the DPC system to accurately identify the morphological abnormalities that are responsible for the anisocytosis. Since anisocytosis could be a result of a variety of disorders such as thalassemia (decreased globin synthesis) and myelodisplastic syndrome (preleukemia) [103], more detailed information on the cause will aid in a quick and early automatic diagnosis of these conditions.

In summary I have demonstrated the ability of the DPC system to operate as an automatic blood analyzer, which recovers the parameters provided by current clinical instruments. We
showed that the additional set of parameters measured by DPC offers insight into the nature of the numerical abnormalities used to identify morphological disorders. Using this type of analysis may aid in an automatic diagnosis of conditions that currently require manual smear analysis. Even though the current DPC system has lower throughput and speed than state-of-the-art impedance counters, these are practical issues which can be overcome due to the rapid advances in automated image acquisition and processing technologies.

The strong dependence of our results on the cell hemoglobin content indicates that an accurate measurement of individual cell protein content needs to be made. A previous method entails measuring the cells in two solutions with different refractive indices [112]. Though this decoupling method is an effective way to calculate the refractive index, it may be impractical in a clinical setting, due to throughput considerations and because exposing the cells to different solutions may affect their properties. It has recently been shown that DPC can directly measure single cell hemoglobin concentration by either utilizing a broadband source [113] or performing DPM at different wavelengths [114]. Both of these techniques rely on the dispersion properties of hemoglobin to infer the protein concentration. In Chapter 4.2 I show how it is possible to measure both hemoglobin and thickness by combining absorption measurements at one wavelength with QPI data. This new method frees DPC from relying on any external measurements and thus greatly add to both is practical application in a clinic and its power in aiding differential diagnosis.

4.2 Simultaneous Measurement of Morphology and Hemoglobin*

As discussed above QPI is capable of providing detailed, quantitative, morphological analysis as well as several novel clinically relevant parameters for red blood cells [105, 115-125] at the

---

*The work shown here has appeared in its entirety in M. Mir, K. Tangella and G. Popescu, Blood testing at the single cell level using quantitative phase and amplitude microscopy, Biomed. Opt. Exp., 2 (12), 3259 (2011). This material is reproduced with permission of the publisher.
single cell level. However, since the optical phase shift through a red blood cell is a function of both thickness and refractive index, the demonstrated morphological analysis has depended on a priori knowledge of the hemoglobin concentration [105, 126]. Other groups utilizing phase measurements have addressed this problem in various manners. One popular approach utilizes two different immersion media to decouple the thickness and refractive index [112, 127, 128]. This approach requires that cells be kept in place as the media is changed and thus requires coating of the cover slips and a profusion setup. Thus, such a technique may not be well suited for clinical measurements especially with the goal of developing a point-of-care diagnostic tool in mind. More recently, it has been demonstrated that by acquiring diffraction phase microscope (DPM) measurements at different wavelengths, hemoglobin concentration may be quantified at the single cell level [114]. This technique, dubbed Spectroscopic Phase Microscopy, is highly stable and utilizes a relatively simple experimental setup and is therefore well suited for adoption as a clinical method. However, DPM requires modifications to the illumination path of a microscope and is not easily integrated with other popular modalities such as fluorescence measurements.

Here I provide the proof of principle of a novel combination of the quantitative phase information measured using a Spatial Light Interference Microscope (SLIM) [32, 129], with a bright field absorption measurement acquired in the Soret band. We show both theoretically and experimentally, that this combination can be used to quantitatively determine both hemoglobin concentration and cell morphology at the single cell level. SLIM is a new QPI modality which utilizes broadband illumination (400-700 nm, center wavelength of 530 nm) in common path geometry. Due to this, SLIM provides the ability to measure optical path length with unparalleled sensitivities of 0.28 nm spatially and 0.029 nm temporally [16]. Furthermore, SLIM
is designed as an add-on module to a commercial microscope and can easily be integrated with other commonly used modalities.

The method described here may be deployed as a standalone blood smear analyzer in a clinical setting without relying on external measurements of hemoglobin concentrations. The additional set of measured parameters may offer insight into the nature of morphological abnormalities used to identify various disorders and will likely automate the diagnosis of conditions that currently require manual smear analysis. Such a method may be implemented for a fraction of the cost of current analyzers, requires no reagents or complicated sample preparation and has the potential to easily be adapted to a compact and portable platform [105, 122]. The technique presented here may be also utilized with any QPI instrument, provided that it meets the resolution and sensitivity requirements for single erythrocyte analysis.

The phase measured by SLIM is related to the refractive index and thickness of the sample as

$$\Delta \phi(x, y) = k_0 \Delta n(x, y) t(x, y)$$  \hspace{1cm} (4.1)

where $k_0=2\pi/\lambda$ and $\lambda$ is the mean wavelength, $t$ is the thickness and $\Delta n=(\beta C + n_w)-n_s$. Here $\beta$ is the refractive increment of protein in mL/g, $C$ is the concentration in g/mL, $n_w$ is refractive index of water and $n_s$ is the refractive index of the surrounding media. The refractive increment is defined as the increase in refractive index per one percent increase in the concentration [130-133]. It was shown in the 1950s that the refractive increments of a wide range of proteins lie within the range of 0.17 and 0.20 [130-133]. Furthermore, this is also true for other cellular components such as lipids and carbohydrates to the point that we may assume that the bulk refractive index of a living cell is a good measure of the total dry mass of the cell [17-21]. Given the small variations in refractive increment, Eq. 4.1 can be rewritten in terms of the concentration and refractive increment as:
\[
\Delta \phi(x, y) = k_0 \left[ \beta C(x, y) + \Delta n_{ws} \right] t(x, y),
\]

where \( \Delta n_{ws} = n_w - n_s \) is the difference between the refractive index of water and the surrounding media. The absorption measurements may be described according to the Lambert-Beer law:

\[
A = -\ln \left( \frac{I}{I_0} \right) = \sigma t N,
\]

where \( A \) is absorbance, \( \sigma \) is the absorption cross section perpendicular to the optical axis and \( N \) is the density of absorbers. The relationship between absorption cross section, density and refractive index is \( \sigma N = 2k_0 n'' \), where \( n'' \) is the imaginary part of the refractive index which describes the absorption phenomenon. For liquid solutions the absorbance is typically expressed in terms of a molar extinction coefficient:

\[
A'(x, y) = -\log_{10} \left( \frac{I}{I_0} \right) = \frac{\varepsilon t(x, y) C(x, y)}{M},
\]

where \( \varepsilon \) is molar extinction coefficient in \( \text{L} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1} \) at the wavelength being used, \( M \) is the molar mass (g/mol) and \( C \) is the concentration (g/L). It can be seen that the only unknowns in Eq. 4.2 and Eq. 4.3 are the thickness, \( t \) and concentration \( C \). Therefore we may simply solve for both:

\[
C(x, y) = \frac{\Delta n_{ws}}{\varepsilon \frac{\Delta \phi(x, y)}{M k_0 A(x, y) - \beta}},
\]

\[
t(x, y) = \frac{MA'(x, y)}{\varepsilon C(x, y)}.
\]
Thus, for a single molecular species case, as in the case of the almost homogeneous red blood cell, only one absorption and one phase measurement is necessary to determine both the thickness and the concentration.

To verify this method blood samples were prepared as described in Section 4.1 [126]. For measuring the quantitative phase map, SLIM was used, which has been described in detail previously [32, 129]. To ensure that the phase measured is integrated over the entire thickness of the cell I used a low numerical aperture (NA) objective. Thus, for both the SLIM and absorption measurements a 10x/0.3 Ph1 objective was used. For measuring the absorption map a bandpass filter centered at 430 nm (+/- 10 nm) is introduced into the light path after the condenser field diaphragm as shown in Fig. 4.4. In principle any bandpass filter could be used, provided the SNR is high enough. The choice of 430 nm was made since it is strongly absorbed by both oxygenated and deoxygenated hemoglobin. The phase contrast annulus in the condenser is also swung out of position so that the illumination is set for bright-field measurements. In order to optimize the absorption measurement, both the NA of the condenser and the objective must be taken into account. As for the SLIM measurement the NA of the objective must be chosen such that the depth of field is greater than the thickness of the red blood cells. The optimal NA for the condenser was determined experimentally by varying NA between 0.55 and 0.1 and measuring the absorption. It was found for multiple objectives (data not shown) that the absorption continues to increase as the NA is decreased and peaks at a value close to the NA of the objective being used. After this point, aberrations become clearly observable in the image. For the 10x/0.3 objective used here, the value for the condenser NA which gave the greatest contrast was determined to be 0.2.
Figure 4.4. Experimental setup. The SLIM system is built as an add-on module to a commercial phase contrast microscope. The back focal plane of the objective is projected onto a spatial light modulator which is calibrated to impart a phase shift to the un-scattered light (yellow lines) relative to the scattered light (shown in red). Four intensity images are recorded corresponding to 4 phase shifts in increments of $\pi/2$, the quantitative phase map is reconstructed from these 4 intensity images as detailed in Ref. [129]. For the SLIM measurements the illumination type is set to phase contrast and the filter wheel is set to an open position such that the entire spectrum of the halogen lamp is passed. For the absorption measurements the illumination type is set to bright field and a 430 nm filter is used in the filter wheel. The inset for the filter wheel shows the normalized intensity of the white light spectrum, the spectrum of the 430 nm bandpass filter (right axis) and the extinction coefficients of oxygenated and deoxygenated hemoglobin (left axis) as a function of wavelength from Ref. [134].

Although in principle any QPI technique could be coupled with an absorption measurement to yield results similar to those shown here, we used SLIM for two main reasons. First, the white light illumination used for SLIM allows for easy integration of a filter wheel into the setup to
perform absorption measurements. Second, SLIM provides the lowest noise and highest sensitivities out of any QPI technique that we are aware of.

Figure 4.5. Image analysis (a) Quantitative phase map acquired using SLIM, color bar is in radians. (b) Absorption map acquired at 430 nm, color bar is in 16-bit gray scale values. (a-b) Insets show an example of a single RBC from the maps. (c) Overlay of 1 line profiles drawn through the center of a single cell, the phase values are shown in red against the left axis and the corresponding intensity from the absorption maps are shown in black against the right axis. (d) Average absorption vs. phase for each of the 7 patients analyzed in this study, a strong linear relationship (dotted line) indicates the feasibility of this method.

To analyze the images, a semi-automatic image routine was developed in MATLAB. A user selects several cells in every image, avoiding cells that are turned on their side or appear otherwise damaged. Once a cell is picked the region occupied by the cell is identified by generating a binary mask and the center of mass of this mask corresponds to the center of the cell. For each cell a horizontal and vertical line profile are then measured through the center, for both the absorption and phase map (Fig. 4.5c). This approach assumes that the cells are radially symmetric, which is a reasonable approximation for red blood cells, although for future clinical
work a pixel by pixel comparison will be ideal. From the phase profiles the peak values were chosen and from the intensity profile the minima are chosen. As can be seen in Fig. 4.5c, the phase and absorption profiles are in good agreement except in the dimple region of the blood cell. For this reason, to demonstrate the feasibility of this technique just the peak values were chosen for the analysis presented here. Of course, for clinical translation, this mismatch must be understood and addressed; Fig. 4.5d shows that the phase and absorption measurements are in fact linearly related and proves the feasibility of this approach.

The peak values from the horizontal and vertical profiles are averaged and plugged into Eq. 4.5 and Eq. 4.6 to calculate the un-calibrated concentration and thickness values. For this analysis it was assumed that the blood cells are oxygenated and we obtained the absorption spectrum for hemoglobin from Ref. [134]. The volume for each cell was calculated by multiplying the average thickness, calculated from the line profiles, by the projected area of the cell. For calibration, the measured mean cell volume (MCV) and mean cell hemoglobin concentration (MCHC) values were plotted against the values reported by the CBC and a best fit line is calculated to provide a calibration function. In principle this analysis could be completely automated as previously shown [105, 126], but is not necessary for the proof of principle of this technology.

For this study, samples from a total of 7 patients were measured and total of 651 cells were analyzed with an average of 93 cells per patient. The comparison between the calibrated mean values from our measurements and the CBC values is shown in Fig. 4.6. It can be seen that both the measured MCV and MCHC agree well, with $R^2$ values of 0.86 and 0.79, respectively. The discrepancies are likely due to two major reasons. First, the MCHC reported by the clinic is calculated by lysing all the blood cells to make a solution of hemoglobin. An absorption
measurement was then made on this solution to calculate the hemoglobin concentration. Therefore, this measurement does not take into account any variability in hemoglobin concentration between cells. Secondly, the number of cells measured in this study is relatively low compared to the large numbers measured by the clinical impedance counters. Since the comparison between our measurements and the clinic relies on calibration, the agreement will likely increase with an increase in throughput.

**Figure 4.6.** Comparison of measured mean values with clinically reported values. (a) Mean Cell Volume, red error bars correspond to the SD reported by the Clinic and black error bars correspond to SD measured by the QPI and absorption measurements. (b) Mean Cell Hemoglobin Concentration, error bars correspond to the measured SD, no SD information on the hemoglobin concentration is available from the Clinic. The dashed black lines have a slope of one.

For the volume measurement, Fig. 4.6a also shows the standard deviations measured by the clinic in red and those measured by our technique in black. The lack of perfect agreement in the distribution widths is most likely due to the higher sensitivity of our method and the difference in the sample sizes measured. Although the clinical counter we are comparing our measurement to does provide a histogram of size distributions for each patient, it does not have the capability to do the same for hemoglobin concentration; Fig. 4.6b thus only shows the standard deviations in the hemoglobin concentration distributions measured by our technique. The fact that the clinical analyzer in a major community hospital does not have this capability illustrates the need for a simple approach to provide this measurement.
In this section I have shown both theoretically and experimentally that by combining quantitative phase measurements with bright field absorption measurements it is possible to calculate both cell morphology and hemoglobin concentration at a single cell level. The method was validated by comparing the values measured with those reported by a state-of-the-art automated clinical blood analyzer. Although in this study a calibration was necessary, in the future a more detailed understanding of the formation of the bright field image may render this step unnecessary. In particular, the measured intensity includes contributions from scattered light and is not a pure absorption map as described by Beer's law. Furthermore, Lambert-Beer's law must be rewritten for the case of convergent illumination as is provided by a typical microscope. The fact that the phase and absorption are linearly related, without taking these effects into account, indicates that the contribution from them is constant for red blood cells.

Although I used SLIM to measure phase, in principle this technology could be utilized in combination with any QPI method. However, SLIM is well suited for this method since it does not require any modifications to a commercial microscope and the filter wheel required for the absorption measurements may easily be integrated with the white light illumination. The fact that SLIM requires 4 intensity measurements is only a practical issue as the advent of fast spatial light modulators, cameras and scanning software means that the speed of the measurement may easily be increased. Furthermore, the high SNR provided by SLIM ensures that data analysis technology can easily be automated to increase throughput. Since SLIM can simply be added as a modality to an existing microscope, minimal re-training will be necessary to use the equipment, especially given that the parameters provided by this analysis are already familiar to pathologists and technicians.
The technology presented here offers a powerful new blood screening tool that may aid pathologists in making differential diagnosis and risk stratification. This technology combined with the morphological analysis described previously [105, 126] provides the ability to analyze red blood cells with unprecedented details and may enable new diagnostic capabilities when monitoring and treating red blood cell disorders. Furthermore, the ability to easily measure single cell hemoglobin concentrations may open new avenues for monitoring blood cell disorders and the effects of treatment.

4.3 Discussion

In this chapter I have shown that QPI offers a powerful new blood screening utility that can be used to aid in making differential diagnosis by an experienced pathologist. QPI instruments can be simply added on as a modality to any existing microscopy, and no special sample preparation is necessary to integrate it into the clinical workflow. Furthermore, the outputs of QPI blood measurements are intuitive morphological characteristics, such as sphericity and skewness, meaning that no new specialized knowledge is necessary to take advantage of the added information. Recently others in our group have shown that blood analysis may be performed in real time, further demonstrating the abilities of this technology to be deployed as a point of care tool. Advancements in spectroscopic measurements, image processing and computing power will continue to augment the abilities presented here, while maintaining its position as a low cost, high throughput and highly sensitive instrument.
CHAPTER 5. CELL GROWTH*  

Single cell growth regulation has been described as "One of the last big unsolved problems in cell biology" [135] and the ability to measure the growth rate of single cells is integral to answering this question [54, 120, 136, 137]. The age-old debate is whether the growth rate is constant through the life-cycle of a cell (linear growth) or grows proportionally with the cell mass (exponential growth) [138-144]. Each growth pattern carries its own biological significance: if the growth is linear, cells do not need machinery to maintain homeostasis; on the other hand, exponential growth requires checkpoints and regulatory systems to maintain a constant size distribution [139]. This can be understood simply by considering two daughter cells of different size: under exponential growth the larger of the two would grow faster and thus the variability would increase with each generation; thus a mechanism to regulate growth must be present. The reason that this debate has persisted despite decades of effort is primarily due to the lack of quantitative methods to measure cell mass with the required sensitivity. In order to distinguish an exponential pattern from a linear one, it has been calculated that a resolution of less than 6% in cell size is required [52].

Until recently, the state-of-the-art method to assess a single cell growth curve was using Coulter counters to measure the volume of a large number of cells, in combination with careful mathematical analysis [52]. For relatively simple cells such as *Escherichia coli* (*E. coli*), traditional microscopy techniques have also been used to assess growth in great detail [53]. In this type of method the assumption is that volume is a good surrogate for mass; however, this assumption is not always valid, for example due to variations in osmotic pressure [120]. Recently, shifts in the resonant frequency of vibrating microchannels have been used to quantify

the buoyant mass of cells flowing through the structures [54, 55]. Using this approach, Godin et al. have shown that several cell types grow exponentially; i.e., heavier cells grow faster than lighter ones [54]. However, while this method is sensitive enough to measure bacteria growth, it cannot be applied to adherent cell lines. Later, Park et al. extended this principle to allow mass measurements on adherent cells [136]. This benefit comes at the expense of sensitivity (e.g., it cannot measure single bacterium growth), and throughput (it measures single cells at a time). An ideal method will perform parallel growth measurements on an ensemble of cells simultaneously and continuously over more than one cell cycle, quantifying possible cell cycle phase-dependent growth, apply equally well to adherent and non-adherent cells, and work in a fully biocompatible environment [52, 54]. Here we demonstrate that a new imaging method developed in our laboratory, Spatial Light Interference Microscopy (SLIM) [145], approaches these ideals.

The principle behind using interferometry to measure cell dry mass was established in the early 1950s when it was recognized that the optical phase shift accumulated through a live cell is linearly proportional to the dry mass (non-aqueous content) of the cell and has since then been used by many groups to monitor cell dry mass [39, 58, 146-148]. In the past decade or so, quantitative phase imaging methods have advanced rapidly (see, for example Refs. [149, 150] and references therein) and new biological applications have been explored [43, 151, 152]. However, despite these advances, two main limitations commonly affect the performance of quantitative phase imaging: (i) reduced contrast due to the speckle generated by the laser sources and (ii) the complexity of the experimental setups limits their adaptation in biological settings. SLIM overcomes these challenges by combining traditional, white light phase contrast microscopy with holography, thus providing speckle-free quantitative phase maps (for details on
the operating principle of white light interferometry and SLIM, see Chapter 2, Refs. [14, 145, 153, 154] and Appendix C).

Recently, it has been shown, both theoretically and experimentally, that the surface integral of the cell phase map is invariant to small osmotic changes [120], which establishes that quantitative phase imaging methods can be used for dry mass measurements. The dry mass density at each pixel is calculated as \( \rho(x, y) = \frac{\lambda}{2\pi\gamma} \phi(x, y) \), where \( \lambda \) is the center wavelength, \( \gamma \) is the average refractive increment of protein (0.2 ml/g) [120] and \( \phi(x, y) \) is the measured phase. The total dry mass is then calculated by integrating over the region of interest. Remarkably, SLIM’s pathlength sensitivity of 0.3 nm spatially (pixel to pixel) and 0.03 nm temporally (frame to frame) [145] translates into spatial and temporal sensitivities of 1.5 fg/\( \mu \)m\(^2\) and 0.15 fg/\( \mu \)m\(^2\), respectively.

5.1 E. coli Growth

In order to demonstrate that SLIM can recover cell growth results on a well-studied model [54], I imaged E. coli cells growing on an agar substrate at 37 °C. The evolution of single cells was tracked using the Schnitzcell semi-automatic software (Michael Elowitz, Caltech). Figure 5.1aA shows the dry mass growth curves for a family of E. coli cells. The negative mass densities are due to the fact that our measurements are always with respect to a baseline value of the surrounding medium which is of zero-average. As a control I also measured fixed cells under the same conditions from which we retrieved a standard deviation of 19.6 fg. Note that, because of the noise introduced by the culture environment, this error is larger than intrinsically allowed by the optical instrument. Figure 5.1b shows the growth rate of 22 single cells as a function of mass, \( dM(t)/dt \). The average of the data (black circles) shows that the growth rate is proportional to
the mass, \( \frac{dM(t)}{dt} = \alpha M(t) \), indicative of exponential growth. Prior to calculating the derivative, the raw data (markers) in Fig. 5.1a is first time-averaged (solid line). These results are in excellent agreement with recent measurements by Godin et al. [54] and demonstrate that SLIM can measure dry mass with the precision necessary for answering such biological questions. Note that my measurements are performed simultaneously on many individual cells and can be performed on adherent cells or bacterial bio-films, unlike the prior approaches which can be either performed on suspended cells only, or on adherent cells but lacking the resolution to interrogate single bacteria. From our images I can also retrieve the cell volume and, thus, extract information about the cell density. I found that the volume increase is also exponential with the same growth constant as for mass, 0.011 min\(^{-1}\) (see Fig. B.1). It can be seen that on average, the volume is linearly proportional to mass, indicating constant average volumetric density. These results confirm the commonly accepted fact that, for this simple organism, in normal growth conditions, the volume and (because of the constant cell cross section) cell length can be used as surrogates for mass [53].
Figure 5.1. SLIM measurements of E. coli growth. (a) Dry mass vs. time for a cell family, growth curves for each cell are indicated by the colored circles on the images. The inset shows the histogram of the dry mass noise associated with the background of the same projected area as the average cell (standard deviation $\sigma=1.9$ fg is shown). The blue line is a fixed cell measurement, with standard deviation of 19.6 fg. Markers indicate raw data and solid lines indicate averaged data. (b) Growth rate vs. Mass of 20 cells measured in the same manner, faint circles indicate single data points from individual cell growth curves, dark squares show the average and the dashed line is a linear fit through the averaged data; the slope of this line, 0.011 min$^{-1}$, is a measure of the average growth constant for this population. The linear relationship between the growth rate and mass indicates that on average, E. coli cells exhibit exponential growth behavior. Images show single cell dry mass density maps at the indicated time points (in min.) and the white scale bar is 2 $\mu$m.
5.2 Cell Cycle Dependency
Next I investigated the cell growth behavior in mammalian cells. In order to test the ability of SLIM to study growth in large populations of mammalian cells over more than a cell cycle, we imaged continuously for a two-day period, a 3.2 x 2.4 mm$^2$ field of view of a U2OS synchronized cell culture (Fig. 5.2). It is important to note here that for bigger cells it is important to select the correct objective to ensure that the integral phase through the entire cell thickness is measured; for more details on this measurement refer to Appendixes A and B. Figure 5.2 shows the results in terms of single cell and ensemble growth curves. The results show that the mean cell mass evolves synchronously in time with the total mass of the entire population during the duration of a (mean) cell cycle, i.e. 22-26 hours, after which it levels off. This indicates that after one cell cycle, the culture loses synchrony and the single cell mass is limited by mitosis. This measurement highlights the problems of using a synchronized population for cell cycle studies, and reiterates the need for measuring single cells through an entire cell cycle in an asynchronous culture. To the best of my knowledge, this type of study, on such broad spatial and temporal scales, is impossible using any other existing method, but feasible by SLIM, as described below.
Figure 5.2. SLIM measurement of synchronized U2OS cell culture over more than 2 days. Black: dry mass vs. time for a synchronized cell population over a 3.2x2.4 mm² field of view obtained by montaging 8x8 microscope images. (10X objective, NA=0.3) Red: cell mean dry mass vs. time. Images show the field of view at 4 and 45 hrs respectively, horizontal edge of image is 2.4 mm.
To study single cell growth in an asynchronous culture and obtain information about cell cycle-dependent growth, I used SLIM in combination with epi-fluorescence imaging. Note that because it interfaces with an existing microscope, SLIM shares the same optical path with all the other channels of the microscope including fluorescence. I imaged YFP-PCNA (Yellow Fluorescent Protein - Proliferating Cell Nuclear Antigen) human osteosarcoma (U2OS) cells, stably expressing YFP-PCNA, which enabled me to monitor PCNA activity and thus progression through S-phase via the fluorescence channel (Fig. B.2). This activity is greatest during the DNA synthesis of the cell cycle and is observed in the localization of the fluorescence signal (its granular appearance), which reveals the S-phase of the cell cycle (Fig. 5.3a-b). This marker has been used extensively in the past to study cell cycle and replication dynamics (see for example [155-157]). Using the fluorescence signal as one marker and the onset of mitosis as the second, it is possible to study cell growth in each phase of the cell cycle separately (Fig. B.2). We measured a culture of U2OS cells for 51 hours, scanning a 1.2 × 0.9 mm² area every 15 minutes and acquiring fluorescence data every 60 minutes as described in detail in the Appendix A. Although a reminiscent halo effect is noticeable in the SLIM images, this does not affect our growth measurements if it is thresholded (Fig. B.6 and B.7). In order to avoid cell damage due to UV exposure, we minimized exposure time and power by using a highly sensitive EM-CCD as discussed in the materials and methods. The consistent growth of the cells and the expected 24 hour cell cycle [158] is a testament to the overall health of the culture. Figure 5.3c shows typical growth curves measured from a single cell as it divides into two cells and then its daughters into four. This ability to differentiate between two daughter cells growing very close together, and to measure their dry mass independently, is a major advantage of SLIM over other methods, including micro-resonators, where such measurements are currently impossible to perform. As a
control, I measured a fixed cell under the same conditions and found a standard deviation 1.02 pg, which is well within the acceptable error range. This error is larger than in the case of the E. coli measurements because the debris that exists in the mammalian cell culture contributes to the measurement noise. This debris is naturally occurring from cellular processes and can occasionally be observed passing through the field of view.

Figure 5.3. SLIM measurement of U2OS growth over 2 days. (a) Dry Mass Density Maps of a single U2OS cell over its entire cycle at the times indicated; yellow scale bar is 25 μm, color bar indicates dry mass density in pg/μm². (b) Simultaneously acquired GFP fluorescence images indicating PCNA activity; the distinct GFP signal during S phase and the morphological changes during Mitosis allow for determination of the cell cycle phase. (c) Dry mass vs. time for a cell family (i.e 1->2->4 cells); the two different daughter cell lineages are differentiated by the filled and empty markers; only one daughter cell from each parent is shown for clarity. Different colors indicate the cell cycle as reported by the GFP-PCNA fluorescence. The dotted black line shows measurements from a fixed cell, which has a standard deviation of 1.02 pg.
The data shows that U2OS cells are typically successful in doubling their mass by the end of G2 and that the daughter cells are typically half of the mass of their parents’ doubled mass following mitosis. One unexpected observation is that the mass continues to increase during mitosis (Fig. B.3). However, after this increase, upon completing cytokinesis, the two daughter cells decrease in mass and begin G1 at exactly half the mass of their parent at G2, which is the generally accepted behavior \[52\]. See Appendix B. and Fig. B.3 for more details on mitosis.

Due to the cell cycle phase discrimination provided by the YFP-PCNA, I can numerically synchronize the population \textit{a posteriori} (Fig. 5.4a). In order to perform this numerical synchronization, I measured the average time of each cell cycle phase and then all the growth curves are re-sampled to fit the respective time windows. The dotted lines in Fig. 5.4a show the results for individual cells and the solid lines indicate the ensemble-averaged data. Although this average is performed on a limited number of cells, clear differences in the growth behavior during the three cell cycle phases can be observed. Figure 5.4b illustrates the differences in the growth rate between the G1, S and G2 phases of the cell cycle. It can be seen that during G2, U2OS cells exhibit a mass-dependent growth rate that is approximately linear and thus indicates an exponential growth pattern. The large standard deviation is to be expected from a small population set growing under heterogeneous conditions in terms of cell confluence. I anticipate that the interaction of a cell with its neighbors must play a role in cell growth and plan to study this aspect with future experiments. Even though further studies are required in order to make universal statements regarding mammalian cell growth, to my knowledge this is the first time that cell cycle dependent mass measurements have been performed.
Figure 5.4. (a) A posteriori synchronization combination of PCNA stain for S-Phase determination and the visual determination of the onset of mitosis allows for the study of cell growth dependence on cell cycle phase, in an asynchronous culture. The figure shows G1, S and G2 dependent mass growth as indicated by color; the cycles of the individual cells were aligned as described above; the x-axis indicates the average time spent in the respective cell cycle phase by all. Open circles indicate single cells data and solid lines indicate ensemble averages by cell cycle phase. It can clearly be seen that the cell growth is dependent on both the cell cycle phase and the current mass of the cell. (b) Dry Mass Growth Rate vs. Dry Mass for the ensemble averages; it can be seen that G2 exhibits a distinct exponential growth rate compared to the relatively low growth measured in G1 and S phases.
5.3 Cell Growth and Motility

Since SLIM provides both imaging and mass measurements simultaneously, it is possible to use it to study the effects of spatio-temporal interactions on cell growth, an ability that is not shared by any other dry mass measurement technology. This ability was demonstrated by measuring the motility of single cell in conjunction with dry mass [159]. As mentioned above, the fundamental processes of developmental biology are differentiation, growth and morphogenesis. Morphogenesis (beginning of shape) is the process that organizes the development spatially and temporally to provide a complex and functional three-dimensional structure. To achieve morphogenesis, cell motility is crucial for positioning in space and time before undergoing growth or differentiation. Thus to truly understand a proliferating and developing cellular system it is necessary to measure both growth and motility. Such a measurement can be achieved with no extra effort using SLIM due to its imaging nature.

Drosophila Schneider S2 cells were transferred onto a glass bottom dish coated with poly-L-lysine (PLL) and the sample was scanned every 10 minutes for a total of 47 hours. PLL promotes cell adhesion through electrostatic interactions and thus inhibits cell motility. The cells were analyzed using image segmentation as described above; in addition to the mass and morphology, the centroid position of each cell at each time point was also recorded. The centroid positions were then used to calculate the mean square displacement (MSD) as:

\[
MSD(\tau) = \left\langle \left[ r(t+\tau) - r(t) \right]^2 \right\rangle,
\]

\[
= \left\langle \left[ x(t+\tau) - x(t) \right]^2 \right\rangle + \left\langle \left[ y(t+\tau) - y(t) \right]^2 \right\rangle,
\]

where \( |r(t+\tau) - r(t)| \) is the mean distance travelled by the cell over the time interval \( \tau \) and angular brackets denote time averaging. This MSD analysis allows for evaluation of how PLL affects cell growth over time. It was found that the cell motility increases with each generation (Fig.
5.5a) and that the average growth rate increases with the increase in motility (Fig. 5.5b). In addition to the single cell analysis, entire cell clusters were also measured to determine the bulk growth properties of S2 cells. Figure 5.5c shows the growth of the clusters in the 3rd and 4th generations once the cells are becoming non-adherent. By studying the relationship between a MSD and growth rate it was shown that S2 cells do not grow normally when attached to PLL substrate. However, the effects of the PLL wear off by the 3rd generation of cells, after which the cells exhibit normal growth trends as quantified by the measurements on cell clusters.

The results on the various cell types discussed above establish that SLIM provides significant advantages over existing cell mass measurement systems: (1) SLIM can perform parallel growth measurements on an ensemble of individual cells simultaneously; (2) spatial and temporal correlations, such as cell–cell interactions, can be explored on large scales; (3) in combination with fluorescence, specific chemical processes may be probed simultaneously; (4) the environment is fully biocompatible and identical to widely used equipment; (5) the imaging nature of SLIM offers the ability to directly monitor cells and their surroundings, elucidating the nature of any artifacts and providing morphological information simultaneously; (6) a lineage study is possible, i.e., a cell and its progeny may be followed; and (7) measurements can be performed on cells ranging from bacteria to mammalian cells [13].
Figure 5.5 Growth rate and motility. (a) Semilogarithmic plot MSD vs. time for all the individual cells tracked. It can be seen that the MSD increases by 3–4 orders of magnitude between the 1st and 4th generations. (b) Semilogarithmic plot of the maximum MSD vs. the approximated linear growth rate for each cell. (c) Dry mass vs. time for cell clusters in the 3rd and 4th generations. Each colored time series corresponds to a single cluster, the solid black line is the average exponential fit for each cluster, with the average time constant, $\tau$, shown for each fit.

5.4 Discussion

Although population level measurements on various cell types reveal exponential or linear growth patterns, we can expect large variability in results from different cell types. My experiments on *E. coli* show that, on average, the cells follow an exponential pattern although there is large variation among single cells in the same population. These types of variations are expected from a biological system and are of scientific interest in themselves; by studying the variations in the growth patterns of single cells under varying conditions we may help elucidate some of the underlying regulatory processes. Since SLIM is an imaging technique we may also simultaneously calculate the volume of regularly shaped cells such as *E. coli*. This allows us to explore questions of cell density and morphology and their roles in mass regulation. For *E. coli* we found that the density is relatively constant, which is consistent with the exponential growth model for this organism [143]. SLIM is also a powerful tool to study the relationship of cell cycle stage, growth and mass measurement in complex mammalian cells.

By taking advantage of the ability of SLIM to be implemented as an add-on to a commercial microscope, we can utilize all other available imaging channels. By combining
SLIM with fluorescence it is possible to combine the quantitative nature of interferometry with the specificity provided by fluorescent molecular probes. In conclusion, the results presented here establish that SLIM provides a number of advances with respect to existing methods for quantifying cell growth: (i) SLIM can perform parallel growth measurements on an ensemble of individual cells simultaneously; (ii) spatial and temporal correlations, such as cell-cell interactions can be explored on large scales; (iii) in combination with fluorescence, specific chemical processes may be probed simultaneously; (iv) the environment is fully biocompatible and identical to widely used equipment; (v) the imaging nature of SLIM offers the ability to directly monitor cells and their surroundings, elucidating the nature of any artifacts and providing morphological information simultaneously (vi) a lineage study is possible, i.e. a cell and its progeny may be followed; and (vii) measurements can be performed on cells ranging from bacteria to mammalian cells.
CHAPTER 6. BREAST CANCER GROWTH KINETICS*

“Throughout the centuries, the sufferers of this disease have been the subject of almost every conceivable form of experimentation. The fields and forests, the apothecary shop and temple have been ransacked for some successful means of relief from the intractable malady. Hardly any animal had escaped making its contribution to hide or hair, tooth or toenail, thymus or thyroid, liver or spleen, in the vain search for a means of relief”-W. S. Bainbridge[160]

The history of cancer treatment [161] has witnessed a tragically slow progression from extreme disfiguring surgeries (for example super-radical mastectomies) [162], to bringing patients to the brink of death with chemotherapy treatments [163], to our current finer understanding of the fundamental molecular and genetic basis [161, 164] of the disease. For example, breast cancer, which accounts for 30% of all diagnosed cases and for 14% of all cancer related deaths in women [165] has been subject to a dramatic decrease (7%) in incidence from 2002 to 2003. This decrease can be directly attributed to a better understanding of the link between estrogen and the growth of breast cancer which was first established more than a century ago [166]. This knowledge led to the development of a class of agents that directly modulate the estrogen receptor (ER) and are now the linchpin of treatment and prevention in ER positive patients [167]. Despite our improved understanding of cancer at the molecular level, screening methods for anti-cancer compounds have remained essentially unchanged for decades.

In the 1950s the realization of the benefits of chemotherapy resulted in the commencement of large scale drug development programs at both research institutes and pharmaceutical companies. Initially, murine models and transplantable tumors were adopted by the National Cancer Institute (NCI) as their primary screen and by 1974 over 40,000 compounds

* The work presented in this chapter was performed in collaboration with Prof. Benita S. Katzenellenbogen and Dr. Anna Bergamaschi in the school of Molecular and Cellular Biology at the University of Illinois at Urbana-Champaign.
were being tested annually [163, 168]. In 1990, NCI adopted a panel of 59 human cancer cell lines grown \textit{in vitro} as its primary assay [169, 170]. To measure cell growth and viability in this new screen, metabolic assays were developed and explored many of which are still in use today [169, 171-175].

One form of these metabolic assays is based on the reduction of a colorless terazolium salt to yield a colored formozan proportional to the number of viable cells. Although these assays are useful for measuring the overall cytotoxic effectiveness of a compound, large numbers of cells have to be used to avoid incorrect conclusions from effects such as variable doubling times [169]. Non-tetrazolium based assays were also developed that are based on dyes that bind electrostatically to basic amino acids [174] and the measured signal is thus linear with the cell count. Despite the practical difficulties involved, one such reagent known as sulforhodamine B (SRB) was eventually adopted for routine screenings. However, both assay types provide indirect measurements of growth; the tetrazolium based assays measure metabolic activity whereas the SRB assay essentially measures total protein concentration. Both methods rely on using large numbers of cells, only provide bulk measurements and are unable to measure time-dependent responses to drugs at the cellular level. Typically, growth assay data is supplemented with fluorescence activated cell sorting (FACS) experiments to provide additional statistical information, study gene expression, and study cell cycle progression. For FACS experiments, cells are removed from normal culture conditions, clusters are separated, and cells are often subjected to various other treatments and labelling procedures. This drastic change in environment is undesirable and its effects on the population phenotype are unknown.

It is becoming increasingly clear that to truly understand the nature of cancer cell proliferation and to develop personalized adjuvant therapies to treat it, quantitative information
on cell growth and morphology is required at the single-cell level. The ideal drug screen would be sensitive enough to rapidly assess the response of a small number of cells to a variety of potential therapies in order to directly evaluate the response of a patient or the efficacy of a particular drug. The major reason such a technology has not been developed is due to the practical difficulties in measuring cell growth at the single-cell level. Here we show that spatial light interference microscopy (SLIM) can be used to address this technological gap [12]. SLIM has femtogram level sensitivity to changes in dry mass and can simultaneously provide this information at both the single-cell and population level [13]. SLIM can also be combined with fluorescence microscopy to measure cell cycle dependent growth [13]. In this work we use the MCF-7 cell line as a model system to demonstrate that SLIM can be used as a highly sensitive drug proliferation assay.

The MCF-7 cell line is a widely used model for studying hormonal influence on breast cancer growth as it is the first ER positive [176, 177] and estrogen responsive [178, 179] human breast cancer cell line to be developed. We measured the growth of MCF-7 cell clusters in standard cell culture media (Veh) under the influence of estradiol (E2), the predominant form of estrogen during reproductive years, and ICI, 182, 780 [180], a complete antagonist of the ER used to treat metastatic breast in postmenopausal women. The results shown here establish that in addition to being a superior proliferation assay, SLIM also provides biologically relevant information at the cellular and cluster level that is not accessible through other methods. Such measurements, in combination with existing molecular assays, have the potential to improve drug design and characterization and also to bridge the connection between our molecular understanding of cancer and its effects on cell growth.
6.1 Effects of Estrogen on MCF-7 Growth Kinetics

Figure 6.1. Measuring cancer cell proliferation using SLIM dry mass measurements. (a) Schematic of experimental setup. A fully automated commercial phase contrast microscope equipped with stage top incubation control and x, y, z scanning capabilities was used to scan a 1.5 mm x 1.2 mm area in each well of a 2-well slide every 30 minutes. The components in the dotted line comprise the SLIM add-on module: Fourier Lens 1 (FL1) projects the pupil plane of the phase contrast microscope onto a Liquid Crystal Phase Modulator (LCPM) which provides control over the phase delay between the scattered and un-scattered light; Fourier Lens 2 projects the phase modulated image onto a CCD. All components of the instrument were synchronized using the CPU. (b) Representative images of a scanned field of view in one of the chambers at 0 hours and 94 hours, the area in the dashed yellow line is enlarged and shown at each time point (yellow scale bar is 50 microns). (c) Average normalized surface area for clusters in each group in the labelled time periods. (d) Average normalized mass for clusters in each group in the labelled time periods. (c-d) Square markers indicate mean, center line is median, top of box is 25th percentile line, bottom is 75th percentile line, whiskers indicate 5th and 95th percentiles, significance was tested using an un-paired t-test, o: p>0.05, *: p<0.05, **: p<0.01, ***: p<0.001. (e) WST-1 proliferation assay measurement at 72 hours.
Measurements were performed in three conditions (see Appendix A for more details on cell culture and treatment): first typical cell growth media as the control vehicle (Veh), second with 10 nM Estradiol (E2), and finally with 1 µM ICI + 10 nM E2 (E2+ICI). For the E2+ICI treatment, cells were grown under E2 conditions for 10 hours before ICI was added. The 10 hour point was chosen to administer the drug since that is the earliest point at which a significant difference between the growth rates of Veh and E2 populations was observed (Fig. B.8). In each experiment a two-well slide was used, with one well under the Veh or E2+ICI treatments and the other under the E2 treatment. A 1.55 x 1.16 mm² area of each well was scanned every 30 minutes using a commercial phase contrast microscope equipped with a SLIM add-on module. As shown in Fig. B.10 the sensitivity of the mass measurement is on the order of picograms for all experiments.

A schematic of the instrument and representative SLIM images from one well are shown in Fig. 6.1 a and b respectively. SLIM maintains subcellular resolution (Fig. 6.1b) over a large area, by scanning each chamber in a mosaic pattern (for more details on the measurement refer to Appendix A). From the large mosaic images, the edges of individual clusters (composed of 2-3 cells at t=0 hours) were traced at each time point and the surface area and total dry mass were measured. In this manner we can analyze both the overall growth trends of each group and the heterogeneity at the cluster level within each population. It should be noted that this type of measurement is currently impossible to perform with any existing proliferation assay.

First, we establish the capabilities of SLIM as a proliferation assay by measuring the effects of estrogen on the relative changes in growth, which is qualitatively similar to the information provided by conventional assays. The quantities of interest here are the relative amounts of growth in size and mass, not the absolute values. To perform this analysis, the mass
and surface area of each cluster is normalized relative to its initial size \((M(t)/M(t=0))\) and \((\text{Area}(t)/\text{Area}(t=0))\). The normalized area and mass for all analyzed clusters were separated into 15 hour bins as shown in Figs. 6.1c and d (see Fig. B.9 for data at all time points and clusters).

It can be seen that although there is not a significant difference in the normalized area at every time point, the differences in the normalized mass are detectable throughout the measurement period. This highlights the importance of measuring mass rather than simply the size of a cluster. It can also be seen that the ICI treatment takes effect rapidly as the E2 group exhibits greater relative growth in the mass within 30 hours. A difference between the E2+ICI and control group can be detected by 60 hours. These results establish that SLIM can detect the effects of estrogen on proliferation rate (size and mass growth) earlier than can be detected with colorimetric assays conducted on large samples. Furthermore, current proliferation assays rely on using a large number of cells whereas the SLIM measurements were performed at the individual cluster level.

For comparison, measurements from a WST-1 assay taken after 72 hours of treatment are shown in Fig. 6.1e. It can be seen that there is a good qualitative agreement between the WST-1 data, which indirectly measures proliferation rate, and the normalized area measurements after 75 hours. However, the normalized mass is higher in the control than the E2+ICI group after 60 hours. These differences are likely due to the fact that the WST-1 assay simply measures the reduction of a tetrazolium dye outside the cell. Although the level of this reduction is related to the metabolic activity of the cells and reflects the number of viable cells in the population, it is not a direct measurement of cellular mass or size. Furthermore, such assays are restricted to providing one number for a bulk population and provide no practical way to study the heterogeneity in the population.
In addition to providing a quantitative understanding of how various treatments affect relative changes in size and mass, SLIM can also measure changes in the growth rate of small cell clusters as a function of time or size. Measuring the growth rate with high sensitivity is more informative than simply measuring relative changes in mass as it provides an understanding of when treatments begin to take effect and how long the effects persist. Dry mass density maps of typical clusters from each group over time are shown in Fig. 6.2a. Figure 6.2b shows the growth rate of clusters in each group vs. time. A significant difference in the growth rate between all three groups can be detected as early as 15 hours (5 hours after ICI treatment was administered), which is 15 hours earlier than the detectable change in both the area and mass. Furthermore, although the normalized mass is greater for the E2+ICI group than the control up to 60 hours, the growth rate of the control exceeds the E2+ICI group by 45 hours. It can also be seen that although clusters in the E2 group achieve much larger relative masses and areas than the control there is no significant difference in the growth rates between the two groups after 90 hours.
Figure 6.2. Cluster growth rate analysis. (a) Dry mass density maps of representative clusters from each group at every 22 hours. The colors indicate the dry mass density at each pixel as shown on the color bar. The yellow scale bar is 50 microns. Note that in the E2 + ICI group, ICI was added to each sample at 10 hours. (b) Cluster growth rate in each group in the shown time period. (c) Cluster growth rate in each group as a function of normalized mass. Solid lines are shown as a guide to the eye to determine how the growth rate is changing as a function of mass growth. (b-c) Square markers indicate mean, center line is median, top of box is 25th percentile line, bottom is 75th percentile line, whiskers indicate 5th and 95th percentiles, significance was tested using an un-paired t-test, o: p>0.05, *: p<0.05, **: p<0.01, ***: p<0.001.
By plotting the growth rate as a function of the normalized mass (Fig. 6.2c), the growth trend (exponential or linear) of the groups can be determined. It can be seen that the mean growth rate of clusters in the E2 and Veh groups continues to increase until a 4-fold increase in mass is achieved, after which the growth rate is either stable or decreases. This trend implies that for approximately two mass doublings both groups exhibit exponential growth, after which the growth is linear. In contrast, the E2+ICI group exhibits exponential growth until a 2-fold increase in mass is achieved, after which the growth is linear. It is important to note here that a doubling in the mass does not necessarily correspond to a complete cell cycle as both estrogen and ICI are known to result in changes in how a cell progresses through the cell cycle; i.e. the doubling time and size and division both may be affected.

To determine how changes at the single-cell level contribute to the measurable changes in relative size, mass, and growth rates, we measured the doubling time and percent change in mean cell size for individual cells that compose the clusters (Fig. 6.3). The doubling time is calculated simply as 

\[
\text{doubling time} = \frac{t_f}{\log_2 \left( \frac{N_{cell}(t_f)}{N_{cell}(0)} \right)}
\]

where \(t_f\) is the last time point, \(t_0\) is the initial time point and \(N_{cell}(t)\) is the cell count at time \(t\). The doubling times for the E2 group were found to be significantly lower than both the Veh and E2+ICI groups (Fig. 6.3a). This data shows that estradiol causes cells to divide at almost twice the rate as the control group and that treatment with ICI almost completely reverses this effect. This data confirms the suspicion that estrogens result in cells “rushing” through the cell cycle, a well-established hallmark of a fast growing tumor. It should be noted that the increase in the doubling time following ICI treatment does not imply that the cell cycle is returned to normal conditions and could result from the cells spending a larger amount of time in a specific phase of the cell cycle.
Figure 6.3. Estrogen modulated changes in proliferation kinetics. (a) Doubling time in each group; the mean doubling time is reduced by 12 hours in the E2 group compared to the Veh and E2 + ICI groups, indicating that adding ICI returns the doubling time to control levels. (b) Percent change in the mean cell mass over the measurement period for each group. A significant decrease in the cell mass is observed in both the E2 and E2+ICI groups compared to the control. (c) Measured doubling time vs. change in mean cell mass for each cluster that was measured; these two parameters can be used to separate the three groups completely and can serve as a growth signature.

The change in mean cell mass over time was calculated by dividing the total mass of each cluster by the number of cells in the cluster. Figure 6.3b shows the percent change in the mean cell mass between the initial and final time points for each cluster. The data shows a significant decrease in the mean cell mass over time in the E2 and E2+ICI cells as compared to the control. This decrease in cell mass and doubling time shows that compared to the control, estrogen results in smaller, faster dividing cells, and that adding ICI results in longer doubling times along with smaller cells. The smaller cells in the E2+ICI groups imply that although the doubling time has
returned to control levels, ICI is affecting the cells’ metabolic activity and ability to grow normally. As shown in Fig 6.3c, the doubling time and change in mean cell mass provide a reliable “growth signature” for each group and suggest that the levels of expression of the estrogen receptor or presence of an ER modulator may be assessed simply by examining these two parameters. To the best our knowledge, these are the first measurements that elucidate how estrogen affects MCF-7 growth kinetics at the cellular level.

6.2 Discussion

The results shown here establish that SLIM measurements of dry mass density can be used as highly sensitive proliferation assays. The main advantages over existing techniques are that SLIM can detect changes in growth kinetics on fewer cells, in less time, and can be used to study differences within a population rather than just providing a number for the bulk growth of a culture. As a comparison, the WST-1 assay works with cell numbers in $10^3$-$10^5$ range [181] whereas SLIM is sensitive to changes in the proliferation of even a single cell. Furthermore, SLIM provides the capability to analyze the growth rate of individual cells and clusters as a function of their mass, providing insight on the mechanism of growth regulation (e.g., whether a size or age checkpoint is being utilized). This information is inaccessible to any existing proliferation assay. Although we have demonstrated these capabilities on just one cell type and growth modulation scheme, this system can be readily used to measure any cell type and treatment.

Our results also demonstrate that measuring growth at the cellular and cluster level not only provides advantages in terms of improved sensitivity and reduced sample sizes, but also provides additional information that is not accessible by existing methods. In particular, we show that under the influence of E2, MCF-7 cells divide faster and achieve lower masses, resulting in
an increased number of cells that are on average smaller. Due to the reduced doubling time, this still results in an overall increase in total mass for E2 when compared to the control group. For cells grown in E2 and subsequently treated with ICI, the doubling times returned to those found in the control; however, the reduction in cell size is still greater than in the control group.

The effects of E2 and anti-estrogens on cell-cycle progression have previously been studied in detail [182-185]. Both rapid and transient effects have been observed due to functional activity in both the nucleus (genomic effects) and cytosol (non-genomic effects) [186]. The transient effects on growth are clearly observable in our data as the differences in the growth rate between the E2 and Veh are observable after 10 hours (Fig. B.8). After the addition of ICI at this time, the differences in growth rate between the E2 and E2+ICI group diverge slowly over time (Fig. 6.2a). The transient effects of E2+ICI are also manifested in how the growth rate changes as a function of increase in cell size (Fig. 6.2b); a clear break in the growth rate can be observed when the ICI clusters approximately double in size.

Estrogens are known to activate transduction cascades which regulate several genes—both positively and negatively—that play key roles in cell proliferation [182, 184, 187-190]. It has also been shown that estrogens cause non-cycling cells (G0 phase) to enter the cell cycle and to rapidly progress through the G1 to the S phase [182, 184, 185]. This rapid progression through the cell cycle accounts for both the decreased doubling time and reduction in average cell mass observed in the E2 group (Fig. 6.3). The greater variance in the growth data for E2 clusters is also likely caused by progressing haphazardly through the cell cycle, resulting in increased mutations and lack of size regulation. On the other hand, ICI a pure-antagonist, has been shown to significantly block MCF-7 cells in the G1 phase. This inhibitory action on the progression through G1 is manifested in the increased doubling time of the E2+ICI group when compared to
the E2 group. ICI also affects transcription of several genes responsible for growth in all phases of the cell cycle. The down-regulation of genes known to be responsible for proliferation, in combination with the increased time spent in G1, is likely responsible for the reduction in the cell size observed in the E2+ICI group [184].

As in the case of the estrogen receptor, all cellular proliferation is ultimately controlled through the modulation of regulatory signaling cascades by growth factors; measuring growth at the cellular level has the potential to bridge the gap between the molecular understanding of cancer growth and the actual tumor growth. Thus it is of great interest is to combine these growth measurements with fluorescence markers for cell cycle phase (as was done previously for U2OS cells [13]) or other proteins known to play key roles in regulating proliferation. Measurement of changes in the growth kinetics as a function of the cell cycle, or more specifically gene activation, will allow for better understanding of the particular action of a compound/drug on cell cycle progression.

In summary, we have demonstrated the potential of SLIM to be used as a highly sensitive proliferation assay for drug screening applications. Although we have focused on a specific model system here, the experimental setup does not need to be altered to measure other cell types and treatments. In addition to measuring growth kinetics, SLIM also simultaneously provides information on cellular morphology and motility [159], and can also be readily combined with other microscopy modalities. A subject of future study will be to understand and characterize the morphological differences that arise as a result of modulating the ER. The biological insights provided by SLIM measurements in combination with other molecular assays will undoubtedly improve our understanding of cancer cell growth in general, and have the potential to lead to improvements in the drug design and characterization process.
CHAPTER 7. NEURAL NETWORK FORMATION*

The emergence of network self-organization in the developing central nervous system is a highly complex and insufficiently understood process [191]. Neurons use a diverse variety of internal and external guidance cues to direct their organization into functional units. In a developed nervous system the spatial arrangement (i.e., location) of individual neurons is known to be closely linked to specific functional activity, a phenomenon which has been studied extensively in the visual cortex [192-194]. This spatial arrangement ultimately determines how neurons connect to each other to form neural circuits. Once a connection is made, neurons communicate with each other using a complex system of neurotransmitters, neurotrophins, peptides, and cytokines [191, 195-203]. The spatiotemporal interaction among individual neurons within a network is the basis of all functional activity in the nervous system and higher level cognitive functions such as sensory perception, memory, and learning. Thus, understanding the formation, stabilization, and behavior of neural networks is a key area of study in neuroscience, developmental and synthetic biology, and regenerative medicine. In order to gain insight into how functional neural networks form and develop, it is necessary to quantify how individual neurons grow and spatially organize with respect to each other, and to understand the dynamics of how mass is transported within connected units, preferably through longitudinal measurements within the same neural cultures.

Neural network formation has been studied by various models, ranging from whole brain animal studies [199, 204] to in vitro dissociated neuron cultures [201, 205]. However, there are many limitations to each of these models. For example, the analysis of in vitro neuron cultures is limited by the relative lack of data that can be obtained from conventional live-cell imaging.

*This work was performed in collaboration with Prof. Steve Stive and Dr. Anirban Majumder at University of Georgia, Athens and Prof. Martha Gillette and Chris Liu in the Department of Cell and Developmental Biology. Ryan Tapping and Mike Xiang contributed image analysis.
techniques such as phase-contrast or differential interference contrast (DIC), which only report on morphology and must be coupled with other methods such as fluorescence to provide functional information. Fluorescence imaging techniques have been used to image vesicle transport and synapse formation [206]. More recently, fluorescent voltage-sensors have been used to optically measure electrical activity [207, 208]. However, the limitations inherent to fluorophores, such as photo-bleaching and photo-toxicity, do not allow for continuous monitoring of these processes during dynamic neural-network function on the relevant time scales of days to weeks.

Recent advances in micro-fabrication methods have led to several innovative approaches to study in vitro neural cultures. For example, multi-electrode arrays have been extensively used for measuring electrical activity of neural cultures [209, 210]. However, due to their limited spatial resolution they only provide an incomplete electrophysiological picture of the system. Micro-patterning methods have enabled the design of spatial order that generally mimics what is found in live neurons [201, 211-213]. Furthermore, recently developed microfluidic approaches are amenable to rapid, high content analysis of various cell properties [191, 200, 202, 214-218]. Such methods provide important information on how cells behave when spatial cues are provided but are inherently limited in their capabilities to address how spatial order naturally emerges in a culture.

Little is known about the neuron culture in terms of spatial organization, energy use, and mass transport. Our current understanding of these phenomena is limited by the lack of available technologies to quantitatively measure a forming neural network at multiple temporal and spatial scales, in a high throughput and minimally invasive manner. In this work we show that by using a recently developed optical interferometric technique known as spatial light interference
microscopy (SLIM) [12, 32] we can measure several fundamental properties of the developing network on a broad range of spatial (sub-cellular to millimeter) and temporal scales (seconds to days) in a completely label-free and non-invasive manner. The results indicate that measurements on cell growth, intracellular transport, and scattering structure function are quantitative indicators of neural network emergence.

As described in the previous chapters, SLIM is a quantitative phase imaging (QPI) modality which measures the pathlength shift distribution of an optical wave front as it passes through a cell [2]. Due to its common-path geometry, broadband illumination, and ability to interface with existing phase contrast microscopes, SLIM is a highly sensitive and versatile QPI modality [6]. It has been demonstrated that SLIM has spatial and temporal path length sensitivities of 0.3 nm and 0.03 nm, respectively [12]. When translated into dry mass this sensitivity corresponds to changes on the order of femtograms. In combination with SLIM’s multimodal capabilities this has allowed for the measurement of single-cell dry mass growth in a cell cycle dependent manner [13]. When this measurement is performed with high temporal resolution it can be used to quantify changes in inter- and intra-cellular mass transport characteristics. This method is known as Dispersion Phase Spectroscopy (DPS) [42, 64]. While the mass growth measurements provide information on the overall metabolic activity of the system, DPS reports on whether energy is being used to transport materials in a deterministic manner. Our results show that in a developing neural network, changes in the overall growth rate and network morphology are intrinsically linked to the mass transport behavior of the system.

In addition to dry mass growth and transport, the measurement of the complex optical field allows for direct measurements of the angular scattering spectrum from the sample [100, 219-221]. It has recently been shown that despite the complexity of the network formation
process *in vivo*, neurons will self-organize in a non-random manner *in vitro* without the provision of any additional external environmental cues [218], that is, relying only on chemical gradients. Previous work on analyzing this organization relied on detecting the location of individual neurons in an image and was, thus, time-consuming and prone to error. The angular scattering data measured by SLIM allows for monitoring how spatial correlations in the culture evolve over time at various spatial scales simultaneously, allowing for a way to quantify organization at all spatial scales simultaneously without the need for image segmentation. It has been shown that the shape of the angular scattering power spectrum reports on the large scale spatial organization of tissue [222] and on the fractal properties of the spatial organization of the clusters and cells within the clusters. Performing this measurement on a developing neuronal network revealed that changes in spatial correlations at certain scales are linked to the overall growth behavior of the culture.

The ability to simultaneously measure the metabolism, transport characteristics and spatial correlations of an evolving neural network provides a unique and efficient way to quantify the behavior of such systems. In this work we show that changes in the trends of these fundamental properties are linked temporally and across broad spatial scales. Since all these measurements are obtained from the same quantitative phase data using a completely non-invasive and label-free method, they allow for monitoring the evolution of an *in vitro* neural network in an unprecedented manner.

### 7.1 Measuring a Forming Network

The human embryonic stem-cell-derived neurons used in this study [223, 224] hold therapeutic potential [225, 226] and express multiple, well-established neuronal markers, including MAP2 [227]. The development of the neuronal network was characterized using
several analysis techniques, both to understand the dynamic behavior at the single-cell level and to characterize the metabolic activity and spatial organization of the system population. First, the system was characterized in terms of the total dry mass of the entire field of view (Fig. 7.1). This procedure provides a broad view of the metabolic activity of the culture. Second, mass transport in the system was characterized using DPS (Fig. 7.2). Finally, the angular light scattering spectrum was calculated at each time point in the 24 hour movie to characterize the spatial organization of the culture (Figs. 7.3 and 7.4).

**Dry Mass Growth**

The total dry mass of the 0.87 mm × 1.16 mm area was calculated at each time point (every 10 minutes), as described in Appendix A. Figure 7.1a shows that the distribution of mass is drastically different between 0 hours and 24 hours, changing from compact isolated clusters to a highly connected network. After an initial period of growth that lasts 10 hours, the mass growth levels off and then begins to decline after 20 hours. The images suggest that the mass increase during the first 10 hours is largely caused by the extension of neurites from each cluster; once the connections are well established, there is no significant increase in the total dry mass of the culture. In terms of the culture’s metabolic activity, this time development indicates a shift from using energy to form connections between clusters, to consuming energy for spatial reorganization and deterministic transport of materials. This hypothesis is supported by the mass transport and scattering measurements reported below.
Figure 7.1. Dry mass growth of the human neuronal cell culture. (a) Dry mass density maps acquired at 0, 10 and 24 hours. The yellow scale bar corresponds to 200 µm. (b) Total dry mass vs. time of the entire field of view. Grey markers are raw data, the solid black line is the average over 1 hour, and the error bars show the standard deviation of the averaged data. The majority of the mass growth occurs between 0 and 10 hours, the time during which the cells are extending processes most actively.

Mass Transport

To quantify changes in the mass transport characteristics at the single cell and cluster level, DPS analysis was performed on the 40×, 0.5 Hz movies taken at t=0 and t=24 hours. Six and 8 movies were acquired and analyzed at t=0 hours and 24 hours, respectively.

The data acquired at t=0 hours shows that the mass transport is primarily diffusive. In sharp contrast, when the culture is highly connected after maturing for 24 hours, a large amount of vesicular transport occurs in the processes that connect the clusters. The dispersion analysis shows that the mass transport is dominated by directed motion, which can be attributed to bidirectional transport that occurs along defined tracks, facilitated by molecular motors such as dynein and kinesin [228, 229]. These results support the hypothesis that, initially, the culture
consumes energy primarily to extend processes towards other clusters. After 24 hours, a statistically significant increase in the mean advection velocity was found, indicating a shift toward deterministic transport of mass. Interestingly, there is no significant change in the mean diffusion coefficient. This result indicates that, at small scales where diffusive transport is dominant, the overall mass transport is not affected by network formation. The spread of the diffusion coefficients is much larger at 24 hours, suggesting that in network formation, local transport becomes more inhomogeneous. These mass transport measurements provide a way to quickly quantify and characterize the morphological connectivity of a network, which is a prerequisite for the emergence of functional activity.
Figure 7.2. Mass transport results for the human neuronal cell culture, obtained from analysis of dispersion phase spectroscopy (DPS) data. (a) Radial profiles of the dispersion maps calculated from high resolution movies acquired at a rate of 0.5 Hz at 0 (red) and 24 (black) hours. Faint lines are the profiles from individual movies and the darker lines are the averages calculated from these movies. A clear shift from a quadratic to a linear behavior can be seen as indicated by the dashed blue lines. (b) Comparison of mean advection velocities obtained from DPS analysis. There is a statistically significantly difference between the mean values at 0 hours and 24 hours; indicating a shift from diffusive to deterministic transport. (c) Comparison of mean diffusion coefficients obtained from the DPS analysis. There is no statistically significantly difference between 0 hours and 24 hours, however, there is an increase in the range of the measured values. The whiskers indicate one standard deviation above and below the mean (gray dot).

Angular Scattering

When measuring mostly transparent, optically thin samples, such as neurons, we can assume that the amplitude of the optical field is left unperturbed and that only the phase measured by SLIM is altered by the sample. In this case, SLIM provides a measure of the complex optical field,
\[ U(r,t) = |U(r,t)|e^{i\phi(r,t)}, \] at the sample plane. This field may then be numerically propagated to the far-field or scattering plane by simply calculating its spatial Fourier transform [100] as \[ \tilde{U}(q) = \int U(r)e^{-iqr}d^2r, \] where \( q \) is the scattering wave vector. The modulus square of this function, \( P(q) = |\tilde{U}(q)|^2 \), is related to the spatial auto-correlation of the measured complex field through a Fourier transform, \[ \int P(q)e^{iq\cdot r}d^2q = \int U(r')U(r - r')d^2r', \] and thus describes the spatial correlation of the scattering particles in the sample. Since the signal is measured and reconstructed in the image plane, rather than in the far field as in traditional scattering experiments, all the scattering angles that are allowed by the numerical aperture of the microscope objective are measured simultaneously. This greatly enhances the sensitivity to scattering compared to the traditional approach of goniometric measurements [100].

Since SLIM is an imaging modality it is possible to connect changes in the scattering intensity at certain angles to morphological changes at relevant spatial scales. Figure 7.3a shows the scales of features such as the cell body diameter, cluster size and distance. Once the angular scattering spectrum is calculated as described above, the intensity is averaged over rings of constant scattering angle or scattering wave vector. This radial average, calculated at each time point, is shown in Fig. 7.3. The dashed lines in Fig. 7.3b divide the profile plot into different spatial scale regions, which are associated with different structures in the culture (see Fig. 7.3a). Figure 6.3b shows that, as the culture matures, the profile slope changes in different spatial ranges. The greatest changes are observed in spatial scale regions corresponding to the size of the clusters (605- 315 \( \mu \text{m} \)), the distance between clusters (210-105 \( \mu \text{m} \)), and the distance between cells (90- 35 \( \mu \text{m} \)). Specifically, from 0-24 hours, the power spectrum narrows monotonically, which indicates a broadening of spatial correlations. Remarkably, our label-free imaging is able
to monitor the temporal evolution of the neuron culture and quantify the enhancement of spatial correlations over time. Note that the slopes in spatial ranges corresponding to the size of individual soma (30-15 μm) remain constant, which suggests that local morphology remains essentially constant.

Figure 7.3. Angular scattering analysis of SLIM images. (a) Dry mass density map of the culture at 24 hours. Various spatial scales have been labeled to aid in the interpretation of the angular scattering maps. (b) Radially averaged profile plots of the angular light scattering map from each time point in the data. The plots are color coded such that the color corresponds to the time point shown on the color bar. The dashed lines and corresponding labels indicate the various spatial ranges into which the profiles were divided. The slopes in each region change with time, with the greatest changes in slope occurring at the spatial scales corresponding to the size of the clusters, the inter-cluster distance, and the inter-soma distance.
In order to evaluate these changes in the slope of the angular scattering profiles quantitatively, the profiles were analyzed in the various spatial scale regions indicated by the dashed lines of Fig. 7.3b. Each region was then fit to a power law function of the form \( P(q) = cq^\alpha \). The power of the exponential relationship, \( \alpha \), for each of the spatial ranges vs. time resulting from these fits is shown in Fig. 7.4 for each spatial region. Figure 7.4 a-c show that \( \alpha(t) \) exhibits the greatest changes in the 35 mm to 630 mm spatial scale regions. Furthermore, the power law behavior associated with the largest spatial scales (Figs. 7.4a, b) exhibits the greatest changes after 10 hours, as the larger clusters begin to merge. By contrast, the power law behaviors associated with spatial scales corresponding the inter-soma distance (Fig. 7.4 c) and the width of individual neurites (Fig. 7.4 f) stabilize after 10 hours. Figures 7.4d and e, which correspond to the size of individual soma, suggest that the cellular and subcellular morphology remain essentially unchanged. Notably, the changes in spatial correlations and the stabilization of the growth rate (Fig. 7.1) occur at approximately the same time. This suggests that there is active reorganization of the system once the neurons have already extended neurites to connect to neighboring clusters.
Figure 7.4. Power law behavior at various spatial scales of the angular scattering profiles. (a) 605-315 µm range, corresponding the largest clusters, (b) 210-105 µm, corresponding to smaller clusters and inter-cluster distances, (c) 90-35 µm, corresponding to distances between cell soma, (d) 35-15 µm, size of individual soma, (e) 15-4 µm, features smaller than the soma, (f) 4-2.5 µm, width of individual processes.

7.2 Discussion

In summary I have shown that, using quantitative phase imaging it is possible to characterize several fundamental properties of a forming human stem cell derived neuronal network. To my knowledge this is the first time that mass growth in a neural culture has been measured and linked to changes in transport dynamics and spatial organization. I found two distinct phases in the growth rate of the culture, the first of which is marked by mass accumulation and the second by a relatively stable total mass. From the imaging data, I identified that the first phase corresponds to extension and growth of neurites and that the second phase is characterized by the
aggregation of clusters and spatial reorganization of cells. By performing mass transport analysis I determined that there is a significant increase in deterministic transport after 24 hours, suggesting that the energy consumption of the culture has shifted from extending neurites to transport of materials along these extensions. Finally, by measuring the angular scattering from the culture I quantified the temporal evolution of spatial correlations in the system. Interestingly, the data shows that the increase in spatial correlations at the scales of cluster size and inter-cluster distances is temporally coincident to the shift in growth rate and dominant mode of intra-cellular transport. This observation suggests that in this system the majority of spatial organization occurs after clusters in the network are already well connected.

The methods and analysis that have been developed and proven here can be applied to a variety of studies in neuroscience and beyond, with the potential to transform how basic behavior such as growth, spatial organization, and transport are measured and quantified. Since SLIM is easily interfaced with existing microscopes it is also possible to incorporate commonly used tools to provide external perturbation such as through microelectrode arrays, optical tweezers, microfluidics and micropipettes. My analysis provides a practical way to characterize the spatial correlations in neural networks at all relevant spatial scales simultaneously. Combining external stimulation with the technology demonstrated here, it will be possible to quickly assess the effects of various forces on the natural evolution and maturation of a neural culture, opening the door to develop novel experimental strategies and, potentially, new therapeutic methods. The linkage of informative SLIM measurements with the *in vivo* outcomes of the novel transplanted cells, such as level of sensory and motor function, will likely expedite efficacy and optimization for newly developed neural stem cell therapies.
CHAPTER 8. SUMMARY AND OUTLOOK

Since their inception, the fields of cell biology and microscopy have been progressing hand in hand. Unfortunately, as far as most biologists are concerned, the state of the art for intrinsic contrast imaging remains Zernike’s phase contrast or DIC, with the majority now using fluorescence imaging to meet their needs. In this thesis I have demonstrated that quantitative phase imaging not only provides superior contrast but also provides unprecedented information about fundamental properties of a biological system. In particular, by measuring a dry mass density map important insight is gained into the basic behavior of cells. It is not being suggested that QPI systems replace fluorescence imaging, but rather that they add complementary information and provide a more detailed and biologically relevant picture of the phenomena being studied. In some cases, such as in measuring single cell mass, the capabilities of QPI are unique and provide the capability to ask questions and develop technologies which were previously unimaginable. It is clear that in the near future QPI methods will play a significant role in advancing biomedical imaging and will aid in the transformation of biology from an empirical to a quantitative science. In this chapter I will briefly summarize the results presented in this thesis and also discuss potential future directions.

Over the past decade QPI has been well established as an important tool for quantitative biology. In Chapter 2, I described the general principles for building QPI instruments and described the advantages and disadvantages of various approaches. These trade-offs between various optical configurations and reconstruction methods have been thoroughly explored and characterized. Furthermore the theory behind the measurements is well understood and has led to important advances in analyzing the data as I described in Chapter 3. The demonstration of these tools and analysis methods on various applications has shown that QPI can serve as an important
tool in biological imaging. However, the exploration of biological applications has remained shallow and thus QPI methods have so far failed to gain a foothold as an indispensable tool in any scenario. The goal of my thesis was thus to explore various applications of QPI in detail and prove that QPI measurements provide biologically relevant information that is unobtainable with any other method. In accomplishing this goal I have developed new hardware and analysis tools and made several discoveries. In each application area that I explored, I have demonstrated how QPI can be used to complement existing methods and also identified future areas of study that would not be possible with any existing method.

The first application that I explored was using QPI for red blood cell cytometry (Chapter 4). The goal of this work was to develop a highly sensitive and extremely stable point-of-care blood screening tool that has the capability to be deployed in field settings. While working on this instrument I also developed new analytical tools that characterize the morphology of individual red blood cells and connect this information to various commonly observed pathological conditions. The combination of the new imaging tool and analysis has the potential to quantify and automate blood smear analysis. This would save money in terms of reagents and time spent on analysis while also providing previously unavailable quantitative morphological information. I later validated this approach in the clinic in a trial involving 32 patients that compared the parameters that are common between my analysis and those available from a clinical hematology analyzer. This trial established that my method is both more sensitive than the currently used clinical tools and also provides additional clinically relevant information. In my work on morphological analysis of red blood cells I realized that one parameter that was consistently ignored by others working in the field is the single-cell hemoglobin concentration. To address this problem I developed a new imaging modality that combines micro-absorption
spectroscopy with quantitative phase imaging (QPI) to provide oxygenation state, hemoglobin concentration and morphological information at the single-cell level [48]. My ground work on blood screening is now being followed up by several new members of our and other groups (42 citations to my 3 papers as of May 2013), indicating that it is indeed a viable approach, which with further effort and development has the potential to transform the practice of hematopathology.

The second application I discussed in Chapter 5 is using QPI to study cell growth at the single-cell level. Determining the growth patterns of single cells is still an open and highly active area of research with several high impact publications on almost a monthly basis. The reason that studying this phenomenon in detail has remained elusive is due to the fact that cells weigh in on the order of picograms and only double their mass over their cell cycle, which makes it necessary to measure their mass with femtogram accuracy. I showed that such a measurement is possible using SLIM and that SLIM is superior to other contemporary methods such as microresonators since it has the capacity to measure adherent and non-adherent cells, all different cell types, provides simultaneous information on morphology and motility and can easily be combined with any other microscope modality such as fluorescence to provide additional dimensions of information. I have leveraged all these capabilities in different cell models, establishing SLIM as an ideal instrument for studying cell growth with the ability to answer several open, important biological questions. My initial work on cell growth was on measuring single $E.\ coli$ cell and their progeny. I found evidence of exponential growth at the single cell which agreed well with previous measurements. This study established SLIM as a tool for studying single cell growth. Next, I turned my attention to a more complex system, namely human osteosarcoma (U2OS) cells. The goal of the study was to develop a method to study cell growth as a function of cell
cycle phase. To accomplish this I used an YFP-PCNA transfected version of the U2OS cell line that provided information on the cell cycle phase at the single-cell level through simultaneous fluorescence imaging. This eliminates the need to synchronize the cell population and allows for studying how single cells transition between various phases of the cell cycle. Using this method I found that the growth rate varies throughout the life cycle of the cell, with the highest growth rate in the G2 phase. This overturns the previous commonly accepted wisdom of constant exponential growth patterns.

It is clear that using QPI to study cell growth has the capacity to answer many open questions such as how cells transition between various phases of the cell cycle, how motility, adherence and density affect these patterns, the effects of nutrient availability and gradients among many others. Answering these questions will have a far-reaching impact on both basic knowledge of cell behavior and clinical implications in terms of how various therapeutic agents alter growth at the single-cell level. One important potential application of the cell growth measurement capabilities is a cell proliferation assay. Since I believe that this application holds the highest potential for immediate impact, I explored it in detail by using a model system of breast cancer cells.

In Chapter 6, I showed that SLIM measurements of dry mass can be used as highly sensitive cell proliferation assays with several advantages of existing techniques. Namely, the SLIM based assays are more sensitive and can thus measure changes in growth at the single cell level, compared to the tens of thousands of cells required by currently used assays. The higher sensitivity also leads to detection of effects in shorter times. Furthermore, SLIM can be used to analyze the heterogeneity within a population and how that affects its response to a drug. Since SLIM provides information at the single-cell level and can also measure cell cycle dependent
growth, the specific mechanism of drug action may also be determined. In the MCF-7 model system I showed that adding estradiol results in increased overall growth resulting from smaller, faster dividing cells and that adding an estrogen receptor antagonist results in smaller slower cells with long doubling times. This method can be readily adapted to test the response of any of the NCI primary screen cell lines to any compound.

The final application I discussed in Chapter 7 is neural network formation. A neural culture is a type of system that lends itself extremely well to the quantitative and highly sensitive nature of QPI methods. Using SLIM I investigated self-organization of a network in human stem cell derived neural cultures. To accomplish this I adapted various analytical tools that provide information on the spatial organization, mass transport characteristics and overall mass growth. In the stem cell derived neurons I showed that two phases of growth and development exist and they are linked. In particular the mass of the culture grew for 12 hours after which it stabilized, once the mass growth was stabilized clear changes in the spatial organization and mass transport were observed. Since there is no existing method that provides such information it was difficult to put this knowledge in the context of a biological phenomenon. To address this I am now conducting controlled studies in which various aspects of the culture environment are modulated and the effects are measured using SLIM. In this manner I will establish a link between SLIM data and physiological conditions in a neural culture.

As in the case of neural networks, I believe that the next step for QPI is to integrate with existing methods that allow for precise control of the cellular microenvironment. These include microfluidics for creating chemical gradients and simulating physiological conditions, substrate modification for providing mechanical stimulus, micro-electrodes for stimulation and detection,
and of course combing fluorescence microscopy and genetic engineering for examining gene expression and cell phenotype.

In the future I hope to leverage the knowledge and experience I have gained here to solve key problems in biology and medicine. Based on the results I have presented in this thesis I believe that it is clear QPI can be used in both clinical and research lab settings. As I have demonstrated through various applications the unique data acquired by QPI can help improve our understanding of any biological system. As biology continues to become a more quantitative science I believe that QPI technologies will play an essential role in answering key questions in the areas of cell growth, dynamics, organization, differentiation and beyond.
APPENDIX A. LIVE CELL IMAGING METHODS AND MATERIALS

E. coli

E. coli MG1655 cells were cultured overnight in LB (Luria Broth). The overnight cultures were sub-cultured by dilution (100x) into commercial M9CA media with Thiamine (Teknova M8010). After the culture reached an optical density (OD) of ~0.1 the cells were concentrated to an OD of ~0.4 and 2 µl of cell culture were pipetted onto a glass-bottom dish (In Vitro Scientific D29-20-1-N). The cells were covered by an agar slab (1.5% Agarose, M9CA media, 1mm thickness), 70 µL of H₂O was pipetted onto the edge of the dish (never in contact with the sample) to mitigate drying of the agar. The dish was then covered with a circular cover slip to reduce evaporation, and transferred to the microscope for imaging. For the fixed cell measurements 1 ml of ~0.2 OD cell culture is centrifuged, the resulting cell pellet is then mixed 1 ml of 3.7% paraformaldehyde (Fisher-Reagents p531-100), after 20 minutes the cells are washed with PBS twice (diluted from 10X stock Teknova p0195).

For imaging, cells were kept at 37°C with an incubator XL S1 W/CO₂ kit (ZEISS catalog #1441993KIT010). Time-lapse SLIM images were acquired once a minute with a Zeiss Plan-Apochromat 63x/1.4 Oil PH3 M27 (ZEISS catalog # 4207819910000). The sample is also scanned in z with a slice spacing of 0.280 µm and a total of 10 slices. The exposure time was 35 ms for each image at full lamp power (3200K, or 10.7V), the transmission shutter was closed before and after each scan.

U2OS

U2OS cells were grown in Dulbecco’s Modified Eagle Medium (DMEM) containing high glucose, supplemented with penicillin-streptomycin and 10% fetal bovine serum (Hyclone). Lipofectamine 2000 (Invitrogen) mediated transfection was carried out in U2OS cells as per the manufacturer’s instructions, followed by G418 selection (600µg/ml) to generate
the YFP-PCNA stable cell line. For the synchronized population measurements, cells were arrested at the G1/S boundary, by adding 2mM thymidine. After 24 hrs, cells were washed thrice with fresh medium, grown for 12 hrs, and incubated with 2mM thymidine for an additional 24 hrs. Cells were then released for live cell imaging. For the fixed cell measurements, cells were fixed in 2% formaldehyde for 15 min at room temperature and then washed twice by phosphate-buffered saline (PBS).

For the synchronized U2OS measurements, cells were transferred to a "closed" cultivation chamber (POC-R cell cultivation system, Zeiss) and kept at 37°C with an incubator XL S1 W/CO2 kit (ZEISS catalog #1441993KIT010) and a heating insert P S1/Scan stage (ZEISS catalog #4118609020000) in L-15 medium (minus phenol red) containing 30% FBS. The medium was automatically refreshed every 4 hours using a syringe pump (Harvard pump 11 plus advanced dual syringe with dual RS-232, Harvard Apparatus) controlled by a Labview program developed in house. The pumping rate was set to 150 μl/min and a total of 600 μl was pumped, which is larger than the volume of the perfusion chamber, to ensure complete replacement of the growth media. The imaging details are the same as the cell cycle study described below.

For the cell cycle study, cells were transferred to MatTek dish (35mm dishes, No.1.5 glass thickness and 10mm well diameter) and kept at 37°C with an incubator XL S1 W/CO2 kit (ZEISS catalog #1441993KIT010) and a heating insert P S1/Scan stage (ZEISS catalog #4118609020000) in L-15 medium (minus phenol red) containing 30% FBS. The dish was filled with culture medium (7ml) and covered with a cover glass (diameter 42mm) to prevent possible evaporation. No noticeable medium loss was observed during the imaging interval of two days, due to the cover glass on top of the dish and the continuously supply of moisturized CO2 gas into
the chamber. Time-lapse SLIM images were acquired with a Zeiss EC Plan-Neofluar 10×/0.3 PH1 M27 objective (ZEISS catalog #4203419911000) and the corresponding fluorescence images were recorded using a Zeiss EC Plan-Neofluar 40×/0.75 PH2 objective (ZEISS catalog #4203619910000). It is important to note that for the SLIM measurements a lower numerical aperture was used here in order to ensure that the entire dry mass of the cell is captured as explained in detail in the Appendix B and Fig. B.4 and B.5. Excitation light for the fluorescence measurements was provided by a X-Cite 120XL package (120W HBO/Halide fluorescence illumination, ZEISS catalog #4108092050000) and a FITC filter set (ZEISS catalog #4236060000000). Every fifteen minutes, the sample was scanned in an 3 × 3 tile pattern to achieve a total field of view of 1.2 × 0.9 mm², while a z-stack of 7 slices was taken with slice spacing 4 μm which is optimal selected by ZEISS Axiovision software (ZEISS catalog #4101300300000). The exposure time was 8 ms for each image at full lamp power (3200K, or 10.7V) and the total scanning time for the multidimensional acquisition was 57 seconds. The transmission shutter was closed before and after each scan. At least 52 hours of data was acquired in this manner for each experiment. The maximal projection was used for the processed z-stack phase images in order to minimize the phase oscillatory behavior due to the defocusing effect, which is due to either the focus drift of the system or the movement of the cell. The fluorescence images were taken every hour in a 6 × 5 tile pattern to get a total field of view 1.2 × 1.0 mm, centered on 3 × 3 tile pattern. A highly sensitive EMCCD camera (Princeton Instruments, PhotonMAX 512B) located at the bottom port of the microscope was used for fluorescence image acquisition. The exposure time for each fluorescence image was 60 ms, the lamp power was set at 12.5% of the maximum lowest available for X-Cite 120XL (120W HBO/Halide fluorescence illumination, ZEISS catalog #4108092050000) and the total scanning
time was 21 seconds. The reflection shutter was controlled by the ZEISS Axiovision software so that individual cells were exposed to the excitation light for only 60ms at a time. A careful adjusted reflection illumination field aperture assures only minimum light leakage exists on the neighboring cells during mosaic scanning.

**MCF-7**

Commercially available MCF-7 cells were obtained from the American Type Culture Collection (Manassas, VA) and were cultured in MEM (Sigma-Aldrich Corp, St. Louis, MO) supplemented with 5% calf serum (HyClone, Logan, UT), 100 µg/ml penicillin / streptomycin (Invitrogen, Carlsbad, CA), and 25 µg/ml gentamicin (Invitrogen). Cells were then seeded in phenol-red free MEM containing 5% charcoal dextran treated calf serum to incubate for 4 days. Two chamber slides (Lab-Tek) with glass bottom coverslips were used to allow for side by side imaging of the control and treated samples. The media was changed on day 2 and 4 prior to treatment with the control vehicle or ligand treatments (Estradiol (E2, 10 nM) and ICI 182780 (ICI Fulvestrant, 1 µM)). During imaging the cells were kept at 37 °C and in a 5% CO₂ atmosphere with an incubator and heated stage insert. Each well was scanned every 30 minutes in a 4x4 tile pattern with a Zeiss EC Plan-Neofluar 10×/0.3 PH1 objective providing a total field of view of 1.55 x 1.16 mm. The exposure time was 15 ms for each image at full lamp power (3,200 K, or 10.7 V). For the colorimetric measurements a WTT-assay (Roche, Basel, Switzerland) was used, absorbance was measured at 450 nm using a BioRad 680 Microplate Reader.

**Stem cell derived neurons**

Commercially available differentiated human neuronal cells (hN2 from ArunA Biomedical, Athens, GA) were used. Cells were grown in 35 mm Poly-l-lysine coated glass bottom dishes (20mm well diameter, MatTek) which were first coated with Matrigel. AB2 Basal Neural
Medium was supplemented with ANS supplement (both from ArunA Biomedical), Penicillin-streptomycin, GlutaMAX (both from Invitrogen) and Leukemia Inhibitory Factor (LIF, from Millipore). Cells were moved from the incubator to the imaging system 6 hours after plating. During imaging the cells were kept at 37 °C and in a 5% CO₂ atmosphere with an incubator and heated stage insert (Zeiss). For the 24 hour dataset, the sample was scanned every 10 minutes in a 3x3 tile pattern with a Zeiss EC Plan-Neofluar 10×/0.3 PH1 objective providing a total field of view of 1.2 x 0.9 mm. The exposure time was 15 ms for each image at full lamp power (3,200 K, or 10.7 V). Higher resolution datasets were recorded at 0 and 24 hours using a Zeiss EC Plan-Neofluar 40×/0.75 at a frame rate of 0.5 Hz for 5 minutes with a 50 ms exposure time.

**Primary Rat Neurons**

Primary postnatal day 1 rat hippocampal neurons were plated in a 35mm poly-L-lysine coated glass bottom dish, with a 22mm working diameter (World Precision Instruments). Neurons were grown in Neurbasal-A (Invitrogen) supplemented with L-Glutamine, penicillin/streptomycin, and B27 (Invitrogen). Media was changed every other day. Cells were imaged once every 24 hours for 5 days and were incubated in a Forma-Scientific 1000 series water-jacketed incubator at 37 °C and in a 5% CO₂ atmosphere. Every 24 hours, the cultures were removed from the incubator, imaged, and then returned to the incubator. During imaging the cells were kept at 37 °C and in a 5% CO₂ atmosphere with an incubator and heated stage insert. Each well was scanned in a 7x7 tile pattern with a Zeiss EC Plan-Neofluar 10×/0.3 PH1 objective providing a total field of view of 2.71 x 2.03 mm. The exposure time was 5 ms for each image at full lamp power (3,200 K, or 10.7 V). In order to image the same area every day, a marker was placed on the bottom of each dish using a permanent marker.
Figure B.1. E. coli Volume and Density. (a) Volume Growth Rate vs. Volume for 20 cells; faint circles indicate single data points from individual cell growth curves, dark markers show the average and the dashed lines is a linear fit through the averaged data; the slope of this line, $0.011 \text{ min}^{-1}$, is a measure of the average growth constant for this population. (b) Volume vs. Dry Mass for the cells shown in a; it can be seen that the relationship is linear, indicative of a constant volumetric mass density.
Figure B.2. YFP-PCNA for detecting S-Phase. Images show typical DAPI and YFP-PCNA fluorescence images at the indicated cell cycle phases. It can be see that S-Phase is clearly recognizable from YFP-PCNA signal, whereas the distribution of the DAPI nuclear stain remains constant throughout the cell cycle.

**U2OS Growth During Mitosis**

The kinetics of mass growth during mitosis is extremely interesting and merits further investigation, especially because the cell undergoes significant shape changes. For now, in order to demonstrate that SLIM can maintain accuracy during such extreme morphological changes, we studied the mass change during mitosis. Figure B.3 shows the growth curve for U2OS cells undergoing mitosis. As evident in the figure, cell morphology changes drastically during mitosis; on the other hand, cell mass continues to grow monotonously during all of mitosis. The cell mass is also relatively conserved during prophase, prometaphase and metaphase, where the morphology of the cell changes from flat to spherical, which confirms that our measurement is robust and not susceptible to drastic changes in cell morphology. For the cells shown in Fig. B.3, the measured growth rate is 1.6 pg/min. We hypothesize that, while this change in cell geometry does not affect our optical measurement, it must play an important role in cell growth regulation since the rounding up and flattening down of the cell takes place with significant changes in the ratio of the volume to the surface area. It is apparent in our SLIM data that occasionally cells
release micron-size vesicles (blebs), which potentially can function as negative feedback for cell growth and would be an interesting direction for future research.

**Figure B.3. SLIM measurement of U2OS mass change over mitosis.** Blue dot lines show 8 mitotic cells and the red solid line shows their average. All the cells have been synchronized according to the metaphase *a posteriori*. Colorbar indicates dry mass density in pg/μm². Scale bar: 20μm.

**Measuring the integrated phase delay through a thick object**

When measuring thick objects such as the U2OS cells shown in this thesis, it is important to consider the depth of field of the objective being used. In order to ensure that the phase that we measure is the axially-integrated phase delay through the cell, we used a 10x/0.3 NA objective which has a depth of field of ~8.2 microns according to the manufacturer (ZEISS catalog
Furthermore in order to compensate for possible focus drifts, we also perform a z-scan and project the maximum phase value, as detailed in Appendix A.

In order to estimate the U2OS cell thickness, we used three different methods: high-NA SLIM (Fig. B.4a) and deconvolution fluorescence microscopy (Fig. B.4 b, c). The fluorescence measurements were taken with a DeltaVision microscope using an Olympus 60x/1.42 oil, Plan Apo N objective (UIS2,1-U2B933) and a CoolSnap (HQ2-ICX285) camera. Figure B.4b shows the YFP-PCNA marker and Fig. B.4e shows a cytoplasmic (tubulin) YFP-Stain. These measurements show that a typical cell is between 7-8 µm thick, which is within the depth of field of the 10x objective being used.

To demonstrate that the 10X/0.3 NA objective is indeed sufficient to accurately measure U2OS cells we measured 7 µm silica beads immersed in 50 % glycerol by weight in water (Fig. B.5 a and b). The refractive index difference between the beads (1.431) and the glycerol solution (1.401) is 0.03, as provided by the manufacturers. Thus, the expected phase delay is 2.489 radians, which agrees very well with our measured value of 2.49. Furthermore Fig. B.5c shows that the even if the object is located 4.08 µm above or below the focus, the error in phase is only 0.6 %. For the planes 12.24 µm below and 9.16 µm above the focus, the error is 6.8%. This indicates that our phase integration is around 20 µm for weakly scattering objects such as the cells we are measuring.
Figure B.4. U2OS Cell Thickness. (a) SLIM measurement of a U2OS cell showing that thickness is approximately 7.6 μm, measured from when the membrane is first visible at 2.4 μm, to the last particle in focus on top of the cell at 10 microns; color bar is in radians; scale bar: 8 μm. (b-c) Deconvolution fluorescence images also showing that the 4 cells imaged are approximately 7 μm in thickness, (b) YFP-PCNA measured from when the membrane is first in focus at 4 μm to when the last piece of the nucleus is in focus at 11 μm, (c) Cytoplasmic (tubulin) YFP stain measured from when the membrane is first in focus at 3 μm to the last in focus image at 10 μm. Scale bars: 30 μm, white arrows indicate particles or areas of the membrane that are in focus.
Figure B.5. Measuring the integrated phase delay. (a) 7 μm beads immersed in a mixture of 50% glycerine by weight with water. Scale bar: 10 μm. Colorbar indicates phase shift in radians. (b) Histogram of the selected area in (a) showing that the phase shift is close to the expected value of 2.4896. (c) Mean value of the central area of the bead (dotted circle in (a)) as a function of position in z. It can be seen that the error for the two slices 4.08 μm above and below the focus is less than 0.6%, and for the slices 12.24 μm below and 9.16 μm above is just 6.8%.

Figure B.6. Thresholding for removal of reminiscent halo artifact. Images show a typical cell image before and after setting negative phase values to zero. The graph shows a comparison of the histograms of the two images. These values were removed in order to prevent underestimation of the cell dry mass.
Figure B.7. Comparison with phase contrast and effects of halo. (a) Phase images at two points from a typical *E. coli* colony. *SLIM* is the raw phase image, *No –ve* is with halo remove as shown in Fig. B.6, *Halo* is map with positive values removed, scale bar is 2.5 μm. (b) Corresponding Phase contrast images. It can be seen that halo is noticeably worse. (c) Line profiles (through dotted line in (a)) for SLIM and PC images, it can be noted that the halo is significantly reduced in the SLIM image. (d) Comparison of growth curves with and without halo removal; without halo removal no growth can be observed in the PC data. The slopes of the non-negative and untreated SLIM data are identical. (e) Normalized growth clearly illustrating the advantage of SLIM measurements over PC.
**MCF-7 Estrogen experiments**

**Figure B.8. E2 vs. Veh growth.** (a) Average dry mass (left axis, solid lines) and WST-1 assay data (right axis, dashed lines). (b) Growth rate vs. Time, a significant shift between E2 and Veh can be seen at 10 hours.

**Figure B.9. Growth data for all clusters.** (a) Normalized mass vs. Time for all clusters that were analyzed. (b) Normalized area vs. time for all clusters. (a-b) Dotted lines show individual cluster data and solid lines show averaged data. Dashed lines indicate where the differences between groups becomes significant.

**Figure B.10. Sensitivity of dry mass measurement.** Due to debris in the field of view, the noise in the mass measurement is higher (~pgs) than SLIM’s capabilities (~fgs)
APPENDIX C. SLIM DESIGN DETAILS

The SLIM setup is based on a Zeiss Axio Observer Z1 motorized inverted research imaging microscope which optimized for bright-field, phase, DIC and fluorescence contrast techniques. Axio Observer Z1 base (ZEISS catalog # 431007901000) includes motorized focus drive (minimum step width 10nm), TFT touch screen, motorized 3-position optovar turret, keys for switching TL/RL illumination, circular operation key unit right and left, light and contrast manager, interfaces 4X CAN RS232, USB, and TCP/IP, trigger socket (In/Out) for shutter and connecting socket for external uniblitz shutter. A three position beam splitter (ZEISS catalog # 4251540000000) allows to redirect light to left port (SLIM), right port (DPM) or front port of the microscope. The epi-fluorescence components include X-Cite 120XL package (120W HBO/Halide fluorescence illumination, ZEISS catalog # 4108092050000), FL/HD light train observer (ZEISS catalog # 4236060000000), six position motorized turret (ZEISS catalog # 4249470000000), 31000DAPI/HOECHST set (ZEISS catalog #4108121050000) and CZ917 FITC filter set (ZEISS catalog # 4108121203000). The transmitted light path is equipped with tilt-back illumination carrier (ZEISS catalog #4239200000000), a lamp housing (12V 100W with collector, ZEISS catalog #4230000000000), a bulb (12V 100W Hal, square filament, ZEISS catalog #3800799540000), an interface F/0.55 motorized shutter (ZEISS catalog #4239210000000) that enables SLIM, phase contrast or DIC image overlays with single or multichannel fluorescence image acquisitions, and motorized LD Condenser 0.55 with bright field, Ph1, Ph2, Ph3, DIC and aperture diaphragm (ZEISS catalog #4242440000000).

Objectives used for this study are Zeiss EC Plan-Neofluar 10×/0.3 PH1 M27 (ZEISS catalog #4203419911000), Zeiss EC Plan-Neofluar 40×/0.75 PH2 (ZEISS catalog #4203619910000), and Zeiss Plan-Apochromat 63×/1.4 Oil PH3 M27 (ZEISS catalog #4203619910000).
The intermediate image right after the objective and tube lens has been directed to left port for SLIM, phase contrast and epi-fluorescence imaging.

In order to match the illumination ring and the size of the LCPM, the intermediate image is relayed by a 4f system with a focal length 150mm doublet (Thorlabs, AC508-150-A1-ML) and a focal length 200mm doublet (Thorlabs, AC508-150-A1-ML). Fourier lens L1 (doublet with focal length 300mm, Thorlabs, AC508-300-A1-ML) and Fourier lens L2 (doublet with focal length 500mm, Thorlabs, AC508-500-A1-ML) forms another 4f system. The LCPM (array size 7.68mm×7.68mm, Boulder Nonlinear, XY Phase series, Model P512-0635) is placed at the back focal plane of L1 and thus overlays the back focal plane of the objective and the illumination ring. A polarizer (Edmund Optics, Stock # NT47-316) is placed in front of the LCPM to make sure it works in phase modulation mode. The camera is ZEISS AxioCam MRm (1388×1040 pixels, pixel size 6.45μm×6.45μm, ZEISS catalog #4265099901000).

Overall, SLIM has an additional 2.22× magnification outside the microscope. For a 40× objective, the overall magnification will be 88.89×, which results in 13.78 pixels/μm in the image plane. Thus, our CCD is oversampling the diffraction spot by a safe margin. The microscope is equipped with live cell environmental controls optimized for 4+ hour time studies, including incubator XL S1 W/CO2 kit (ZEISS catalog #1441993KIT010), heating insert P S1/Scan stage (ZEISS catalog #4118609020000) and POC-R Cell cultivation system. The whole microscope is controlled by Axiovision (ZEISS catalog #4101300300000) with multi-channel, time-lapse, mosaic and Z-stack acquisition. The LCPM is controlled by the Labview based software development kits (Boulder Nonlinear). A data acquisition system based on Labview (National Instruments) and NI-DAQ (National Instruments, NI USB-6008) is also developed in
house to synchronize the LCPM and Axiovision. Matlab and ImageJ are used for phase image processing and visualization.
APPENDIX D. LIST OF PUBLICATIONS

BOOK CHAPTERS

JOURNAL PUBLICATIONS
Under review and in preparation
3. G. Popescu, K. Park, M. Mir, and R. Bashir, “Weighing on Cells” (Commentary), Nat. Meth. (under review)

In Print
REFERENCES


