SPATIAL LIGHT INTERFERENCE MICROSCOPY AND APPLICATIONS

BY

ZHUO WANG

DISSERTATION

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Doctoral Committee:

Assistant Professor Gabriel Popescu, Chair
Professor Stephen A. Boppart
Professor Brian T. Cunningham
Associate Professor Paul Scott Carney
Professor M. Taher A. Saif
ABSTRACT

Phase contrast microscopy has revolutionized cell biology by rendering detailed images from within live cells without using exogenous contrast agents. However, the information about the optical thickness (or phase) is qualitatively mixed in the phase contrast intensity map. Quantifying optical path-length shifts across the specimen offers a new dimension to imaging, which reports on both the refractive index and thickness distribution with very high accuracy. Here I present spatial light interference microscopy (SLIM), a new optical method, capable of measuring optical path-length changes of 0.3 nm spatially (i.e. point to point change) and 0.03 nm temporally (i.e. frame to frame change). SLIM combines two classic ideas in light imaging: Zernike’s phase contrast microscopy and Gabor’s holography. The resulting topographic accuracy is comparable to that of atomic force microscopy, while the acquisition speed is 1,000 times higher.

I exploit these features and demonstrate SLIM’s ability to measure the topography of a single atomic layer of graphene. Using a decoupling procedure for cylindrical structures, I extract the axially-averaged refractive index of semiconductor nanotubes and neurites of a live hippocampal neuron in culture. Owing to its low noise and temporal stability, SLIM enables nanometer-scale cell dynamics. Further, the linear relationship between the cell phase shift and its dry mass enables cell growth measurements in mammalian cells. The SLIM/fluorescence multimodal imaging allows for cell cycle dependent growth measurement, revealing that the G2 phase exhibits the highest growth rate and an exponential trend. Due to the micron-scale coherence length of the illuminating field, SLIM provides high axial resolution optical sectioning. Based on a 3D complex field deconvolution operation, tomographic refractive index distributions of live, unstained cells are obtained.
Further, the optical field is numerically propagated to the far-zone and the scattering properties of tissue and cells have been studied. A scattering phase theorem was developed to bridge the gap between scattering and imaging. Other optical degrees of freedom associated with the sample, such as polarization measurement, are also demonstrated.

Finally, SLIM renders the refractive index map of unstained histopathology slides to a quantitative color-coded image which is further proved to report onsite the carcinomas for prostate biopsies and calcifications for breast biopsies. The imaging signatures of SLIM report different properties of the tissue and cells compared to the gold standard of stained histopathology, which relies on a subjective practice and is sensitive to variations in the fixation and staining processes. The spatial correlations of refractive index indicate that cancer progression significantly alters the tissue organization. In particular, tissue refractive index exhibits consistently higher variance in prostate tumors than in normal regions. From the refractive index maps, I further obtained the spatially resolved scattering mean free path and demonstrated its direct correlation with tumor presence. I also studied small intestine tissue with amyloid and tonsil tissue with actinomyces. The results show that refractive index is an intrinsic marker for cancer diagnosis.
To Father and Mother
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CHAPTER 1: INTRODUCTION

Most living cells do not absorb or scatter light significantly; i.e. they are essentially transparent, or phase objects. The phase contrast method proposed by Zernike represented a major advance in intrinsic contrast imaging, as it revealed inner details of transparent structures without staining or tagging [1]. While phase contrast is sensitive to minute optical path-length changes in the cell, down to the nanoscale, the information retrieved is only qualitative. Quantifying cell-induced shifts in the optical path-lengths permits nanometer-scale measurements of structures and motions in a non-contact, non-invasive manner [2]. Thus, quantitative phase imaging (QPI) has recently become an active field of study and various experimental approaches have been proposed [3-12]. Advances in phase-sensitive measurements enabled optical tomography of transparent structures, following reconstruction algorithms borrowed from X-ray computed imaging, in which scattering and diffraction effects are assumed to be negligible [13-18]. Further, QPI-based projection tomography has been applied to live cells [19-21]. However, the approximation used in this computed tomography fails for high numerical aperture imaging, where scattering effects are essential, which drastically limits the depth of field that can be reconstructed reliably in live cells [22].

Despite all these technological advances, the range of QPI applications in biology has been largely limited to red blood cell imaging [23, 24] or assessment of global cell parameters such as dry mass [4, 25], average refractive index [26], and statistical parameters of tissue slices [27, 28]. This limitation is due to two main reasons. First, because of speckle generated by the high temporal coherence of the light used (typically lasers), the contrast in QPI images has never matched that exhibited in white light techniques such as phase contrast and Nomarski. Second,
the experimental setups tend to be rather complex, of high maintenance, which limits their in-depth biological applicability.

Toward this end, I developed SLIM as a novel, highly sensitive QPI method, which promises to enable unprecedented structure and dynamics studies in biology and beyond. SLIM combines Zernike’s phase contrast method of revealing the intrinsic contrast of transparent samples [1] with Gabor’s holography [29] by rendering quantitative phase maps across the sample. Because of the extremely short coherence length of this illumination light, approximately 1.2 μm, SLIM provides label-free optical sectioning, allowing a three-dimensional view of live cells, which reflects the scattering potential distribution. With all these features, SLIM advances the field of quantitative phase imaging in several ways: i) provides speckle-free images, which allows for spatially sensitive optical path-length measurement (0.3 nm); ii) uses common path interferometry, which enables temporally sensitive optical path-length measurement (0.03 nm); iii) offers high-throughput topography and refractometry measurements; iv) renders 3D tomographic images of transparent structures; v) due to the broadband illumination, grants immediate potential for spectroscopic (i.e. phase dispersion) imaging; vi) is likely to make a broad impact by implementation with existing phase contrast microscopes; and vii) inherently multiplexes with fluorescence imaging for multimodal, in-depth biological studies.

The operation theory of SLIM is discussed in Chapter 2, followed by applications in Chapter 3. Tomography capability of SLIM is studied in Chapter 4, including the theory and applications. Because the phase is measured quantitatively, the field is known at the imaging plane, which can be numerically propagated to the scattering plane. This modality is coined as Fourier Transform Light Scattering (FTLS) and discussed in Chapter 5. The scattering and the
imaging are further unified by the scattering phase theorem in Chapter 6, where the scattering properties such as scattering coefficient and anisotropic factor are directly connected with the spatially resolved phase map. Further, clinical applications of SLIM are demonstrated in Chapter 7 with prostate tissue with cancers and breast tissue with calcifications. Other possible applications such as polarization and spectroscopy are also explored in Chapter 8 and Chapter 9 respectively.
CHAPTER 2: SPATIAL LIGHT INTERFERENCE MICROSCOPY (SLIM)

A schematic of the instrument setup is depicted in Fig. 2.1a. SLIM functions by adding spatial modulation to the image field of a commercial phase contrast microscope. Besides the conventional $\pi/2$ shift introduced in phase contrast between the scattered and unscattered light from the sample [1], I generated further phase shifts by increments of $\pi/2$. The objective exit pupil, containing the phase shifting ring, is imaged via lens $L_1$ onto the surface of a reflective liquid crystal phase modulator (LCPM, Boulder Nonlinear). The active pattern on the LCPM is calculated to precisely match the size and position of the phase ring image, such that additional phase delay can be controlled between the scattered and unscattered components of the image field. In this setup, four images corresponding to each phase shift are recorded (Fig. 2.1b) to produce a quantitative phase image that is uniquely determined. Figure 2.1c depicts the quantitative phase image associated with a cultured hippocampal neuron, which can be approximated by

$$\phi(x, y) = \frac{2\pi}{\lambda} \int_0^{h(x,y)} \left[ n(x, y, z) - n_0 \right] dz.$$  \hspace{1cm} (2.1)

In Eq. 2.1, $n-n_0$ is the local refractive index contrast between the cell and the surrounding culture medium, $h$ the local thickness of the cell, and $\lambda$ the central wavelength of the illumination light. The typical irradiance at the sample plane is $\sim 1$ nW/μm². The exposure time was 10-50 ms for all the images presented in the manuscript. This level of exposure is 6-7 orders of magnitude less than that of typical confocal microscopy and, therefore, limits phototoxicity during extended live-cell imaging [30].

As a comparison, I evaluated background images (i.e., no sample) from SLIM and diffraction phase microscopy (DPM) [10], an established laser-based technique that was
interfaced with the same microscope and which provides sub-nanometer path-length temporal stability (Fig. 2.1d-e). Due to the lack of speckle effects granted by its broad spectral illumination, SLIM’s spatial uniformity and accuracy for structural measurements is substantially better than DPM’s. To quantify the spatiotemporal phase sensitivity, I imaged the SLIM background repeatedly to obtain a 256-frame stack. Figure 2.1f shows the spatial and temporal histograms associated with the optical path-length shifts across a 10×10 μm² field of view and over the entire stack, respectively. These noise levels, 0.3 nm and 0.03 nm, represent the limit in optical path-length sensitivity across the frame and from frame to frame.

**Figure 2.1 | SLIM principle.** (a) Schematic setup for SLIM. The SLIM module is attached to a commercial phase contrast microscope (Axio Observer Z1, Zeiss, in this case). The lamp filament is projected onto the condenser annulus. The annulus is located at the focal plane of the condenser, which collimates the light toward the sample. For conventional phase contrast microscopy, the phase objective contains a phase ring, which delays the unscattered light by a quarter wavelength and also attenuates it by a factor of 5. The image is delivered via the tube lens to the image plane, where the SLIM module processes it further. The Fourier lens L1 relays the back focal plane of the objective onto the surface of the liquid crystal phase modulator (LCPM). By displaying different masks on the LCPM, the phase delay between the scattered and unscattered components is modulated accurately. Fourier lens L2 reconstructs the final image at the CCD plane, which is conjugated with the image plane. (b) The phase rings and their corresponding images recorded by the CCD. (c) SLIM quantitative phase image of a hippocampal neuron. The color bar indicates optical path-length in nanometers. (See figure continuation on the following page.)
It is worth noting that usually monochromatic light sources are assumed in phase shifting interferometry. For a broadband light source, the meaning of the phase still needs justification. In the next section, the quantitative phase imaging with broadband light source will be discussed first, followed by characterization of light source, calibration of LCPM, and optical design considerations. The details of the design with part numbers can be found in Appendix A.

2.1 Quantitative Phase Imaging with White Light Illumination

Review of phase contrast microscopy

SLIM exploits the concept of imaging as an interference phenomenon, which is also the basis for Zernike’s phase contrast microscopy, as illustrated in Fig. 2.2a.

The description of an arbitrary image as an interference phenomenon was recognized more than a century ago by Abbe in the context of microscopy: "The microscope image is the interference effect of a diffraction phenomenon" [31]. Further, describing an image as a (complicated) interferogram has set the basis for Gabor’s development of holography [29].
Figure 2.2 | Imaging as an interference effect. (a) Principle of phase contrast microscopy. (b) Each pixel detects the interference between the scattered and unscattered light. (c) The image field $U$ as the vector sum between the scattered and unscattered light.

Under spatially coherent (plane wave) illumination, the light passing through the specimen is decomposed into a scattered ($U_1$) and unscattered ($U_0$) field. In the Fourier plane of the objective, these two components are spatially separated, with the unscattered light being focused on axis. The tube lens generates at the CCD plane the image field $U$, which is identical in phase and amplitude with the field at the sample plane, except for a scaling factor given by the magnification of the system. As shown in Fig. 2.2b, each pixel of the CCD detects the interference between $U_0$ and $U_1$,

$$U(x, y) = U_0 + U_1(x, y) = |U_0|e^{i\phi_0} + |U_1(x, y)|e^{i\phi(x, y)}.$$  \hspace{1cm} (2.2)

The coherent superposition in Eq. 2.2 is represented graphically in the phase space in Fig. 2.2c. Note that the system is equivalent to a large number of interferometers (one per pixel), all using
the same unscattered field as reference. The intensity, i.e. bright field image, is obtained by taking the modulus squared of Eq. 2.2,

\[ I(x, y) = I_0 + I_1(x, y) + 2\sqrt{I_0 I_1(x, y)} \cos[\Delta \phi(x, y)], \]

where \( \Delta \phi(x, y) \) is the phase difference between \( E_0 \) and \( E_1(x, y) \). Clearly, for small values of \( \Delta \phi \), the cosine function varies slowly, as the first order Taylor expansion yields a quadratic function, \( \cos(\Delta \phi) \approx 1 - (\Delta \phi)^2 / 2 \). This result explains why the bright field images show low contrast of optically thin objects such as live cells. To enhance contrast, Zernike came up with a powerful, yet simple, approach: to apply a \( \pi/2 \) phase shift to the unscattered field, such that \( \cos(\Delta \phi) \) becomes \( \sin(\Delta \phi) = 1 - \Delta \phi \), which now is a rapidly varying function. In other words, the phase information was coupled to the intensity modulation. Additional attenuation of unscattered light \( E_0 \) is also used to enhance the contrast.

Such contrast enhancement method is widely used for observation of transparent samples. However, for conventional phase contrast microscopy, no quantitative phase information can be obtained. By introducing the LCPM into the optical path, I did additional phase modulation with four different masks. The quantitative phase information is thus retrieved by phase shifting interferometry.

**From phase contrast to SLIM**

As can be seen from Eq. 2.3, from only one measurement of \( I(x, y) \) the information regarding \( \Delta \phi \) cannot be uniquely retrieved; i.e. phase contrast microscopy is a qualitative method. Here, I propose a general experimental approach for retrieving the phase information over the entire image obtained with a broadband field. In optics, quantitative phase imaging has become a dynamic field, especially due to its potential for nanoscale cell and tissue imaging [2]. Note that
knowledge of the phase in the image plane as defined by an imaging system allows
reconstructing the field in the far field zone with very high accuracy [27]. The experimental
demonstration, which follows the theoretical solution proposed by Wolf [32], was performed
with optical fields, but the scheme is generally applicable to other electromagnetic fields,
including x-rays.

The principle relies on the spatial decomposition of a statistically homogeneous field \( U \)
into its spatial average (i.e. unscattered) and a spatially varying (scattered) component
\[
U(\mathbf{r}; \omega) = U_0(\omega) + U_1(\mathbf{r}; \omega)
= |U_0(\omega)| e^{i\Phi_0(\omega)} + |U_1(\mathbf{r}; \omega)| e^{i\Phi(\mathbf{r}; \omega)},
\]
where \( \mathbf{r} = (x, y) \). Here the field is assumed to be fully spatially coherent.

Using the spatial Fourier representation \( \tilde{U}(\mathbf{q}; \omega) \) of \( U \), it becomes apparent that the
average field \( U_0 \) is proportional to the DC component \( \tilde{U}(0; \omega) \), whereas \( U_1 \) describes the non-
zero frequency content of \( U \). Thus, the image field \( U \) can be regarded as the interference
between its spatial average and its spatially-varying component as discussed previously.

The cross-spectral density can be written as
\[
W_{01}(\mathbf{r}; \omega) = \langle U_0(\omega) \cdot U_1^*(\mathbf{r}; \omega) \rangle,
\]
where the angular brackets denote ensemble average and * stands for complex conjugation. If
\( \omega_b \) is the mean frequency of the power spectrum \( S(\omega) = \langle |U_0(\omega)|^2 \rangle \), \( W \) has the factorized form
\[
W_{01}(\mathbf{r}; \omega - \omega_b) = |W_{01}(\mathbf{r}; \omega - \omega_b)| e^{i [\Delta \Phi(\mathbf{r}; \omega - \omega_b)]}.
\]

It follows that the temporal cross-correlation function is obtained by Fourier transforming
Eq. 2.6 [33].
\[ \Gamma_{01}(\mathbf{r}; \tau) = |\Gamma_{01}(\mathbf{r}; \tau)| \cdot e^{i[\omega_0 \tau + \Delta \phi(\mathbf{r}; \tau)]}, \]  

(2.7)

with \( \Delta \phi(\mathbf{r}) = \phi_0 - \phi(\mathbf{r}) \) the spatially varying phase difference of the cross-correlation function.

Equation 2.7 indicates that, for spatially coherent illumination, the spatially varying phase of the cross-correlation function can be retrieved through measurements at various time delay \( \tau \). This phase information is equivalent to that of a purely monochromatic light at frequency \( \omega_0 \). Using Eq. 2.7, one obtains the following irradiance distribution in the plane of interest as a function of the time delay \( \tau \):

\[ I(\mathbf{r}; \tau) = I_0 + I_1(\mathbf{r}) + 2|\Gamma_{01}(\mathbf{r}; \tau)| \cos[\omega_0 \tau + \Delta \phi(\mathbf{r})]. \]  

(2.8)

When one varies the delay \( \tau \) between \( U_0 \) and \( U_1 \), interference is obtained simultaneously at each point of the image. The average \( U_0 \) is constant over the entire plane and can be regarded as the common reference field of an array of interferometers. In addition, \( U_0 \) and \( U_1 \) traverse similar optical paths. Thus, the influence of inherent phase noise due to vibration or air fluctuations is inherently minimized, allowing for a precise retrieval of \( \Delta \phi \).

By modifying the delay \( \tau \), I can get the phase delay of \(-\pi\), \(-\pi/2\), \(0\) and \(\pi/2\) (many more combinations exist, e.g. the above four frames plus \(n\pi/2\) where \(n\) is an integer).

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

(2.9)

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

(2.10)

Thus as long as the relationship

\[ \tilde{\Gamma}(0) + \tilde{\Gamma}(-\pi) = \tilde{\Gamma}(\frac{-\pi}{2}) + \tilde{\Gamma}(\frac{\pi}{2}) \]  

(2.11)

holds, e.g., for modifications of the time delay around \( \tau = 0 \) that are comparable to the optical period, the spatially varying phase of \( \Gamma \) can be reconstructed as...
\[
\Delta \phi(r) = \tan^{-1} \left[ \frac{I(r; -\pi/2) - I(r; \pi/2)}{I(r; 0) - I(r; -\pi)} \right].
\] (2.12)

If \( \beta(r) = \frac{|U_i(r)|}{|U_0|} \), then the phase associated with the image field \( U = U_0 + U_i \) can be determined as

\[
\phi(r) = \arg \left[ \frac{\beta(r) \sin(\Delta \phi(r))}{1 + \beta(r) \cos(\Delta \phi(r))} \right].
\] (2.13)

Equation 2.13 shows how the quantitative phase image is retrieved via 4 successive intensity images measured for each phase shift. It is worth noting that even if Eq.2.11 does not hold for broader band illumination, one can still calibrate their ratio and thus correct such errors as long as the spectrum is measured.

Figure 2.3 | Phase measurement of 1 μm diameter polystyrene beads in water. (a) Quantitative phase image of 1 μm. Color bar indicates phase in radians. (b) Histogram of the selected area of (a). The average phase shift through beads is shown.

Figure 2.3 shows an example of such an image and demonstrates the principle of phase retrieval using broadband fields. Polystyrene beads of 1 μm in diameter immersed in water were imaged by the SLIM system. The quantitative phase map is shown in Fig. 2.3a and the histogram of phase shifts in Fig. 2.3b. The maximum phase shift through the beads (\( n=1.59 \)) with respect to
surrounding medium (water, $n_w=1.33$) is expected to be $\delta \phi = \frac{2\pi}{\lambda_0^2} \Delta n d$, with $\lambda_0 = \omega_0/c$, $\Delta n = n - n_w$, and $d = 1 \mu m$. Thus, the measured value of $3.08 \pm 0.15$ compares very well with the expected $\delta \phi = 3.07 \pm 0.15$, where the error is due to the size distribution provided by the manufacturer.

**2.2 Temporal Coherence of the Illumination White Light**

![Figure 2.4](image)

**Figure 2.4 | Temporal coherence of the illumination.** (a) Spectrum of the white light emitted by the halogen lamp. The center wavelength is 531.8 nm. (b) Resampled spectrum with respect to frequency. (c) The autocorrelation function (blue solid line) and its envelope (red dotted line). The four circles indicate the phase shifts produced by LCPM. The refractive index of the medium is 1.33.

In order to apply the procedure outline above, I calculated the temporal autocorrelation function, which was the time-dependent part of $\Gamma (\Delta \phi = 0)$. Using a spectrometer (USB 2000, Ocean Optics, USA), the optical spectrum of the illuminating white light at the CCD plane was measured. As a result of the Wiener-Kintchin theorem, the autocorrelation function was obtained from the power spectrum via a Fourier transform, $\Gamma(\tau) = \int S(\omega) e^{-i\omega \tau}$, with $\omega$ denoting angular frequency. The spectrum provided by the spectrometer is sampled in wavelength, as shown in Fig. 2.4a. In order to obtain the spectrum vs. frequency, which then can be Fourier transformed, I performed resampling of the data, as shown in Fig. 2.4b. From these data, the temporal autocorrelation function of the light was retrieved via a Fourier transform (Fig. 2.4c). In a medium of refractive index $n=1.33$ (water), the coherence length defined at full-width half-
maximum is $I_{\text{FWHM}}^\mu \mu m$. This coherence length is at least an order of magnitude shorter than that of other light sources such as lasers, light emitting diodes and superluminescent diodes. However, within this coherence length there are still several full cycle modulations. Thus, the envelop varies slowly over one period near the central peak, which enables the application of the phase shifting procedure described in Section 2.1. In other words, the major difference between broadband illumination and laser, in our context, is the coherence length. For white light the envelop drops fast from center peak as shown in Fig. 2.4c and for laser it is constant. However, if the envelop does not vary too much within the modulation (e.g. from $-\pi$ to $\pi/2$), intuitively there is no difference between laser and white light source in terms of phase reconstruction.

### 2.3 Calibration of the Liquid Crystal Phase Modulator (LCPM)

The LCPM (XY Phase Series Model P512–635, Boulder Nonlinear Systems, Inc., USA) was calibrated to decide the relationship between pixel grey values fed via a VGA signal to the liquid crystal array and the final phase delay introduced to the unscattered field.

![Figure 2.5](image.png)

**Figure 2.5** | LCPM Calibration for white light source. (a) Intensity modulation obtained by displaying different grayscale values on the LCPM. (b) Phase vs. gray scale calibration curve obtained by Hilbert transform of the signal in (a). (c) Corresponding spectrum measured at CCD plane. In order to test the stability of the instrument in time, we show two measurements performed at different dates, as indicated.

The LCPM was placed between two polarizers, and its intensity transmission was recorded. I first changed the polarizer and analyzer by 45° so that SLM would work in
“amplitude modulation” mode. Then I scanned through the grayscale value from 128 to 255 (i.e. 8 bits). The modulation from pixel value 0 to 127 and from 128 to 255 is symmetric. Thus, I only need to scan half of the pixel values. The intensity transmitted through the LCPM vs. the grey value is shown in Fig. 2.5a. From the amplitude response of the modulator, I obtained its phase response via a Hilbert transform, as shown in Fig. 2.5b.

2.4 Optical Design Considerations

After several hundred years of refinement, the objectives from major companies in the market are diffraction limited; i.e. the root mean square (RMS) wavefront aberration is less than \( \frac{\lambda}{4} \) or even \( \frac{\lambda}{8} \) in some cases. The SLIM module, on the other hand, needs to be carefully designed in order to deliver diffraction limited images. Fortunately, at the image plane (Fig. 2.1a), the field of view and NA are quite limited. It is possible to use the off-the-shelf components to achieve diffraction limited imaging even with white light illumination.

After an initial layout design based on the field of view, NA, magnification, matching of the Fourier plane ring size and the LCPM, the first order parameters of the system are obtained. Two 4F systems with an overall magnification of 20/9 are used for the current design, with focal lengths of the first 4F system 150 mm and 200 mm, and those of the second 4F system 300 mm and 500 mm. The 200 mm and 300 mm pair will magnify the back focal plane by 1.5 times to match the size of the LCPM.
Figure 2.6 | Aberration analysis for SLIM system. (a) Spot diagrams. (b) Wavefront aberration. (c) RMS (root mean square) wavefront error. (d) MTF (modulated transfer function). (e) Field curvature and distortion. (f) Lateral color.

For white light imaging systems, usually wavelengths 0.4861 μm (F line), 0.5876 μm (d line) and 0.6563 μm (C line) are used for the design. Doublet is chosen to minimize the color aberration. The spot diagram of the system is shown in Fig. 2.6a. Since all the rays fall into the
Airy circle, the optical path length difference is calculated with respect to the reference sphere, which shows the system's maximum wavefront aberration is within $\lambda/8$. The RMS wavefront error in waves (Fig. 2.6c) indicates that, for center field of view, the aberration is within $\lambda/50$ and for full field of view it is $\lambda/25$. Figure 2.6d shows the MTF (modulated transfer function) of the system, which is approaching the diffraction limit. In reality, due to the manufacturing errors and the misalignments, the performance is worse, but can still be diffraction limited with carefully chosen elements and hearty alignments.

Since the system has no negative focal power, the field curvature is not corrected as shown in Fig. 2.6e. The distortion is limited at 0.003% at full field, which is negligible. The lateral color is also well controlled as shown in Fig. 2.6f.

In sum, the SLIM module is capable of delivering diffraction limited images. The aberrations at the frequency plane are also controlled to minimize the frequency mixing. In the alignment process, precise control of spacing between the lenses is essential. The spacing between lenses can be found in Table A.1. Both the careful alignment and the flawless design are critical for the success of the whole system.
CHAPTER 3: SLIM APPLICATIONS

3.1 Topography

Amorphous carbon film

To assess the spatial accuracy of SLIM, I imaged an amorphous carbon film and compared the topography measurements against atomic force microscopy (AFM) (Fig. 3.1). The two types of measurement agree within a fraction of a nanometer. Note that both SLIM and AFM are characterized by much smaller errors than suggested by the widths of the histogram modes, as these widths also reflect irregularities in the surface topography due to the fabrication process itself. Compared to AFM, SLIM is non-contact, parallel, and faster by more than 3 orders of magnitude. Thus, SLIM can optically measure an area of $75\times100 \, \mu m^2$ in 0.5 s compared to a $10\times10 \, \mu m^2$ field of view measured by AFM in 21 minutes (Fig. 3.1b). Of course, unlike AFM, SLIM provides nanoscale accuracy in topography within the diffraction limited transverse resolution associated with the optical microscope. Compared to AFM, another hallmark of SLIM is its insensitivity to base curvature of the sample; i.e. if the sample is sitting on another curved surface with constant thickness, as long as it is within the depth of field of the objective (usually up to a micron), it will not affect SLIM measurement.

The noise levels for SLIM are 0.3 nm across the frame and 0.03 nm from frame to frame. Several error sources can potentially be diminished further: residual mechanical vibrations in the system that are not “common path”, minute fluctuations in the intensity and spectrum of the thermal light source, digitization noise from the CCD camera (12 bit), and the stability (repeatability) of the liquid crystal modulator (8 bit). The LCPM maximum refresh rate is 60 Hz, in principle allowing for 15 SLIM images per second, but throughout the manuscript I report imaging at 2.6 frames/s, as the camera has a maximum acquisition rate of 11 frames/s.
Acquisition speed could be increased to video rate by employing a faster phase modulator and camera.

**Figure 3.1 | Comparison between SLIM and AFM.** (a) SLIM image of an amorphous carbon film (40×/0.75NA objective). (b) AFM image of the same sample. The colorbar indicates thickness in nm. (c) Topographical histogram for AFM and SLIM, as indicated.

**Single atomic layer of graphene**

In order to demonstrate the capability of SLIM for imaging single atomic layers, I performed measurements on graphene flakes. Graphene is a two-dimensional lattice of hexagonally arranged and $sp^2$-bonded carbon atoms, i.e. a monolayer of the bulk material graphite. The graphene sample was obtained here by mechanically exfoliating a natural graphite crystal using
adhesive tape [34]. The exfoliated layers were deposited on a glass slide which was then cleaned using isopropanol and acetone to remove excess adhesive residue. Single-layer (graphene) and few-layer graphite flakes are routinely obtained in this process, with lateral dimensions up to several tens of microns.

![Figure 3.2](image_url)

**Figure 3.2** | SLIM topography of graphene. (a) Quantitative phase image of a graphene flake. (b) Topography histogram for the various regions indicated in (a).

Figure 3.2a shows the SLIM image of such a graphene flake. Qualitatively, it can be seen that the background noise is below the level of the sample itself. To quantify the nanoscale profile of this structure, I transformed the phase distribution $\phi$ into thickness $h$, via $h = \frac{\phi \lambda}{2\pi(n-1)}$, with $n=2.6$ the refractive index of graphite [35]. Thus, I generated the topography histogram of the entire sample and individual regions, as shown in Fig. 3.2b. The overall histogram exhibits local maxima at topography values of 0 nm (background), 0.55 nm, 1.1 nm, 1.65 nm. These results indicate that the topography of the graphene sample has a *staircase* profile, in increments of 0.55 nm. This is comparable with reported values in the literature for the thickness of *individual atomic layers* of graphene via atomic force microscopy (AFM) in air (~1 nm step size) or scanning tunneling microscopy (STM) in ultra-high vacuum (~0.4 nm step size) [36, 37]. The difference between air and vacuum measurements indicates the presence of ambient species (nitrogen, oxygen, water, organic molecules) on the graphene sheet.
in air. Thus, SLIM provides topographical accuracy that is comparable with atomic force microscopy, but its acquisition time is much faster and, of course, it operates in non-contact mode.

**Red Blood Cell Volume**

![Image of blood smear and topographical plots]

**Figure 3.3** Red blood cell volume. (a) Quantitative phase image of blood smear. Color bar in radius. (b) 3D surface plot of the sample in (a). (c) Another blood smear containing echinocytes. Color bar in radius. (d) Histogram of red blood cell volume of sample (a) (red solid line) and (c) (black dashed line).

I also extended the topography measurement to biological samples such as red blood cells, as shown in Fig. 3.3. Unlike other cells, red cells do not contain nuclei and are often considered homogeneous within. Thus from the measured phase and known refractive index contrast for haemoglobin and cytoplasm, cell topography and then cell volume can be measured. Figure 3.3a shows the measured phase map for the blood smear. A 3D surface plot can be obtained as shown in Fig. 3.3b. If the blood smear is left standing longer, the salt concentration will increase due to
evaporation and the normal discocytes shown in Fig. 3.3a will become echinocytes as shown in Fig. 3.3c. The volume histogram of the cells in Fig. 3.3a shows a bimodal distribution, indicating that some of the discocytes are in transition to echinocytes, which feature a small cell volume.

3.2 Refractometry

Using a decoupling procedure developed for cylindrical structures, I extract the axially-averaged refractive index of semiconductor nanotubes (SNT) (see Appendix B for fabrication details) and neurites of live hippocampal neurons in culture (see Appendix C for cell cultures).

Nanotubes

Figure 3.4 | SLIM refractometry of nanotube. (a) Tube structure with refractive index and thickness of layers shown in (b). (c-d) SEM images of nanotubes. (e) Optical path-length map; color bar in nanometers. (f) Distance map; color bar in microns. (g) Histogram of the refractive index contrast, \( n-1 \), of the selected area in the inset. Inset: distribution of refractive index contrast, \( n-1 \).

SLIM was first applied to image semiconductor nanotubes. SNT is a new type of nanotechnology building block [38]. It is formed by a combination of bottom-up and top-down approaches through self-rolling of residually strained thin-films that are epitaxially grown and
lithographically defined. The tube diameter is determined by the total layer thickness and the mismatch strain in the epitaxial layers (bottom-up aspect). The top-down aspect allows feasible large area assembly and integration with existing semiconductor technologies. Heterojunctions include structures with active light emitters embedded in the wall of the tube [38, 39]. For this study, clusters of such rolled-up tubes consisting of InGaAs/GaAs coated with Cr/Au (see Fig. 3.4 for structure and SEM images) are randomly distributed on glass slides and imaged by SLIM.

Figures 3.4e-g show the results of SLIM investigation of such nanotube structures. I used the prior knowledge of the tube cylindrical shape to decouple the thickness and refractive index, as demonstrated on the 15×20 μm² SLIM image of Fig. 3.4e. This procedure operates on the principle that the tube thickness, generally unknown, can be obtained for cylindrical structures from the projected width, which is directly measurable in the image. Of course, the refractive index information reports on the chemical composition of the nanotube and its optical behavior. Using thresholding and binary masking of the SLIM image, I measured the distribution of the tube projected width, which is illustrated in Fig. 3.4f. This distance map, shows the distance from the center of the tube to its edge; thus the diameter is twice the number indicated by the color bar. This process was implemented via an automated routine in ImageJ, an image processing platform based on Java. Assuming the tube thickness $h$ and width are equal, I extracted the average tube refractive index, $(n-1) = \phi \lambda / 2 \pi h$. Note that for each tube, SLIM provides refractive index information that is spatially resolved. Thus, in Fig. 3.4g, I present the histogram of the refractive index measured along one of the nanotubes. The average value, $n_{av}-1=0.093$, compares very well with the estimated value $n_{est}-1=0.087$ resulting from averaging the refractive index for the layered structure shown in Fig. 3.4b, $n_{est}^2 = \left( \sum_i n_i^2 h_i \right) / h$. The fluctuations in the refractive index along the nanotube are mostly due to physical
inhomogeneities in the tube itself. I believe that SLIM may offer a high-throughput screening method for nanofabricated structures.

Neuron processes

I employed this refractometry procedure to extract the refractive index of neuron processes, i.e. axons and dendrites, which are also characterized by cylindrical shapes. Refractive index is an intrinsic measure of cell content and also defines how light interacts with tissues. Dendrites are the principal recipients of incoming chemical messengers from axon terminals. On dendritic shafts, specialized structural elements (dendritic spines) initially emerge as collateral filopodia, then mature into spiny synapses or filopodia are pruned. The mechanisms by which dendrogenesis leads to spine formation have not been resolved. Thus a label-free, non-invasive method for imaging such structures in detail is very beneficial.

Figure 3.5 shows the SLIM image of a live rat hippocampal neuron in culture, i.e. immersed in culture medium during imaging. Following the routine applied to nanotubes, I retrieved the distance map of the axon (Fig. 3.5b) and its refractive index distribution. The average refractive index contrast obtained is $\Delta n=0.034$. Thus, by using the refractive index of the
culture medium of 1.34, I obtain an average value for the neuronal structure that is comparable with what has been measured before on other live cells [26]. Besides providing the absolute values for the refractive index of cellular structures, which is crucial for predicting the light-tissue interaction, SLIM can quantify the spatial inhomogeneities of the neurites structure. Thus, the discrete regions of enhanced refractive index are most likely related to the development of synaptic connections. The ability to image these dynamically without the need for fluorescence tagging may open the door of cell-to-cell communication. Another example of neuritis refractive index extraction can be found in Fig. 3.6.

Figure 3.6 | SLIM refractometry of neuron processes. (a) Phase map (optical path length difference), colorbar unit: nm. (b) Phase map of selected area, colorbar unit: nm. (c) Binary mask. (d) Distance map (unit: μm). (e) Refractive Index map. Field of view for (a) is 100 μm × 75 μm; field of view for (b-e) is 25 μm × 14 μm. Scale bar 2 μm.
3.3 Multimodal Imaging

One distinct feature of SLIM is that the quantitative phase image is overlaid with all the other imaging channels of the microscope, such as epi-fluorescence, differential interference contrast (DIC), and, obviously, phase contrast. In addition, since SLIM is directly measuring the phase, other qualitative phase measurement such as DIC can be simulated numerically.

**SLIM and fluorescence**

For **a** and **b**: SLIM (Red); MAP2 (Green); Dapi (Blue);

![Multimodal Imaging Example](image)

**Figure 3.7 | SLIM-fluorescence multimodal imaging.** (a-b) Combined multimodal images of cultured neurons (19 DIV) acquired through SLIM (red) and fluorescence microscopy. Neurons were labeled for somatodendritic MAP2 (green), and nuclei (blue). (c) Optical path-length fluctuations along the dendrites (lines) and axon (markers) retrieved from the inset of (a). (d) Synaptic connections of a mature hippocampal neuron (33 DIV) immunochemically labeled for synapsin (green), MAP2 (blue), and f-actin using rhodamine phalloidin (red). All scale bars are 20 μm.
Simultaneous fluorescence imaging complements SLIM’s unique structural information with the ability to study cellular constituents with molecular specificity. In Fig. 3.7a-b, I show SLIM imaging of axons and dendrites identified by fluorescent staining for somato-dendritic microtubule associated protein 2 (MAP2) of primary hippocampal neurons cultured for 19 days in vitro (DIV). Fine axonal processes are also distinguishable from dendrites by SLIM where the quantitative phase imaging channel reveals changes in the local refractive index of structures reminiscent of actin-rich synaptic connections (Fig. 3.7b). As shown in Fig. 3.7c, these inhomogeneities are observed along dendrites where the spines develop. In order to quantify these structural differences observed by SLIM, I traced individual neurites using NeuronJ, a semi-automatic algorithm implemented in Java [40]. The results demonstrate the optical path-length fluctuations for each trace, wherein I found that the standard deviation of the path-length fluctuations along the axons, $\sigma=25.6$ nm, is the lowest among all neurites. This result indicates that subtle inhomogeneities are associated with the connecting synaptic structures, which can be revealed by SLIM as path-length changes. By 3 weeks in dispersed culture, the majority of dendritic spines mature to form presynaptic boutons [41] on the dendritic shafts of hippocampal neurons [42]. These are comparable to synaptic elaborations on a mature hippocampal neuron (33 DIV) with labeled f-actin, synapsin, and MAP2 (Fig. 3.7d). Thus, SLIM may offer a window into studying the dynamic processes associated with the formation and transition of collateral filopodia into spines, and the dynamics of plasticity-related changes in spine structure [43-45]. Note that SLIM can be used to image cellular dynamics over extended periods of time without loss in performance or damage to the specimen.
From the quantitative phase map, other representations of the information can be obtained numerically. Figure 3.8a shows two cardiac myocytes captured by SLIM. Phase contrast is also obtained naturally as one channel of SLIM as seen in Fig. 3.8d. The halo effect is clearly seen in the phase contrast image. The spatial gradient of the SLIM image simulates DIC microscopy in Fig. 3.8b. The shadow artifact can be seen clearly. Further, I show that the Laplacian of the image, a second order derivative operation, is even more powerful than DIC in revealing fine
structures within the cell, as it does not contain shadow artifacts. It will also emphasize the high frequency component in the images, which is evident in Fig. 3.8c where the small particles in the cardiac myocytes are seen clearly. It is known that heart cells are very active, i.e. energy-consuming; therefore this type of cell is rich in mitochondria, which are responsible for the energy supply of the cell metabolism [46]. Mitochondria are most likely the predominant type of visible particle, especially in the area surrounding the cell nucleus. Because phase contrast will mix the intensity information with phase information, suffers from phase ambiguity and is qualitative (e.g., some of the particles in Fig. 3.8d are bright spots and some are dark spots due to the larger phase than $\pi/2$, while all the particles in Fig. 3.8a are bright spots), the Laplacian of a phase contrast image is much worse than the Laplacian of SLIM, which is termed as Laplacian phase microscopy (LPM), detailed in Section 3.5.

3.4 Cell Dynamics

Two-dimensional SLIM dynamic imaging of live cells has been performed over various time scales, from a fraction of second to several days. Figure 3.9 summarizes the dynamic measurements obtained via 397 SLIM images of a mixed glial-microglial cell culture over a period of 13 minutes. In order to illustrate microglial dynamics, I numerically suppressed the translation motion via an algorithm implemented in ImageJ [47]. Phase contrast images, which are part of the measured data set, are also presented for comparison (Fig. 3.9b-c). These results demonstrate that phase contrast cannot provide quantitative information about dynamic changes in optical path-length, because the light intensity is not linearly dependent on phase. In addition, the cell size is significantly overestimated by phase contrast due to the well known halo artifact which makes the borders of the cell appear bright (Figs. 3.9b, d show the same field of view) [1]. By contrast, SLIM reveals details of intracellular dynamics, as evidenced by the time-traces (Fig.
3.9e, f). Path-length fluctuations associated with two arbitrary points on the cell reveal an interesting, periodic behavior (Fig. 3.9f). At different sites on the cell, the rhythmic motions have different periods, which may indicate different rates of metabolic or phagocytic activity. This periodicity can be observed in the coordinated cell behavior as the cell extends broad, dynamic filopodial ruffles under, and above, the neighboring glial cells.

**Figure 3.9 | SLIM dynamic imaging of mixed glial-microglial cell culture.** (a) Phase map of two microglia cells active in a primary glial cell culture. Solid line box indicates the background used in (g), dashed line box delineates a reactive microglial cell used in (b-e) and dotted line box indicates the glial cell membrane used in (g) (see figure continuation on following page). (b) Phase contrast image of the cell shown in (a), psuedocoloration is for light intensity signal and has no quantitative meaning for phase contrast. (c) Registered time-lapse projection of the corresponding cross-section through the cell as indicated by the dash line in (b). (d) SLIM image of the cell in (b); the fields of view are the same. The arrows in (b) and (d) point to the nucleus which is incorrectly displayed by PC as a region of low signal. (e) Registered time-lapse projection of the corresponding cross-section through the cell, as indicated by the dash line in (d). The color bar indicates path-length in nm. (f) Path-length fluctuations of the points on the cell (indicated in d) showing intracellular motions (blue- and green-filled circles). Background fluctuations (black) are negligible compared to the active signals of the microglia. (g) Semi-logarithmic plot of the optical path-length displacement distribution associated with the glial cell membrane indicated by the dotted box in (a). The solid lines show fits with a Gaussian and exponential decay, as indicated in the legend. The distribution crosses over from a Gaussian to an exponential behavior at approximately 10 nm. The background path-length distribution, measured from the solid line box, has a negligible effect on the signals from cells and is fitted very well by a Gaussian function. The inset shows an instantaneous path-length displacement map associated with the membrane. (h) Dry mass calculated from micro-glia cell in dashed line box and background in solid line box.
I studied glial cell membrane fluctuations. Due to the extremely low noise level of SLIM, the probability distribution of path-length displacements between two successive frames was retrieved with a dynamic range of over 5 orders of magnitude (Fig. 3.9g). Note that these optical path-length fluctuations, $\Delta s$, are due to both membrane displacements and local refractive index changes caused by cytoskeleton dynamics and particle transport. Remarkably, this distribution can be fitted very well with a Gaussian function up to path-length displacements $\Delta s=10$ nm, at which point the curve crosses over to an exponential decay. The normal distribution suggests that these fluctuations are the result of numerous uncorrelated processes governed by equilibrium. On the other hand, exponential distributions are indicative of deterministic motions, mediated by metabolic activity.

Using the procedure outlined in [25], I use the quantitative phase information rendered by SLIM to extract the non-aqueous, i.e. dry mass, of this microglia cell. The approximately linear mass increase of 4.6 fg/s evidenced in Fig. 3.9h is most likely because the cell is continuously scavenging the neighboring glia cells. A further study of cell mass dynamics can be found in later section.

In sum, SLIM studies of cell dynamics will likely reveal previously unknown information regarding membrane motions, cytoskeleton mechanics, and particle transport within the cell.
3.5 Laplacian Phase Microscopy (LPM)

Particle tracking has drawn a lot of attention as a useful tool for rheology and micro-rheology studies [48, 49]. It is a popular way to evaluate the viscoelastic properties of micro-environments such as the cell cytoplasm, cell membrane, polymer solutions, etc. For cellular studies the probing beads are inserted into the cells or attached to the membrane, under the general assumption that they do not alter the normal physiology of the cell. Alternatively, fluorescence markers can be used for particle tracking. However, the fluorescence signal is usually weak and suffers from photobleaching and may produce phototoxicity under long-term observation. Therefore, it is valuable to develop label-free methods for tracking intrinsic particles within cells. Since the cells are usually transparent, or termed as phase object, a method based on phase information is required for imaging.

A number of QPI based tracking methods were reported recently [50-52]. However, in these previous reports, large beads or the cells themselves were tracked. This current limitation is due to two main reasons. First, because of the speckle generated by the high coherence of the illuminating light (typically lasers), the contrast-to-noise ratio in QPI images has never matched that exhibited in white light techniques such as phase contrast microscopy. Second, like all interferometric methods, the experimental arrangements tend to be rather complex, of high maintenance, limiting their in-depth biological applicability. On the other hand, qualitative methods such as phase contrast or DIC provide better contrast-to-noise ratio, but their application to intrinsic particle tracking is limited as well.

In this section, I exploit the exquisite spatial sensitivity of SLIM and demonstrate that the Laplace operator can be used to reveal a high-detail quantitative phase image without gradient artifacts common to differential interference contrast (DIC) microscopy. In particular, this
imaging approach, termed Laplace phase microscopy (LPM), allows us to quantify intracellular transport without the typical need for exogenous contrast agents. This opens up new avenues for particle tracking, which has been largely limited to fluorescently labelled tracers [48]. I show that LPM can study transport in live cells over very broad time scales, unlimited by photobleaching or photoxicity, as demonstrated by tracking organelles in hippocampal neurons and cardiomyocytes. The measurements indicate a diffusive regime for the particle motion, from which the diffusion coefficient can be extracted quantitatively.

Figure 3.8 shows an example of a quantitative phase image, $\phi(x, y)$, of cardiomyocytes, which were obtained from 2-day-old neonatal Sprague-Dawley rats (Harlan Laboratories, Inc.) using an approved protocol by the Institutional Animal Care and Use Committee (IACUC). Briefly, whole hearts were excised [53] in ice-cold Hanks balanced salt solution (HBSS) buffer, the atria were removed, and the remaining ventricles were quartered and digested in 0.05% (w/v) trypsin (Worthington Biochemicals) with gentle rotation (4 °C, 16 hours). To inhibit trypsin digestion, growth media (DMEM with 10% fetal bovine serum) was added for 5 minutes at 37 °C. After discarding the supernatant, 0.1% (w/v) purified type II collagenase (Worthington Biochemicals) was added for 45 minutes while rotating at 37 °C. The cardiac tissue was gently triturated to mechanically loosen the cells, filtered through a cell strainer, and centrifuged at 150×g for 10 minutes. The cell pellet was re-suspended in 37 °C growth media and pre-plated for 1 hour to enrich the suspension for cardiac myocytes.

I computed the LPM image (Fig. 3.8b) via the Laplace operator

$$\nabla^2 \phi(x, y) = \frac{\partial^2 \phi}{\partial x^2} + \frac{\partial^2 \phi}{\partial y^2}. \quad (3.1)$$

In order to filter out the high-frequency noise, which is amplified by the Laplace operator, I first convolved the images with a Gaussian kernel that is narrower than the diffraction
spot. Since the Laplacian is a linear operator, this is equivalent to a convolution of the image with the Laplacian of a Gaussian [54]. Here, the input image is represented in discrete pixels, so a discrete kernel that can approximate second order derivatives needs to be used. One possible convolution kernel is a 3 by 3 matrix $[0 \ -1 \ 0; \ -1 \ 4 \ -1; \ 0 \ -1 \ 0]$. As evident from this image, the Laplace operator is able to clearly define the organelle structures within the cell. It is known that heart cells are very active, i.e. energy-consuming; therefore, this type of cell is rich in mitochondria, which are responsible for the energy supply of the cell metabolism [46].

Mitochondria are most likely the predominant type of visible organelles, especially in the area surrounding the cell nucleus (indicated by the circular region in Fig. 3.8a). For comparison, I also computed a “synthetic” differential interference contrast (DIC or Nomarski) image (Fig. 3.8c), via the gradient of the quantitative phase image,

$$
\nabla \phi(x, y) = \frac{\partial \phi}{\partial x} \hat{x} + \frac{\partial \phi}{\partial y} \hat{y}.
$$

(3.2)

where $\hat{x}$ and $\hat{y}$ are the unit vectors along the two coordinates. While DIC can reveal intracellular particles, artifacts (“shadow” effects) are typically introduced due to the rapid change in sign of the first order derivatives, as visible in Fig. 3.8c. By contrast, the LPM image is free of such artifacts because it is based on a second order derivative (Eq. 3.1). Figure 3.8d shows the phase contrast image of the same cell, which reveals the difficulties associated with particle tracking due to the reduced contrast for small particles. Because phase contrast will mix the intensity information with phase information, suffers from phase ambiguity and is qualitative, the Laplacian of a phase contrast image is of much higher quality compared to LPM, as shown in Fig. 3.8e. Thus, LPM offers valuable opportunities for tracking these particles inside live cells, which in turn reports on the statistics of the organelle transport and on their diffusion coefficients. The measured irradiance at the sample plane is about 1 nW/μm². The exposure time
is usually 10-50 ms. Thus, this irradiance is 6-7 orders of magnitude below that of typical confocal microscopy [30]. Unlike imaging fluorescently tagged organelles, in LPM the imaging can be performed over arbitrarily long time scales, without limitations due to photobleaching or phototoxicity.

Figure 3.10 | MSD measurement for particles within the cardiac myocytes. (a) Zoom into the selected area shown in (d). (b) Displacement in Y direction. (c) Displacement in X direction. (d) Laplacian of the phase map. (e) MSD for the particle shown in (a). (f) MSD ensemble-averaged over 15 particles in (d).

Figure 3.10 demonstrates the procedure of quantifying intracellular diffusion via LPM. LPM images of a pair of beating cardiomyocytes in culture were recorded for more than a minute at a rate of 2.6 frames/s. The speed is currently limited by the acquisition camera which has a frame limit of 11 frames/s at full resolution. The organelle diffusion coefficient $D$ is extracted from the measured mean squared displacement (MSD) of the particles, and is defined as

$$\Delta r^2(\tau) = \left\langle \left( x(t+\tau) - x(t) \right)^2 + \left( y(t+\tau) - y(t) \right)^2 \right\rangle. \quad (3.3)$$
In Eq. 3.3, the angular bracket stands for temporal and ensemble average. For a diffusion process, the mean squared displacement grows linearly in time, \( \Delta r^2(\tau) = 2nD\tau \), where \( n \) indicates the dimensionality, i.e. \( n=1, 2, \) and 3 for diffusion in 1, 2, and 3 dimensions, respectively. Thus, the slope of \( \Delta r^2 \) reveals the diffusion coefficient.

Figures 3.10a-d illustrate the tracking of a single particle within the cell. Figure 3.10a shows the magnified particle in the selected area of Fig. 3.10d. Particles are traced using an automatic algorithm implemented in Java. The tracking algorithm may lose the particle at certain frames (e.g., due to the particle going out of focus) and find the particle again in later frames. If the algorithm identifies the same particles and relinks the traces, the link (an indication of skipped frames) is in red and the trace is in yellow (e.g., red in Fig. 3.10a). This will not cause any problem in the calculation of MSD because it is a time and ensemble average and is not sensitive to the individual frame loss. Figures 3.10b-c show the displacement of the particle in Y and X directions respectively. The MSD for the specific particle shown in Fig. 3.10e shows no specific trend at all, as expected. It can be seen that the high frequency component of the signal is due to the cell beating. However, if I analyze all 15 particles in Fig. 3.10d and perform an ensemble average, the MSD is linear which clearly shows a diffusive movement, as summarized in Fig. 3.10f. The results demonstrate that the high-frequency beating signal is averaged out and that a linear dependence is obtained. The resulting diffusion along y is slightly smaller than along x, which might be explained by the beating signal propagation between the two cells, almost parallel to the x-axis. Note that the overall value, \( D=0.14 \mu m^2/min \), obtained for the diffusion coefficient, \( D=(D_x+D_y)/2 \), is approximately 188 times smaller than that predicted by the Stokes-Einstein equation for a 1 \( \mu m \) particle diffusing in water at room temperature [55]. This reduced diffusion of organelles within the cells can be understood by realizing that they occupy a
crowded space, populated by cytoskeletal network and macromolecules, which make the effective viscosity significantly higher than that of water [56]. For comparison, I also captured the cardiomyocytes in culture with diffraction phase microscopy [1], a typical laser technique for quantitative phase imaging. Although the acquisition is faster, no particles can be resolved at all. It is clear that by simply applying a Laplacian operator to a laser based quantitative phase image is not useful for tracking intrinsic particles.

Further, I applied the LPM method to vesicles in hippocampal neurites. Recently, tracking of synaptic vesicles with far-field optical nanoscopy has been demonstrated [57]. Primary neuronal cultures from the postnatal rat were generated on glass bottomed dishes for imaging analysis based on the previously defined protocol. Briefly, postnatal hippocampal neurons were isolated from postnatal rats through enzymatic digestion, tissue rinse, dissociation, and centrifugation, followed by resuspension and plating in supplemented neurobasal media until imaging. Prior to imaging, neuron media was replaced with supplemented, CO₂ independent media Hibernate-A [58].
Figure 3.11 | Particle transport in neurites of a hippocampal neuron processor network. (a) Phase map of the neuron network. The arrows 1-5 show the time-traces of the corresponding points along the dash line. The whole field of view is 100 μm × 75 μm. The objective used is Zeiss Plan-Neofluar 40X/0.75. (b) Optical path length change in time for the 5 points indicated in (a). Peaks in the point traces correspond to phase shifts associated with (fast) organelle traffic. (c) Laplacian of the selected area in (a). The scale bar is 5 μm. (d) Phase map of the same area as in (c), with some particle traces shown in fine lines. (e) Log-log plot of the MSD for 70 individual particles in (d). Since the particles are confined in the Y direction, the diffusion coefficient for this direction is 2 orders of magnitude smaller than for the other direction. The inset shows the same MSD curves in linear representation and two Y axes.
With the high sensitivity of SLIM, the vesicles in neurites can be resolved, as seen from Fig. 3.11. SLIM images were acquired at 4 frames/min. The phase map shown (Fig. 3.11a) for the dashed line includes five temporal traces (Figs. 3.11a-b); trace 1 is the background, 2 and 4 are two large processes, 3 and 5 are two small processes. The optical path length changes for individual traces are shown in Fig. 3.11b, where the background stays very stable, and the peaks in the figure correspond to the particles in transport. Since the optical path length change corresponds to the dry mass, the heights of the bumps are indicators of the protein mass under transport. Evident from Fig. 3.11b, SLIM is sensitive enough to display the optical 10-20 nm path-length changes associated with particles in transport. Fig. 3.11c is the Laplacian of the selected area in Fig.3.11a. The particles with the traces are shown in Fig. 3.11d.

Neuronal processes demonstrate bidirectional transport of cargo (i.e. synaptic vesicle precursors, mitochondria, piccolo-bassoon transport vesicles, signalling endosomal organelles and translation machinery) to and from the soma and distal tips [59-61]. A subset of particles exhibit fast, directed (non-diffusive) motion. However, for the mean squared displacement analysis, I retain only particle trajectories that survive throughout the entire measurement window of 18 min. These long-time trajectories again exhibit diffusive motion, as indicated by the very strong linear dependence shown in the inset of Fig. 3.11e. To my knowledge, these are the first label-free diffusion measurements in neurons. As expected, the displacements parallel to the y-axis are approximately two orders of magnitude smaller than the x-axis displacements because the neurite structures are largely parallel to the x-axis. Because neurons have numerous long neurites, the surface area of a neuron far exceeds that of other cells by as much as 10-1,000 times [62]. Thus, the structure and function of the neuron place high demands on cellular resources, and the active transport of cellular resources is needed to maintain these demands.
Most axonal transport velocities range 0.5-5.0 µm/sec [60]. From the data, the directional transport velocity is about 0.5 µm/sec and the diffusion coefficients $D_x$=6.50 µm²/min and $D_y$=0.046 µm²/min. Thus, the diffusion process in dendrites is largely one-dimensional. Note that the diffusion coefficient in neurons is significantly larger than that in the heart cells, $D$=0.14 µm²/min, which indicates that the transport in neurons is more active. This result is consistent with the neuronal function that involves mass transport over large distances.

In summary, I demonstrated LPM as an efficient method for revealing and tracking organelles within unstained cells such as cardiomyocytes and neurons. To my knowledge, these are the first label-free diffusion measurements in such cells. Based on the advance of Spatial Light Interference Microscopy [63], this approach may help unravel important open questions regarding intracellular transport, which modulates cell development and cell signalling. It will also facilitate the study of the viscoelastic properties of the cells. Laplace phase microscopy, Fourier phase microscopy [11], and Hilbert phase microscopy [12] exploit the ability of mathematical operators (from which they borrow their names) to enhance optical imaging.

### 3.6 Cell Growth

The question of how single cells regulate and coordinate their growth has been described as "one of the last big unsolved problems in cell biology" [64]. The ability to measure the growth rate of single cells is thus integral to answering this question [25, 65-67]. The age-old debate is whether the growth rate is constant through the life-cycle of a cell (linear) or grows proportionally with its mass (exponential) [68-74]. Each growth pattern carries its own biological significance: If the growth is linear, cells will not need machinery to maintain homeostasis; on the other hand, exponential growth requires checkpoints and regulatory systems to maintain a constant size distribution [69]. 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 size is required [75].

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 [75]. For relatively simple cells such as *Escherichia coli* (*E. coli*), traditional microscopy techniques have also been used to assess growth in great detail [76]. 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. Recently, shifts in the resonant frequency of vibrating microchannels have been used to quantify the buoyant mass of cells flowing through the structures [65, 77]. Using this approach, Godin et al. have shown that several cell types grow exponentially; i.e., heavier cells grow faster than lighter ones [65]. Later, Park et al. extended this principle to allow mass measurements on adherent cells [66]. Still, an ideal method will perform parallel growth measurements on an ensemble of cells simultaneously and continuously over more than one cell cycle, quantify possible cell cycle phase-dependent growth, apply equally well to adherent and non-adherent cells, and work in a fully biocompatible environment [65, 75].

Here I demonstrate that SLIM 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 [78, 79]. Recently, it has been shown theoretically and experimentally that the surface integral of the cell phase map is invariant to small osmotic changes, which establishes that quantitative phase imaging methods can be used for dry mass measurements [25]. 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) [25] and \( \phi(x, y) \) is the measured phase. The total dry mass is then calculated by integrating over the region of interest (see Materials and Methods for details on this procedure). Remarkably, SLIM’s path-length sensitivity, of 0.3 nm spatially (pixel to pixel) and 0.03 nm temporally (frame to frame) translates into spatial and temporal sensitivities of 1.5 fg/\( \mu \)m\(^2\) and 0.15 fg/\( \mu \)m\(^2\), respectively. I show that SLIM enables studies of cell growth that benefit from the long-term stability over many days, sub-second temporal resolution, ability to study growth during specific phases of the cell cycle, throughput of 1,000s of cells in a single experiment and, importantly, ease of implementation by interfacing with existing inverted microscopes.

To demonstrate this approach I studied growth in both synchronous and asynchronous proliferating human osteosarcoma (U2OS) cell cultures. A motorized stage, incubator and computer-controlled perfusion system, combined with the long-term stability of SLIM, allowed us to continuously monitor a 3.2 \( \times \) 2.4 mm\(^2\) area of proliferating cell cultures for up to a week, with submicron transverse resolution and automatic refreshment of the growth medium every 4 hours (see Appendix D for details on preparation and analysis). Figure 3.12 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 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. 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 3.12 | SLIM measurement of U2OS cell growth over 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.
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, as discussed. I imaged YFP-PCNA (Yellow Fluorescent Protein - Proliferating Cell Nuclear Antigen) transfected human osteosarcoma (U2OS) cells, which enabled us to monitor PCNA activity via the fluorescence channel (Fig. 3.13). 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. 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 (see Fig. 3.13).

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**Figure 3.13 | 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.
Figure 3.14 | 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.
I 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 Appendix D. Figure 3.14c 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.

Figure 3.15 | 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: 20 μm.
Our data show 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. 3.15). 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.

The kinetics of mass growth during mitosis are extremely interesting and merit further investigation, especially because the cell undergoes significant shape changes. In order to demonstrate that SLIM can maintain accuracy during such extreme morphological changes, I studied the mass change during mitosis. Figure 3.15 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 whole mitosis. The cell mass is also relatively conserved during prophase, prometaphase and metaphase, where the morphology of the cell changes from flat to sphere, which confirms that the measurement is robust and not susceptible to drastic changes due to cell morphology. For the cells shown in Fig. 3.15, the measured growth rate is 1.6 pg/min. I hypothesize that, while this change in cell geometry does not affect the optical measurement, it must play an important role in cell growth regulation. Thus, the rounding up and flattening down of the cell take place with significant changes in volume-to-surface-area ratio. It is apparent in the SLIM data that occasionally cells release micron-size vesicles (blebs), which potentially can function as negative feedback for cell growth.
Due to the cell cycle phase discrimination provided by the YFP-PCNA, I can numerically synchronize the population \( a \ posteriori \) (Fig. 3.16a). In order to perform this numerical synchronization, I find the average time the cells spend in each cell cycle phase and then all the growth curves are re-sampled to fit this time window. The dotted lines in Fig. 3.16a 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 3.16b 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.

In summary, although population-level measurements on various cell types reveal exponential or linear growth patterns, I can expect large variability in results from different cell types. These types of variations are expected from a biological system and are of great scientific interest in themselves; by studying the variations in the growth patterns of single cells under varying conditions we may elucidate some of the underlying regulatory processes. Since SLIM is an imaging technique we may also simultaneously calculate the volume of regularly shaped cells. This allows us to explore questions of cell density and morphology and their roles in mass regulation.

By taking advantage of the ability of SLIM to be implemented as an add-on to a commercial microscope, I can utilize all other available 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 effects, 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 a direct look at the cells, which
can reveal artifacts, cell morphology, etc; vi) a lineage study is possible, i.e. a cell and its progeny may be followed.
CHAPTER 4: SPATIAL LIGHT INTERFERENCE TOMOGRAPHY (SLIT)

A three-dimensional visualization of cellular structure with its spatial relations will greatly improve the understanding of the cellular functioning and mechanics [80]. Most cellular structures cannot be resolved without high-resolution imaging in 3D [81]. For many cases 3D imaging is required for structure and even dynamics study [82].

In the 3D imaging toolbox, different tools satisfy different requirements. Some examples are confocal microscopy [83], 3D-STORM [81], diffraction tomography [21], projection tomography [84], OCT [85], etc. Confocal and 3D-STORM depend on fluorescence, while diffraction tomography, projection tomography, and OCT rely on the intrinsic contrast of the sample, i.e. the refractive index.

Confocal only renders the axial resolution of 500 nm. As a comparison, the transverse resolution of confocal is about 200 nm. There are many new techniques will improve the transverse resolution to about 20 to 30 nm, including STED [86], STORM [87], PALM [88], etc., which are usually coined as far-field nanoscopy [89]. To improve the axial resolution, 4Pi [90] is a choice with axial resolution about 90 nm. Another breakthrough is from 3D-STORM [81], where astigmatism is used for z-positioning.

3D deconvolution microscopy is another choice for 3D reconstruction, and an alternative to confocal microscopy. For confocal microscopy, the out-of-focus light is rejected by a pinhole in front of the detector. The 3D information is gained by a transverse scan (x-y scan) and a longitudinal scan (z-scan). For 3D deconvolution microscopy, no pinhole exists and the 3D information is gained by the acquisition of a stack of images corresponding to different sections of the specimen. In a general model, the original fluorophores’ distribution is denoted as $f(x,y,z)$;
after going through the imaging system, the acquired data (usually blurred) is denoted as \( f_b(x,y,z) \). The deconvolution microscopy restores the original function \( f(x,y,z) \) from the function \( f_b(x,y,z) \).

By contrast, quantifying optical path-lengths permits label-free measurements of structures and motions in a non-contact, non-invasive manner. Thus, quantitative phase imaging (QPI) has recently become an active field of study and various experimental approaches have been proposed [2]. Advances in phase-sensitive measurements enabled optical tomography of transparent structures, following Radon transform based reconstruction algorithms borrowed from X-ray computed imaging [14-18]. More recently, QPI-based projection tomography has been applied to live cells [19-21]. However, the approximation used in this computed tomography fails for high numerical aperture imaging, where diffraction and scattering effects are essential and drastically limit the depth of field that can be reconstructed reliably [22].

Due to the combination of white light illumination, high numerical aperture, and phase-resolved detection, in addition to suppressing the speckle effects that generally degrade laser light imaging, SLIM has the ability to provide optical sectioning as shown in Chapter 4.1. Thus, the out-of-focus blur is suppressed by the micron-range coherence length, which overlaps axially with the plane of focus (Fig. 2.4c). In order to obtain a tomographic image of the sample, I performed axial scanning by translating the sample through focus in steps that are no more than half the Rayleigh range and with an accuracy of 20 nm. Based on first order Born approximation, a linear shift invariant model was developed and the deconvolution in field was demonstrated, which allows for extracting the 3D refractive index distribution associated with extremely transparent specimens, including live cells. Similar to the deconvolution microscopy for fluorescence, the question now is how we can gain knowledge about the original distribution \( f(x,y,z) \) from the measured data \( f_b(x,y,z) \), where the distribution now will be the susceptibility (or
refractive index) other than the fluorophore intensity. This is not a trivial question. In fact, if the answer to this question is yes, it implicates two things: we did gain 3D information of the sample; and we actually achieved superresolution, which is similar to other fluorescence technique such as FIONA, where the position of the fluorophore is obtained through its centre mass.

The organization of the chapter is as follows: first the SLIM sectioning capability is discussed, followed by the theory for 3D reconstruction and resolution analysis; then examples of tomographic reconstruction are examined, including standard samples and neuron collateral with 3D confocal microscopy. At the end, a discussion of possible superresolution is presented with future outlook.

### 4.1 SLIM Depth Sectioning through Live Cells

Due to the combination of white light illumination, high numerical aperture, and phase-resolved detection, SLIM has the inherent ability to provide optical sectioning.

A schematic illustration of 3D sectioning of SLIM is shown in Fig. 4.1a. Assume P2 is the particle in focus and P1 is the out-of-focus particle. After passing P1, most of the incident light is unscattered and forms the field U1, while a small portion forms the scattered field S1. The field at P2 will have a contribution from P1, which is the interference between the scattered field S2 (propagated wavefront of S1) and unscattered field U1. If the coherence length is shorter (e.g. for SLIM it is about 1.2 μm) than the optical path difference S2-S1, the interference term will disappear and only intensity modulation is left. The intensity modulation will be further subtracted by phase-shifting interferometry as discussed. On the other hand, for laser systems where the coherence length is much longer, the field at P2 will be affected by P1 (usually seen as a lot of concentric rings around P1 in both transverse direction and longitudinal direction); for the
common phase contrast microscopy and bright field microscopy, which share the coherence gating with SLIM, the intensity modulation from P1 will contribute to the final image retrieval for P2. As shown in Fig. 4.1, the larger the angle of the P1-P2 path with respect to the optical axis, the larger the path-length difference S2-S1, and thus, the stronger the sectioning; i.e. higher scattering angles result in stronger sectioning. Therefore a high NA objective is preferred for better sectioning and 3D reconstruction.

Figure 4.1 | 3D sectioning of SLIM. (a) Visualization of 3D sectioning. (b-d) SLIM optical sectioning. (b) An x-z cut through a live neuron; the bottom of the image corresponds to the glass surface. The soma and nucleolus are clearly visible. (c-d) Images of the same neuron at the depths indicated by the dash lines in (c).
Figures 4.1b-d show depth-resolved SLIM imaging, where the quantitative phase images were retrieved at various z-positions, separated by 0.2 μm. While there is certain elongation along the z-axis, as indicated especially by the shape of the cell body and nucleolus in Fig. 4.1b, it is evident that SLIM provides optical sectioning, as indicated by Figs. 4.1c,d. The z-axis elongation is due to the details of the image formation in the microscope. Current efforts are aimed at correcting these artifacts and essentially solve the scattering inverse problem as detailed later, which will result in the quantitative 3D distribution of the cell’s refractive index.

There are two main factors that determine the ability of the method to perform 3D sectioning. First, there is coherence gating due to the extremely short coherence length (~1.2 μm) of the white light illuminating field. This optical gating due to the low coherence of light is at the heart of optical coherence tomography, which is now a well established method for deep tissue imaging [85]. However, in SLIM the optical sectioning ability depends also on the numerical aperture of the objective, i.e. depth of focus gating. SLIM provides depth sectioning only in the presence of finite numerical aperture because both the reference and the object beams are traveling through the sample. This aspect adds important versatility to SLIM, as it can adapt from low NA imaging when, for instance, the phase integral through the entire object thickness is needed (i.e. no sectioning, e.g. cell dry mass measurements [25]) for high NA imaging, when only a thin slice through the object is of interest. Needless to say, in SLIM the two optical gates (coherence and depth of field) are inherently overlapped axially because the two interfering fields are derived from the same image field.
Figure 4.2 | Comparison between imaging with different numerical apertures of the objectives. The sample is unstained prostate cancer tissue slice (in xylene). Thickness of tissue slice is 4 μm. Colorbar indicates phase in rad. (a) Phase map obtained by Zeiss EC Plan-Neofluar 40X/0.75. (b) Phase map obtained by Zeiss EC Plan-Neofluar 10X/0.3. Inset: 0.25X zoom of the 40X phase map.
A comparison between low NA SLIM imaging and high NA SLIM imaging is shown in Fig. 4.2. The sample is a prostate tissue slice sealed in xylene. The thickness of the tissue is 4 μm. Both 40×, NA=0.75 objective and 10×, NA=0.3 objective are used to image the same field of view. For 40×, the field of view only covers the center part of that of 10×. As expected, 40× phase map renders much more detail compared to 10× phase map. Remarkably, both phase maps show similar distribution across the sample, as if the 10× phase map were merely a blurred version of the 40× phase map. However, the sectioning is actually evident from the small defocused object (only rings can be seen here) indicated by the arrow in Fig. 4.2a. The same object is evident in 10× and literally contributes to the final phase.

Depending on the application, different objectives can be used for SLIM imaging. I have used extensively 40×/0.75 and 63×/1.4 oil for 3D reconstructions, while 10×/0.3 and 40×/0.75 were used for both dry mass and quantitative phase imaging.

4.2 Theory of Spatial Light Interference Tomography (SLIT)

As mentioned, because white light has very short coherence length (coherence gating), combined with high numerical aperture objective (depth of focus gating), I was able to get 3D information in live cells. The process can be modeled as follows (see also Fig. 4.3a and Fig. 4.1a): plane wave incident into the scattering sample; light was scattered by the sample and the scattered field propagated as spherical wave; unscattered light remains plane wave within first order Born approximation and interfere with the scattered field. Theoretical analyses show the imaging system now behaves like a band pass filter in \( k \) space (Fourier transform of the space vector \( r \)). 3D information of the sample is gained by z slice sectioning with white light, which means tomography reconstruction is possible. For laser based systems, such depth scanning will not
provide any new information of the sample because by measuring the amplitude and phase of the field at any plane, one can always numerically propagate the field to any other plane desired.

As depicted in Fig. 4.3a, the incident field is a plane wave propagating along the $k_0$ direction:

$$U^i(r; k_0) = A(\omega)e^{j\omega t}$$

(4.1)
where \( k \) is the illumination wavenumber and is related to temporal frequency \( \omega \) by the dispersion relation. If the medium is non-dispersive, then \( k = \omega/c \) where \( c \) is the speed of light in the medium.

Scattered field with first order Born approximation:

\[
U^s(\mathbf{r};k) = \int V(\mathbf{r}',k) F(\mathbf{r}',k) \frac{e^{i|\mathbf{r}-\mathbf{r}'|}}{|\mathbf{r}-\mathbf{r}'|} d^3\mathbf{r}',
\]

(4.2)

where the scattering potential of the medium \( F(\mathbf{r};k) = \frac{1}{4\pi} k^2 [n^2(\mathbf{r};k) - n_0^2] = \frac{1}{4\pi} k^2 \chi(\mathbf{r},k); n \) and \( n_0 \) are the refractive index of the sample and medium respectively, and \( \chi \) is the electrical susceptibility. For non-dispersive medium, the wavelength dependence of the electrical susceptibility \( \chi \) can be dropped, i.e. \( \chi \) is only a function of position.

Following the discussion of Born and Wolf [91], we may express the scattered field using angular spectrum representation at plane \( z_0 \):

\[
U^s(x,y,z_0,k_0;k) = \int \int_{-\infty}^{\infty} \frac{jk^2A(k)}{8\pi k_z} \tilde{\chi}(\mathbf{k} - \mathbf{k}_0) e^{i(k_x x + k_y y + k_z z_0)} dk_x dk_y,
\]

(4.3)

where \( \tilde{\chi}(\mathbf{k} - \mathbf{k}_0) = \int V(\mathbf{r}) e^{-i(\mathbf{k} - \mathbf{k}_0) \cdot \mathbf{r}} d^3\mathbf{r} \), i.e. the Fourier transform (3D Fourier transform, \( \chi \) must be non-dispersive, otherwise the Fourier transform cannot be defined here) of the scattering potential and \( k_z(k) = \sqrt{k^2 - k_x^2 - k_y^2} \). From Eq. 4.3, if we define the 2D Fourier transform of \( U^s(\mathbf{r};k) \) as \( \tilde{U}^s(k_x,k_y;z,k) \) (see Appendix E for the definition of Fourier transform used throughout the chapter), we have

\[
\tilde{U}^s(k_x-k_0x,k_y-k_0y;z_0,k) = \frac{j}{\pi} k^2 A(k) k_z^{-1} e^{i(k_zz_0)} \tilde{\chi}(\mathbf{k} - \mathbf{k}_0).
\]

(4.4)
Now if the spatial coherent point spread function of the 4f imaging system is $P(x, y; k)$, the scattered field at image plane will be the convolution of the field $U^i(x, y, z_o; k)$ and $P(x, y; k)$:

$$U^i_f(x, y, z_o; k) = U^i(x, y, z_o; k) * P(x, y; k)$$

$$= \int_{-\infty}^{\infty} \int_{-\infty}^{\infty} U^i(x', y', z_o; k)P(x-x', y-y'; k)dx'dy'$$  \hspace{1cm} (4.5)

By applying 2D Fourier transform on both sides of Eq. 4.5, we have

$$\tilde{U}^i_f(k_x, k_y; k_0, k_0; z_o, k)$$

$$= \frac{j2\pi A(k) e^{j k_0 z_o}}{k_z(k)} \tilde{F}(k - k_0, k) \tilde{P}_u(-k_x, -k_y; k)$$  \hspace{1cm} (4.6)

where $\tilde{P}_u(-k_x, -k_y; k)$ is the pupil function of the system, which is not related to frequency as long as the system is achromatic. Explicitly for such an achromatic system it can be written as $\tilde{P}_u(-s_x, -s_y)$, where $s_x = k_x / k$ and $s_y = k_y / k$. In reality new space variables should be introduced for the image space. However, since we are only considering the field at the front focal plane of the objective, which will be relayed with fidelity to the back focal plane of the tube lens, we use the same notations for image space and object space. It will not cause any problem as they will not appear at the same time.

The incident field will remain as a plane wave:

$$U^i_f(x, y, z_o; k) = A(k)e^{j(-k_0 x - k_0 y + k_0 z_o)}$$  \hspace{1cm} (4.7)

In phase shifting interferometry, a phase modulation SLM is used to introduce additional phase delays, and the corresponding field can be written as

$$U^i_f(x, y, z_o; k) = A(k)e^{j(-k_0 x - k_0 y + k_0 z_o) - jkd}$$  \hspace{1cm} (4.8)
The final intensity is thus expressed as the sum of the interference patterns over all emitting frequencies:

\[
I_f(x, y, z_0) = \int \left[ U_y^+(x, y, z_0; k) + U_y^-(x, y, z_0; k) \right] d\omega .
\] (4.9)

Apply 2D Fourier transform on both sides of Eq. 4.9, and we have

\[
\tilde{I}_f(k_x, k_y, z_0) = \left( \int A^2(k) d\omega \right) \delta(k_x, k_y) + \tilde{B}(k_x, k_y, z_0) + \tilde{C}(k_x, k_y, z_0),
\] (4.10)

where

\[
\tilde{B}(k_x, k_y, z_0) = \int \left[ \int \left[ U_y^+(x, y, z_0; k) \right] d\omega \right] e^{-j(k_x x + k_y y)} dxdy
\] (4.11)

\[
\tilde{C}(k_x, k_y, z_0; d) = j\tilde{P}_u(-s_x - s_{0x}, -s_y - s_{0y})
\]

\[
\int \frac{A^2(k)k^2e^{jkd}}{k_z(k)} \tilde{\chi}[k_x, k_y, (k_z - k_{0z})] e^{-j[k_z(k) - k_{0z}]} d\omega
\] (4.12)

Now we take the 1D Fourier transform on both sides of Eq. 4.12 regarding variable \( z_0 \),

\[
\tilde{C}_\beta(k_x, k_y, \beta; d) = j\tilde{P}_u(-s_x - s_{0x}, -s_y - s_{0y})
\]

\[
\int \frac{A^2(k)k^2e^{jkd}}{k_z(k)} \tilde{\chi}[k_x, k_y, (k_z - k_{0z})] \delta[\beta - [k_z(k) - k_{0z}]] d\omega
\] (4.13)

The delta function will select out \( k = \frac{-\beta^2 - k_x^2 - k_y^2}{2\beta} \). From the property of delta function

\[
\delta[f(t)] = \sum_{i=1}^{n} \frac{1}{|f'(t_i)|} \delta(t - t_i),
\] we get

\[
\tilde{C}_\beta(k_x, k_y, \beta; d) = \tilde{H} \times \tilde{\chi}(k_x, k_y, \beta)
\] (4.14)

where \( \tilde{H} \equiv \frac{jk^2\tilde{P}_u(-s_x - s_{0x}, -s_y - s_{0y})}{k_z(k) - k} \int \frac{A^2(k)e^{jkd}}{dk} d\omega \).

Now we have a recipe to obtain the function \( \chi(r) \):
1. Apply 3D Fourier transform to the complex field to get function \( \tilde{C}_\beta(k_x, k_y, \beta) \).

2. Divide \( \tilde{C}_\beta(k_x, k_y, \beta) \) by the leading factors (with proper regularization) and then obtain \( \tilde{F}(k_x, k_y, \beta) \).

3. Apply inverse 3D Fourier transform to get the 3D susceptibility distribution \( \chi(r) \).

The measurement of the optical field

In order to obtain the term \( \tilde{C}_\beta(k_x, k_y, \beta) \) in Eq. 4.14, the interference term needs to be obtained through the measurement. The principle relies on the spatial decomposition of a statistically homogeneous field \( U \) into its spatial average (i.e. unscattered) and a spatially varying (scattered) component. I assume the light is propagating in the z-direction. If the incident light field (plane wave) is \( U^i(z_0; d, k) \) and the scattered light field is \( U^s(x, y, z_0; k) \) (\( U^i \) and \( U^s \) are the same as \( U^f \) in Eq. 4.8 and \( U^f \) in Eq. 4.5; here I dropped the subscript "f" for simplicity), then for each frequency we have

\[
U(x, y; z, z_i, \omega) = U_0(z_i, \omega) + U_i(x, y; z, \omega) = |U_0(\omega)| e^{i\phi_0(z, \omega)} + |U_i(x, y; z, \omega)| e^{i\phi(x, y; z, \omega)}.
\]  

(4.15)

Using the spatial Fourier representation \( \tilde{U}(k_x, k_y, z_0; d, k) \) of \( U \), it becomes apparent that the average field \( U^i \) is proportional to the DC component \( \tilde{U}(0, 0, z_0; d, k) \), whereas \( U^s \) describes the non-zero frequency content of \( U \). Thus, the image field \( U \) can be regarded as a result from the interference between its spatial average and its spatially-varying component.

By controlling the phase delay between \( U^i \) and \( U^s \) (i.e. change of the \( d \)), I show that the spatially varying phase associated with an effective monochromatic field can be reconstructed.
simultaneously over the entire plane, in a quantitative manner. From Eq. 4.15, the intensity can be written as

$$I(x, y, z_0; d, k) = \left| U^i(k) \right|^2 + \left| U^s(x, y, z_0; k) \right|^2 + 2 \Re \{ \Lambda(x, y, z_0; d, k) \}, \quad (4.16)$$

where $\Lambda(x, y, z_0; d, k) = \overline{U^i(z_0; d, k)} \cdot U^s(x, y, z_0; k)$ and the bar stands for complex conjugation.

It has been shown that if the field is fully spatially coherent, then $C$ factorizes as

$$\Lambda(x, y, z_0; d, k) = S(k) \cdot \left| W(x, y, z_0; k) \right| e^{-j[kd + \Delta \phi(x, y, z_0; k)]}, \quad (4.17)$$

where $\Delta \phi(x, y, z_0; k) = \phi^d(z_0; k) - \phi^s(x, y, z_0; k)$ is the spatially varying phase difference of the cross-correlation function, $S(k) = A^2(k)$ the non-structural $k$ dependence and $W$ the leftover. If no dispersion is considered, the structural frequency dependence of $W(x, y, z_0; k)$ and $\Delta \phi(x, y, z_0; k)$ can be dropped. Integrate Eq. 4.17 over the frequency support, and we have

$$\int \Lambda(x, y, z_0; d, k) d\omega = \left| W(x, y, z_0) \right| e^{-j\Delta \phi(x, y, z_0)} \Gamma(d), \quad (4.18)$$

where $\Gamma(d) = \int S(k) e^{-jkd} d\omega$. If the central frequency of the power spectra $S(k)$ is $k_0$, then we have

$$\Gamma(d) = \tilde{\Gamma}(d) e^{-jkd} \quad (4.19)$$

with the envelop $\tilde{\Gamma}(d) = \int S(k + k_0) e^{-jkd} d\omega$. Thus we can integrate Eq. 4.16 over all frequencies and get rid of the frequency dependency:

$$I(x, y, z_0) = \int A^2(k) d\omega + \int \left| U^s(x, y, z_0) \right|^2 d\omega + C(x, y, z_0), \quad (4.20)$$

where $C(x, y, z_0) = 2\tilde{\Gamma}(d) \left| W(x, y, z_0) \right| \cos[k_0d + \Delta \phi(x, y, z_0)]$. It is obvious that the 2D Fourier transform of $C$ is $\tilde{C}$ in Eq. 4.12. Equation 4.20 indicates that, for spatially coherent illumination, the spatially varying phase of the cross-correlation function can be retrieved through
measurements at various delay $d$. This phase information is equivalent to using purely monochromatic light at frequency $\omega_0$. If one varies the delay $d$ between $U^i$ and $U^s$, interference is obtained simultaneously at each point of the image. The average $U^i$ is constant over the entire plane and can be regarded as the common reference field of an array of interferometers. In addition, $U^i$ and $U^s$ traverse similar optical paths. Thus, the influence of inherent phase noise due to vibration or air fluctuations is inherently minimized, allowing for a precise retrieval of $\Delta \phi$.

From Eq. 4.20, the spatially varying phase can be reconstructed as

$$\Delta \phi(x,y) = \tan^{-1} \left[ P \frac{I(x,y;\Delta z_k) - I(x,y;\Delta z_{k+1})}{I(x,y;\Delta z_{k}) - I(x,y;\Delta z_{k+1})} \right],$$

(4.21)

where $P = \frac{\tilde{\Gamma}(\Delta z_0) + \tilde{\Gamma}(\Delta z_2)}{\tilde{\Gamma}(\Delta z_1) + \tilde{\Gamma}(\Delta z_3)}$, $\Delta z_k$ is given by $\omega_0 \Delta z_k / c = k \cdot \pi / 2$, $k = 0, 1, 2, 3$. For modifications of the time delay around $z - z_i = 0$ that are comparable to the optical period, $\tilde{\Gamma}$ can be assumed to vary slowly at each point and the correction factor $P$ can be ignored. If we define $eta(x,y) = |U_0(x,y)|/|U_0|$, then the phase associated with the image field $U = U_0 + U_1$ can be determined as

$$\phi(x,y) = \tan^{-1} \left[ \frac{\beta(x,y) \sin[\Delta \phi(x,y)]}{1 + \beta(x,y) \cos[\Delta \phi(x,y)]} \right].$$

(4.22)

Equation 4.22 shows how the SLIM image is retrieved via 4 successive intensity images measured for each phase shift. The interference term therefore is the real part of the complex field.

For most of the biological samples (e.g. cells), absorption is usually negligible; i.e., they are phase objects and can be characterized as $U(x,y,z_i) = e^{i\phi(x,y,z_i)}$. Thus the unscattered light is
\( U^i = 1 \) and scattered light is \( U^s(x, y, z_0) = e^{i\phi(x, y, z_0)} - 1 \). The interference term is thus \( U^s \overline{U}^i = e^{i\phi(x, y, z_0)} - 1 \), whose real part is \( C(x, y, z_0) = \cos[\phi(x, y, z_0)] - 1 \). A 3D Fourier transform of the acquired 3D stack \( C(x, y, z_0) \) will thus provide the \( \tilde{C}_\beta(k_x, k_y, \beta) \) in Eq. 4.14 for reconstruction.

The reconstructed 3D refractive index map is shown in Fig. 4.3b-d. Compared to Fig. 4.1b-d, most out-of-focus light is rejected after reconstruction. Because the \( k \) space is band-limited, frequency extrapolation might be useful here to retrieve the lost spatial frequency and thus improve the 3D resolution. A detailed frequency support and resolution analysis can be found in Chapter 4.3.

**4.3 SLIT Resolution Analysis**

It is well known that for coherent fields, as long as at one plane the complex field is recorded, one can always refocus it to any plane desired, including far-field as described in Chapter 5. This cannot be called 3D imaging. Mathematically it is impossible to reconstruct \( \mathbb{R}^3 \) from \( \mathbb{R}^2 \) if you do not perform more measurements. It is ambiguous, especially in the digital holography community where they usually achieve "3D" imaging simply by numerical propagation of the field, which should be termed as refocusing rather than a real 3D reconstruction.

In order to achieve tomography, it is possible to use diffraction tomography proposed by Wolf [91], where the beam direction is scanned in two dimensions and thus is a mapping from \( \mathbb{R}^4 \) to \( \mathbb{R}^3 \), or the CT-like technique described in tomographic phase microscopy [21], also a mapping from \( \mathbb{R}^4 \) to \( \mathbb{R}^3 \). I have introduced SLIT as a new tomography method with a mapping from \( \mathbb{R}^3 \) to \( \mathbb{R}^3 \). In order to fully understand the capability of SLIT, it is desired to have an analysis of its resolution and frequency support.
The start point is Eq. 4.6, which expresses a very basic result that within the accuracy of the first-order Born approximation, the amplitude (complex) of the scattered field depends entirely on the Fourier component of the scattering potential and the pupil function. The Fourier component is labeled by the vector \( \mathbf{Q} = \mathbf{k} - \mathbf{k}_0 \), where I assume the 3D Fourier transform of the scattering potential will be \( \tilde{F}(\mathbf{Q}) \).

**Figure 4.4 | Frequency support analysis for SLIT.** (a) Modified Ewald’s sphere of reflection. (b) Frequency coverage in \( \mathbf{k} \) space. (c) Experimental results for the frequency coverage. A square root is taken to emphasize the small amplitude. (d) Log scale display of (c).
Suppose the object is illuminated by a plane wave in the direction of $s_0$ shown in Fig. 4.4a and Fig. 4.3a; the scattered field is measured in the far zone in all possible directions $s$. All possible vector $k$ will form a circle as shown in Fig. 4.4a, for elastic scattering considered here. The dash line divides the forward scattering and backward scattering. Depending on the numerical aperture of the objective, only the scattered field will be collected when the scattering angle $\theta$ satisfies $n \sin \theta < \text{NA}$, which will further limit the possible $k$. Thus all the possible coverage will be the green arc shown in Fig. 4.4a. The discussion here is an extension of Ewald's sphere of reflection [91].

Now assume the light is incident along the z-direction, as shown in Fig. 4.4b. By varying the wavelength, the centre of the circle will vary from $O_1$ to $O_2$, resulting in continuous frequency coverage shown by the green area. Here I only show one section of the 3D coverage. The whole 3D map is rotation symmetric along the $Q_z$ axis. The radius of the small circle will be $k_{\text{min}}$ and the radius of the large circle will be $k_{\text{max}}$. For wavelength range from 0.4 $\mu$m to 0.7 $\mu$m, $k_{\text{min}}=9.0$, $k_{\text{max}}=15.71$. In order to make full use of the $k$ space coverage and avoid aliasing, the sampling in $z$ direction should be $2\pi / \Delta z \geq 2 \times k_{\text{max}}$, i.e. $\Delta z \leq 0.2$ $\mu$m. Here I assume only the forward scattering angles are collected. If the reflected light is also collected, the sampling will be $2\pi / \Delta z \geq 2 \times 2k_{\text{max}}$, i.e. $\Delta z \leq 0.1$ $\mu$m.

Now let us further consider the ring illumination. In the case of ring illumination, the incident direction of the $s_0$ will form a cone, and the new coverage will be the green area rotated along the cone.

Apparently, the frequency coverage of the 3D susceptibility depends on the bandwidth of the source (coherence gating) and numerical aperture of the objective (depth of focus gating), shown in Fig. 4.4b. For laser system, because of the long coherence length, the frequency
coverage in k-space of the susceptibility will be a sheet. By measuring at any plane the complex field, one can numerically propagate to any another plane, i.e. no 3D information can be acquired by depth scanning for laser system. For white light, the frequency coverage in k-space will be a volume. Additional information will be acquired by depth scanning. In order to extend the frequency coverage, in principle frequency extrapolation can be applied, based on the Plancherel-Polya theorem [91]. Such frequency extrapolation also forms the basis for possible super-resolution, which is discussed in detail later.

4.4 SLIT Applications

Equation 4.14 shows that under first order Born approximation, the measured data will be the convolution between scattering potential and point spread function. Here I provide a detailed description of SLIM 3D reconstruction based on the iterative deconvolution [92].

As discussed, for a transparent sample such as a live cell, the 3D distribution of the real field measured \( U \) is the result of the convolution between the scattering potential of the specimen and the point spread function \( P \) of the microscope,

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

(4.23)

where \( \chi(\mathbf{r}) = n^2(\mathbf{r}) - 1 \) is the scattering potential and \( \mathbf{r} = (x, y, z) \). I retrieved \( P \) experimentally by measuring a set of axially-resolved quantitative phase images of a point scatterer. In order to retrieve \( F \), I performed the inverse operation in the spatial frequency domain as

\[
\tilde{\chi}(\mathbf{q}) = \tilde{U}(\mathbf{q}) / \tilde{P}(\mathbf{q}),
\]

(4.24)

where \( \sim \) indicates Fourier transformed functions.

In order to perform the deconvolution, the PSF needs to be determined beforehand. Both theoretical and experimental methods can be used for such purpose. Theoretical methods are
noise free, but may not be able to model all the parameters such as aberrations. In order to get the model, some detailed information of the system, such as the thickness and refractive index of the coverslip, are required.

Figure 4.5 | Measured PSF (point spread function). Objective: Zeiss EC Plan-Neofluar 40X/0.75. (a) The PSF in the x-z plane. (b) PSF profiles along x- and z-axis.

Another alternative is the experimental method. Microspheres with diameter about one-third of the resolution are usually imaged. This method is noise-limited but counts all contributions (e.g. spherical aberrations in the system), which is usually better for high-NA lenses. I measured the point spread function by scanning through focus a 200 nm diameter polystyrene bead for 40×/0.75 objective (50 nm for 63×/1.4 objective). Phase and amplitude images were measured at each depth position, incremented in steps of 200 nm, and $P$ was obtained as the real part of this measured complex analytic signal. This measured $P$ is shown in Fig. 4.5. The full-width half-maximum of $P(x)$ has a value of 0.36 µm. The full-width half-maximum of the $P(z)$ main lobe, which defines the axial resolution, has a value $\delta z=1.34$ µm. The measured profiles are slightly larger than expected mainly because the bead used as sample had a finite diameter (200 nm).
With the information of PSF, we are able to apply different deconvolution methods, e.g. linear methods including inverse filtering, Wiener filters, Tikhonov filtering, etc., as well as constrained iterative methods including Janson-van Cittert, nonlinear least squares, statistical image restoration including maximum likelihood, maximum a posteriori probability, maximum penalized likelihood, blind deconvolution and more [93]. Linear methods are well understood, generally non-iterative and computationally cheap. For inverse filtering method, the spectrum within the frequency support is divided by the system function and outside the support is set to 0. For Wiener filter a false inverse which depends on the signal-to-noise ratio (SNR) is used. Both methods are sensitive to PSF and noise, and may generate negative intensity. Most importantly, they are unable to extrapolate unmeasured spatial frequencies. Besides linear methods, iterative deconvolution methods are proven effective and advantageous in many senses [93]. Thus the iterative deconvolution is preferred, and the details of the method can be found in Appendix F.

**SLIT of standard samples**

Using the aforementioned procedure, I successfully measured the refractive index associated with polystyrene beads (Polyscience Inc., diameter 1.025 μm). Figure 4.6 shows this map where the refractive index measurement agrees very well with expected value of 1.59.

Further, I made two layers of beads separated by 4.25 μm (Fig. 4.7). The beads are suspended in water and sandwiched by two coverslips. The first layer of beads is attached to the bottom coverslip and the second layer is attached to the top coverslip. The beads on the top layer (e.g. the one pointed by the arrow) disappear in the bottom refractive index map and vice versa. This is a phantom sample relevant for imaging cells, where vesicles and organelles are distributed in 3D.
Figure 4.6 | Refractive index calibration for 1 μm polystyrene beads in water. Objective: Zeiss EC Plan-Neofluar 40X/0.75.

Figure 4.7 | Two layers of 1 μm beads in water. The beads are sandwiched by two coverslips and spaced by 4.25 μm. First layer of beads are attached to the bottom coverslip and second layer of beads are attached to the top coverslip. The beads on the top layer (e.g. the one pointed by the
arrow) will disappear in the bottom refractive index map and vice versa. Objective: Zeiss EC Plan-Neofluar 40X/0.75.

Besides 1 μm beads in water, I measured larger beads (Polyscience Inc., diameter 3.12 μm) immersed in microscope immersion oil (Zeiss Immersol 518F, refractive index 1.518). Figure 4.8 shows the reconstructed phase map of different Z positions. A defect (most likely a pore) is clearly seen at Z=-1.45 μm slice. Also a cleaved edge of the beads can be found at Z=0 μm.

Figure 4.8 | Refractive index map of 3.1 μm polystyrene beads in immersion oil (Zeiss Immersol 518F, refractive index 1.518) at different Z positions. Objective:Zeiss Plan-Apochromat 63X/1.4 oil.
I also measured photonic crystal samples (Fig. 4.9), which are obtained from 1 μm SiO2 spheres (Fiber Optic Center Inc.) dispersed in ethanol (4% w/w). Approximately 6 ml of microsphere suspension was dispensed into a 20 ml scintillation vial (Fisher) with a 1 cm × 2.5 cm cut glass coverslip. The substrate was placed at an angle (about 35°) in the vial. The temperature was set to 50 °C in an incubator (Fisher, Isotemp 125D). The sample is immersed in alcohol and covered with another coverslip upon imaging. As evident in the plot, it is difficult to indentify three consecutive layer of 1 μm silica beads with axial scanning from phase contrast,
while the reconstructed SLIM image shows nice and clear sectioning. Nevertheless, the out-of-focus light still presents in SLIM images, e.g. SLIM image at $Z=0 \ \mu m$ and $Z=1.0 \ \mu m$. The sectioning is further improved with a deconvolution algorithm, as shown in SLIT images at the same focus $Z=0 \ \mu m$ and $Z=1.0 \ \mu m$, where most of the out of focus light is rejected. Due to the notorious halo effect associated with phase contrast [1], the reconstructed SLIM images suffer from alleviated phase. The tomographic reconstruction is also affected, especially when the volume of interest is surrounded by highly scattering structures (the indistinct beads at $Z=2.2 \ \mu m$ of SLIT). The current effort is to minimize this effect and thus further improve the tomographic reconstruction.

**Cell Tomography**

Results obtained from a single neuron are shown in Fig. 4.10. Figures 4.10a-b present the refractive index map of the cell at two different depths, separated by 5.6 $\mu m$. Notably, the refractive index distribution seems to fall below 1.39, which is compatible with previous average refractive index measurements on other cell types [26]. The nucleolus (arrow in Fig. 4.10b) has a higher value, $n\sim1.46$, which matches very well previous measurements on DNA [94]. Fig. 4.10c shows a 3D rendering of the cell lying on a glass substrate. For comparison, I used fluorescence confocal microscopy to obtain a similar view of a different neuron. The neuron was stained anti-PSA IgG #735. The numerical aperture of the confocal microscope was NA=1.2, higher than the NA=0.75 used in SLIM, which explains the higher resolution of the confocal image. This qualitative comparison reveals that the quality of 3D imaging by SLIM is comparable with that obtained via fluorescence confocal microscopy. However, SLIM offers significant advantages over confocal microscopy. The SLIM tomogram in Fig. 4.10c was acquired in less than one minute, but much faster SLIM imaging is possible by simply employing higher frame rate
camera and liquid crystal modulator. Further, SLIM is a label-free method that enables studying cells dynamically over long periods of time, completely non-invasively. Remarkably, SLIM reveals dynamically the high refractive index associated with chromatin during mitosis, as shown in Fig. 4.11. This type of 4D (x,y,z, time) imaging may uncover new science in phenomena such as cell division, motility, differentiation, and growth.

Figure 4.10 | Tomography capability. (a-b) Refractive index distribution through a live neuron at different z positions. The soma and nucleolus (arrow) are clearly visible. The color bar indicates the refractive index. (c) 3D rendering of the same cell. The field of view is 100 μm × 75 μm × 14 μm 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. 3D rendering in (c-d) is done by ImageJ 3D viewer.
Mathematically, the reconstruction is based on the theory of linear shift invariant (LSI) systems. An LSI system exhibits: a. linear superposition (field summation for coherent case, e.g. SLIM, and intensity summation for incoherent case such as fluorescence); b. consistent imaging quality throughout the field of view. In terms of third order aberrations, spherical aberration is independent of FOV, which means a viable system will also correct the blur due to spherical aberrations after deconvolution.

Within the framework of an LSI system, the problem now can be rephrased as follows: the original object function will convolve with PSF and then experience another noise process, which can be productive or addictive, resulting the blurred image I obtained. The deconvolution will reject the blurry from the system and, preferably, suppress the noise.
In order to gain some insights into the possibilities of the reconstruction, I first consider a case where the system is aberration-free and noise-free. If the object is $f(x, y, z)$ and its 3D Fourier transform is $\tilde{f}(q_x, q_y, q_z)$, then the Fourier transform of the acquired data $f_b(x, y, z)$ will be $\tilde{f}(q_x, q_y, q_z) \cdot \hat{h}(q_x, q_y, q_z)$ where $\hat{h}(q_x, q_y, q_z)$ is the system function.

It is obvious that the blur comes from the finite frequency support of the system function. In other words, from the acquired data we only know a small piece of the $\tilde{f}(q_x, q_y, q_z)$. Can we go back to the original function from such a small piece?

A short answer is yes. Since $\tilde{f}(q_x, q_y, q_z)$ is an analytical function due to the finite support of object $f(x, y, z)$, by simple Taylor expansion we can get the whole function from its debris, which is usually called frequency extrapolation. In real applications, because of the noise and the limitation of sampling (its density), the ability of superresolution is usually limited.

In order to gain some insights into and appreciation of super-resolution with iterative deconvolution methods, we start the discussion from a one-dimensional frequency extrapolation example. The material discussed here is known as the Gerchberg method in optics community and the Papoulis method in signal processing community. It has been studied in the context of general theory of superresolution and extrapolation [95, 96].

Previous knowledge of the sample is always helpful. For most real cases we know for sure the signal has finite support, which means $f(x) = 0$ for $|x| > X/2$. Theoretically the spectrum of such space-limited signal will expand through the whole Fourier space. However, due to band-limited system, in frequency domain the acquired data usually have only finite support as shown in Fig. 4.12, where the information beyond Q/2 is lost. Can we recover the information denoted by the red dotted line?
To get the invisible distribution, one method is shown in Fig. 4.12. Starting from the frequency function, IFT (inverse Fourier transform) is applied to the frequency function. Since the frequency is finite supported, after IFT the space domain will no longer be finite supported. Then we truncate it and Fourier transforms it back. Now we gain some frequency information outside the original support (-Q/2, Q/2). However, the frequency information inside the support will no longer be the one we start with. Replace them with the known \( F(q) \) and apply IFT again.
Repeat this process and you will get a converged solution. The frequency extrapolation method can be comprehended mathematically as projection on convex set (POCS).

![Figure 4.13](image)

**Figure 4.13** | Comparison between SLIT after frequency extrapolation with SEM. Cells are echinocytes (bumpy red blood cells). (a) SLIT image after frequency extrapolation. (b) Blowup of the selected area in (a). (c) SEM image of a fixed echinocyte; (b) and (c) share the same scale bar. The SLIM image is acquired with ZEISS EC Plan Neofluar 40X/0.75PH2 objective.

Such frequency extrapolation is very important in several disciplines. One example is in CT, where certain angle information cannot be acquired resulting in missing cones in frequency
domain. Mathematical theory (Plancherel-Polya theorem [91, 97]) assured the convergence and uniqueness of the solution. Compared to linear methods, the main difference is that it can pick up the invisible distribution, though at the cost of extensive computation. A prior knowledge of the sample provides constraints. Sometimes the constraint can be applied easily, such as positivity (the pixel value is always positive) and compact support.

In practice, the above process is limited by noise and instability, and the achievable range of extrapolation is limited. Figure 4.13 shows an example of such extrapolation. Because the signal-to-noise ratio for phase image is very high, frequency extrapolation in k-space is possible and thus superresolution can be achieved as shown for the comparison between SLIM and SEM. Further study is necessary to quantify the gains and limitations for SLIT superresolution.

4.6 Summary

Due to the combination of low coherence and depth of field, SLIM renders 3D tomographic images of transparent structures. The results demonstrate that rich quantitative information can be captured from both fixed structures and cells using SLIT. Because of its implementation with existing phase contrast microscopes, SLIT has the potential to elevate phase-based imaging from observing to quantifying over a broad range of spatiotemporal scales. I anticipate that the studies allowed by SLIT will further our understanding of the basic phenomena related to biological applications as well as material science research.
CHAPTER 5: FOURIER TRANSFORM LIGHT SCATTERING (FTLS)

Elastic (static) light scattering (ELS) has made a broad impact in understanding inhomogeneous matter, from atmosphere and colloidal suspensions to rough surfaces and biological tissues [98]. In ELS, by measuring the angular distribution of the scattered field, one can infer noninvasively quantitative information about the sample structure (i.e. its spatial distribution of refractive index). Dynamic (quasi-elastic) light scattering (DLS) is the extension of ELS to dynamic inhomogeneous systems [99]. The temporal fluctuations of the optical field scattered at a particular angle by an ensemble of particles under Brownian motion relate to the diffusion coefficient of the particles. Diffusing wave spectroscopy integrates the principle of DLS in highly scattering media [100]. More recently, dynamic scattering from probe particles was used to study the mechanical properties of the surrounding complex fluid of interest [101]. Thus, *microrheology* retrieves viscoelastic properties of complex fluids over various temporal and length scales, which is subject to intense current research especially in the context of cell mechanics [102].

Light scattering studies have the benefit of providing information intrinsically averaged over the measurement volume. However, it is often the case that the spatial resolution achieved is insufficient. “Particle tracking” microrheology alleviates this problem by measuring the particle displacements in the imaging (rather than scattering) plane [103, 104]. However, the drawback in this case is that relatively large particles are needed such that they can be tracked individually, which also limits the throughput required for significant statistical average.

I present Fourier transform light scattering (FTLS) as an approach to studying static and dynamic light scattering, which combines the high spatial resolution associated with optical microscopy and intrinsic averaging of light scattering techniques. The underlying principle is to
retrieve the phase and amplitude associated with a coherent microscope image and numerically propagate this field to the scattering plane. The phase measurement is performed in the image plane of a microscope rather than the Fourier plane [105], which offers important advantages in the case of the thin samples of interest here. The signal sampling, phase reconstruction and unwrapping are more robustly performed in the image plane than in the case of Fourier or Fresnel holography, in which the detection is performed at some distance from the image plane, where high-frequency interference patterns and phase discontinuities may occur. Further, in the image plane of a thin and transparent sample, such as live cells, the intensity is evenly distributed, which efficiently utilizes the limited dynamic range of the CCD, as opposed to the common scattering measurement where a very high peak exists at 0° angle (DC).

![Figure 5.1 | SLIM-FTLS of neurite.](image)

(a) Quantitative phase image of a neuronal structure. The color bar indicates optical path-length in nm and the scale bar measures 10 microns. (b) Scattering phase function associated with the structure in (a). The inset shows the scattering map of the neurite indicated in a by the dotted box.

I employed FTLS to measure scattering phase functions of different cell types and demonstrate its capability as a new modality for cell characterization. Figure 5.1 shows an
example of FTLS measurements from substructures of a putative axon of a live neuron, which, to my knowledge, cannot be attained using existing techniques due to extremely weak scattering.

Recently, FTLS is used to extract quantitatively the scattering mean free path \( l_s \) and anisotropy factor \( g \) from tissue slices of different organs [28]. This direct measurement of tissue scattering parameters allows predicting the wave transport phenomena within the organ of interest at a multitude of scales. The scattering mean free path \( l_s \) was measured by quantifying the attenuation due to scattering for each slice via the Lambert-Beer law,

\[
l_s = -d / \ln[I(d)/I_0],
\]

where \( d \) is the thickness of the tissue, \( I(d) \) is the irradiance of the unscattered light after transmission through the tissue, and \( I_0 \) is the total irradiance, i.e. the sum of the scattered and unscattered components. The unscattered intensity \( I(d) \), i.e. the spatial DC component, is evaluated by integrating the angular scattering over the diffraction spot around the origin.

The anisotropy factor \( g \) is defined as the average cosine of the scattering angle,

\[
g = \int \int \cos(\theta) p[\cos(\theta)]d[\cos(\theta)] / \int \int p[\cos(\theta)]d[\cos(\theta)],
\]

where \( p \) is the normalized angular scattering, i.e. the phase function. Note that, since Eq. 5.1 applies to tissue slices of thickness \( d<l_s \), it cannot be used directly in Eq. 5.2 to extract \( g \) since \( g \) values in this case will be thickness-dependent. This is so because the calculation in Eq. 5.2 is defined over tissue of thickness \( d=l_s \), which describes the average scattering properties of the tissue (i.e. independent of how the tissue is cut). Under the weakly scattering regime of interest here, this angular scattering distribution \( p \) is obtained by propagating the complex field numerically through \( N=l_s/d \) layers of \( d=4 \) microns thickness,

\[
p(q) \propto \iiint [U(r)]^N e^{i \mathbf{q} \cdot \mathbf{r}} d^2\mathbf{r}.
\]
Equation 5.3 applies to a slice of thickness $l_s$. It reflects that, by propagating through $N$ weakly scattering layers of tissue, the total phase accumulation is the sum of the phase shifts from each layer, as is typically assumed in phase imaging of transparent structures. The angular scattering distribution, or phase function, $p(\theta)$ is obtained by performing azimuthal averaging of the scattering map, $p(q)$, associated with each tissue sample.

The results above showed that FTLS can quantify the angular scattering properties of thin tissues, which thus provides the scattering mean free path $l_s$ and anisotropy factor $g$ for the macroscopic (bulk) tissue. In the next chapter, I further develop the scattering phase theorem which directly connects the scattering properties of the tissue with the phase images and renders spatially resolved scattering properties.
CHAPTER 6: BRIDGING THE GAP BETWEEN SCATTERING AND IMAGING

Light scattering from tissues has evolved as a dynamic area of study and attracted extensive research interest, especially due to the potential it offers for in-vivo diagnosis [106-117]. Mathematically, the strong light-tissue interaction can be modeled by a radiative transport equation, in complete analogy to the problem of neutron transport in nuclear reactors [118]. With further simplifying assumptions, a diffusion model can be applied to describe the steady state [119] and time-resolved [120] light transport in tissues. The refractive index of biological structures has been modeled both as discrete particle distribution [121] and continuous or fractal [122]. Light propagation in bulk tissue is described by two statistical parameters: the scattering mean free path, $l_s$, which provides the characteristic length scale of the scattering process, and the anisotropy factor, $g$, which scales $l_s$ to higher values, $l_s/(1-g)$, to account for forward scattering. The direct measurement of these scattering parameters is extremely challenging and, therefore, often simulations, e.g. Monte Carlo [123] or finite difference time domain [124], are used iteratively instead.

Recently, Fourier transform light scattering (FTLS) has been developed as the spatial analog of Fourier transform spectroscopy to provide angular scattering information from phase-sensitive measurements [27]. Thus, FTLS was used to measure $l_s$ from angular scattering from tissue slices and the anisotropy parameter $g$ was determined by fitting the scattering pattern with Gegenbauer kernel phase function [28].

In this chapter, I show that quantitative phase imaging of thin slices can be used to spatially map the tissue in terms of its scattering properties. Specifically, mathematical relationships are derived between the phase map $\mathcal{F}(r)$ associated with a tissue slice of thickness
\( L \ll l_s \), and scattering parameters of the bulk, i.e. \( l_s \) and \( g \). First, I show that the scattering mean free path \( l_s \) averaged over a certain area across a tissue slice is directly related to the mean-squared phase (variance of the phase) within that region. Second, I prove that the anisotropy factor \( g \) relates to the phase gradient distribution. These relations, referred to collectively as the \textit{scattering-phase theorem}, are expressed mathematically as

\[
I_s = \frac{L}{\langle \Delta \phi^2(r) \rangle_r},
\]

\[
g = 1 - \left( \frac{l_s}{L} \right)^2 \left[ \frac{\langle \nabla[\phi(r)] \rangle^2}{2k_0^2} \right]_r.
\]

In Eqs. 6.1a-b, \( L \) is the tissue slice thickness, \( \langle \Delta \phi^2(r) \rangle_r = \left[ \phi(r) - \langle \phi(r) \rangle_r \right]^2 \), is the variance of phase distribution over an area denoted by \( r = (x, y) \), \( k_0 = 2\pi / \lambda \), with \( \lambda \) the wavelength of light, and \( \nabla[\phi(r)] \) is the gradient and in rectangular coordinate \( |\nabla[\phi(r)]|^2 = (\partial \phi / \partial x)^2 + (\partial \phi / \partial y)^2 \). I tried to keep the notation in vector form and maintain the equations independent of the coordinate (either rectangular coordinate or polar coordinate) selected.

\[85\]
6.1 Proof of the $l_s$-$\phi$ Relationship

The starting point in proving Eq. 6.1a is the definition of $l_s$ as the characteristic length in the medium over which the irradiance $I_0'$ of the unscattered light drops to $1/e$ of the original value $I_0$, i.e. the Lambert-Beer’s law,

$$I_0' = I_0 e^{-L/l_s}.$$  \hfill (6.2)

In Eq. 6.2, $I_0 = |U_0|^2$ and $I_0' = |U_0'|^2$, where $U_0$ and $U_0'$ represent the incident plane wave and the unscattered light that passed through the slice, respectively, as illustrated in Fig. 6.1. The field after the tissue slice, $U'$, carries information about the spatial phase distribution, $\phi(r)$, which is available for measurement via quantitative phase imaging, $U'(r) = U_0 \cdot e^{i\phi(r)}$. The transmitted field can be expressed as the superposition between the scattered and unscattered components,

$$U'(r) = U_0' + U_1'(r).$$ \hfill (6.3)

Note that $U_0'$ is the zero-frequency (unscattered) component of $U'$ and $U_1'$ is the sum of all high-frequency field components. Therefore, $U_0'$ can be expressed as the spatial average of $U'$,

$$U_0' = \langle U_0 \cdot e^{i\phi(r)} \rangle_r.$$ \hfill (6.4)

For a normal distribution of phase shifts, where the probability density function is a Gaussian function of the form $\exp\left[-\phi^2 / 2\langle \Delta\phi^2 \rangle_r\right] / \sqrt{2\pi \langle \Delta\phi^2 \rangle_r}$, the average in Eq. 6.4 is readily performed as

$$U_0' = \frac{U_0}{\sqrt{2\pi \langle \Delta\phi^2 \rangle_r}} \int_{-\infty}^{\infty} e^{i\phi} e^{-\frac{\phi^2}{2\langle \Delta\phi^2 \rangle_r}} d\phi = U_0 e^{-\frac{\langle \Delta\phi^2 \rangle_r}{2}}.$$ \hfill (6.5)
In Eq. 6.5, $\langle \Delta \phi^2 \rangle_r$ is the variance associated with the phase shift distribution. Since $\left| \frac{U'_0}{U_0} \right|^2 = \frac{I'_0}{I_0}$, combining Eqs. 6.2 and 6.5 yields the expression of the scattering mean free path,

$$I'_s = \frac{L}{<\Delta \phi^2(r)>_r}. \quad (6.6)$$

Note that a similar field average is encountered in dynamic light scattering, where the phase shifts are due to particle displacements in suspension and the Gaussian probability distribution follows from the theory of Brownian motion [125]. Usually Gaussian distribution is a good approximation when the phase value is small [63]. The same result can be readily derived for a Lorentz line shape. Further, it can be shown that such Gaussian distribution is the direct result of a Tailor expansion of the phase function to the second order. In Eq. 6.4, if we assume $\phi(\vec{r}) = <\phi(r)>_r + \delta \phi(\vec{r})$ and expand the phase to the second order, we have

$$U'_0 = e^{i<\phi(r)>_r} \left(1 - \frac{1}{2} \left[ \langle \delta \phi(\vec{r})^2 \rangle_r \right] \right).$$

Since $L >> l_s$, it is safe to expand Eq. 6.2 as $I'_0 = I_0(1 - L/l_s)$, which arrives at the same result shown in Eq. 6.6. This derivation again confirmed that Gaussian distribution is a good approximation when the phase value is small since this is the major assumption in the derivation. By performing the Taylor expansion to the second order, we approximate an arbitrary distribution, including a Gaussian, with a parabola. The assumption of normally distributed phase values is more inclusive in the sense that it covers the small values, where all distributions work, as a particular case. We can also expand to higher terms to improve the precision if necessary. Other phase distributions are explored in Appendix G.
6.2 Proof of the g-\( \phi \) Relationship

By definition, \( g \) represents the average-cosine of the scattering angle for a single scattering event. Recently, this concept is extended to continuous distributions of scattering media, such as tissues [28]. Since \( l_s \) also means the distance over which, on average, light scatters once, \( g \) can be defined by the average cosine of the field transmitted through a slice of thickness \( l_s \),

\[
g = \langle \cos \theta \rangle. \tag{6.7}\]

As illustrated in Fig. 6.1b, the scattering angle connects the incident wavevector \( k_0 \), the scattered wavevector \( k_s \), and the momentum transfer, \( q = k_s - k_0 \), as

\[
\cos \theta = 1 - \frac{q^2}{2k_0^2}, \tag{6.8}
\]
\[
q = 2k_0 \sin \frac{\theta}{2}.
\]

Combining Eqs. 6.7 and 6.8, the average cosine is expressed as

\[
g = 1 - \frac{1}{2k_0^2} \iint q^2 P(q) \, dq \tag{6.9}.
\]

In Eq. 6.9, \( P(q) \) is the angular scattering probability distribution of the field exiting a slice of thickness \( l_s \). \( P \) is the normalized angular scattering intensity and has the form

\[
P(q) = \frac{\left| \tilde{U}'(q) \right|^2}{\iint \left| \tilde{U}'(q) \right|^2 d\mathbf{q}}, \tag{6.10}
\]

where \( \tilde{U}' \) is the spatial Fourier transform of \( U' \). Inserting Eq. 6.10 into Eq. 6.9 we find

\[
g = 1 - \frac{1}{2k_0^2} \iint \left| q \tilde{U}'(q) \right|^2 d\mathbf{q} \iint \left| \tilde{U}'(q) \right|^2 d\mathbf{q}. \tag{6.11}
\]
Using Parseval’s theorem for both the numerator and denominator and applying the differentiation theorem to the numerator, we can express \( g \) via spatial-domain integrals [126],

\[
g = 1 - \frac{1}{2k_0^2} \iint |\nabla U'(\mathbf{r})|^2 \, d\mathbf{r} / \iint |U'(\mathbf{r})|^2 \, d\mathbf{r}.
\]  

(6.12)

Since the spatial dependence of \( U' \) is in the phase only, \( U'(\mathbf{r}) = U_0 \cdot e^{i\phi_0(\mathbf{r})} \), the gradient simplifies to

\[
\nabla U'(\mathbf{r}) = U'(\mathbf{r}) \nabla \phi_0(\mathbf{r}).
\]  

(6.13)

Thus, combining Eqs. 6.12 and 6.13, we arrive at the final formula for \( g \),

\[
g = 1 - \frac{\left\langle |\nabla \phi_0(\mathbf{r})|^2 \right\rangle}{2k_0^2}.
\]  

(6.14)

where \( \left\langle |\nabla \phi_0(\mathbf{r})|^2 \right\rangle \) is the averaged gradient intensity over the area. Equation 6.14 expresses the relationship between \( g \) and the gradient of the phase shift distribution through a slice of thickness \( l_s \). If the phase image, \( \phi(\mathbf{r}) \), is obtained over a thickness \( L \), with \( L < l_s \), then \( \phi_s = \phi l_s / L \). Thus, the anisotropy factor depends on the measurable phase image as

\[
g = 1 - \left( \frac{L}{l_s} \right)^2 \left\langle |\nabla \phi(\mathbf{r})|^2 \right\rangle \frac{1}{2k_0^2}.
\]  

(6.15)

In order to properly define the anisotropic factor \( g \), we must assume the phase function \( \phi(\mathbf{r}) \) is independent of the angle, i.e. \( \phi(\mathbf{r}) \) in Eq. 6.15.
6.3 Implementations

Figure 6.2 | Error estimation due to limited NA. (a) Error in power vs. NA and g. (b) Error in g vs. NA and g. The dash ellipses show the regime of our measurements.

In order to obtain large fields of view, throughout the experiments presented here, I used a 10×, 0.3 NA objective. This limited numerical aperture effectively acts as a low-pass spatial frequency filter. Thus, the spatial averages performed in deriving Eqs. 6.1a-b [127] are expected to be affected by this cut-off. I anticipate that, because tissues scatter strongly forward (g close to unity), the low NA is not a significant error source. However, in order to quantify the effect of the numerical aperture on the overall scattered intensity measured and g estimation, the Henyey-Greenstein angular distribution is used, which is often applied for tissue scattering approximation and simulation, to calculate the respective error functions,

\[ \Delta P(NA, g) = 1 - \int_{\sqrt{1-NA^2}}^{1} P(\cos \theta) d \cos \theta \]  

(6.16)

\[ \Delta g(NA, g) = 1 - \int_{\sqrt{g^2-NA^2}}^{g} \cos \theta P(\cos \theta) d \cos \theta \]  

(6.17)

where \( P \) is the Henyey-Greenstein distribution, normalized to unit area, \( P(\cos \theta) = \text{const.}(1-g^2)/(1+g^2-2g \cos \theta)^{3/2} \). In Eqs. 6.16-17, \( \Delta P \) represents the scattered
power that is not accounted for due to NA, and $\Delta g$ represents the difference between the measured average cosine of the scattering angle. Figure 6.2 shows the two error functions. It can be seen that, in the measurement range set by the NA=0.3 and large $g$ values associated with tissues (ellipses in Figs. 1a-b), the errors are below 10% in power and 5% in $g$.

Figure 6.3 shows $l_s$ map and $g$ map of the unstained small intestine tissue slice with its H&E stained counterpart. It is apparent that the tissue scattering parameters exhibit strong inhomogeneities across the whole biopsy. From the scattering mean free path map (Fig. 6.3c), the blood cells (red staining in H&E) are highly scattering compare to the rest of the tissue. Note that the background $l_s$ values are very high, indicating lack of scattering, as expected. Overall, the scattering mean free path values agree well with those published in literature [121]. The $l_s$ values are typically in the tens of microns and the biopsy slices in the 3-5 $\mu$m range, which is precisely what is required for the theorem to work. The anisotropic factor $g$, on the other hand, is about 0.996 for red blood cells (Fig. 6.3d). This number agrees with other measurement and the calculation from Mie theory [128]. The $g$ associated with the background appears to be very low, which may seem counterintuitive. The explanation is that, although the background noise is very low, i.e. 2-3 orders of magnitude lower than the tissue signal, it has the characteristic of (spatial) white noise, which translates into isotropic scattering. The measurements uniquely underline the significant spread in the measured values for both parameters, which is an important aspect to account for when attempting diagnosis.
Figure 6.3 | Extraction of spatially resolved scattering parameters $l_s$ and $g$ from small intestine biopsy. Scale bar: 4mm. (a) SLIM image of unstained tissue slice cutted for 4 μm, color bar indicates phase in rad. SLIM is stitched from 3375 tile images. (b) Bright field image of H&E stained tissue slice. It is stitched from 660 tile images. (c) $l_s$ map with averaging radius 32 μm. $l_s$ map shows that the blood is highly scattering compare to rest of the tissue. Color bar indicates scattering mean free path in μm. (d) $g$ map with averaging radius of 32 μm. Color bar indicates the anisotropic factor. (e) Histogram of selected area of $l_s$ map in (c). (f) Histogram of dotted area of $g$ map in (d).
I also studied the scattering properties of prostate biopsies with cancers (Fig. 6.4). Similarly, I found the red blood cells are highly scattering with high anisotropic factor. I noticed
the cancer area (red circle in H&E) has shorter scattering mean free path compared to the benign area (green circle in H&E). This will be further discussed in Section 7.

6.4 Summary

The scattering-phase theorem connects the phase image of a thin tissue slice to the scattering properties of the tissue. Note that the tissue can be mapped in terms of $l_s$ and $g$ that are averaged over patches of area S. While the results may seem surprising, it is very intuitive and the physical interpretation is straightforward. The $l_s$-$\phi$ relationship simply establishes that the attenuation due to scattering is stronger ($l_s$ shorter) as the tissue roughness (variance) is greater; i.e. the more inhomogeneous the tissue, the stronger the scattering. For homogeneous tissue, i.e. zero-variance, $l_s$ becomes infinite, which indicates the absence of scattering. On the other hand, the $g$-$\phi$ formula contains the gradient of the phase. This phase gradient relates to a tilt in direction of propagation. The modulus squared of the gradient indicates that the angular average is intensity-based. Thus, the higher the squared-averaged gradient, the higher the probability for large scattering angles, i.e. the smaller the $g$ value (Eq. 6.15). In essence, a thin tissue slice can be assimilated with a (complicated) grating, which is characterized by a certain diffraction efficiency (controlled by $l_s$) and average diffraction angle (reflected in $g$), which is also the spirit of Fourier analysis. I propose quantitative phase imaging as a direct method for extracting $l_s$ and $g$, which is likely to have a significant impact in the biophotonics field.

This idea is demonstrated by mapping the scattering properties of tissues over broad spatial scales without fitting or iterative procedures. The knowledge of $l_s$ and $g$ has great impact on predicting the outcome of a broad range of scattering experiments on large samples. Virtually all optical methods of diagnosis operate on the principle that diseases, especially cancer, affect the architecture and, as a result, the scattering properties of tissues. I envision that this approach
will facilitate building up a large database, where various tissue types, healthy and diseased, are fully characterized in terms of their scattering properties. Furthermore, these measurements will provide important diagnosis value, as they allow studying both healthy and diseased tissue optics from microscopic (organelle) to macroscopic (organ) scales.
CHAPTER 7: CLINICAL APPLICATIONS

Breast cancer and prostate cancer are two of the most widespread cancers in the western world, accounting for approximately 30% of all cases [129]. Following abnormal screening results, a biopsy is performed to establish the existence of cancer and, if present, its grade [130]. The pathologist’s assessment of the histological slices represents the definitive diagnosis procedure in cancer pathology and guides initial therapy [131, 132]. The human evaluation is a combination of subjective and objective assessment and, thus, is subject to inter- and intraobserver variability [133, 134]. Clearly, it is imperative to develop new quantitative methods, combining imaging and computing, capable of assessing biopsies with enhanced objectivity. Such modality, coupled with high-throughput and automatic analysis will enable pathologists to make more accurate diagnoses more quickly.

For the last several decades, tissue-optical interaction was used for both diagnostic [119, 120, 135-137] and therapeutic treatment [138-141]. Absorption and scattering are two physical processes in light tissue interaction. The absorption is determined by the imaginary part of the tissue refractive index, while the scattering is related to the fluctuations of the real part of the tissue refractive index. Both will limit the penetration of light into the tissue, but within optical frequency, scattering is usually the dominant factor [142]. On the other hand, scattering has diagnostic value because it depends on ultra-structure of tissue and cells [109].

Though scattering shows the potential for diagnosis, the process of histopathology has remained largely unchanged for over a century, where the tissue from the patient is usually fixed (e.g. with formalin), embedded (e.g. with paraffin), sectioned as very thin slices (e.g. 4 μm thick for light microscopy and 100 nm for electron microscopy) and then stained for different purposes, such as H&E (hematoxylin and eosin) where the basic dye hematoxylin colors
basophilic structures (e.g. cell nucleus and etc.) with blue-purple hue, and acidic eosin colors
eosinophilic structures (e.g. intracellular or extracellular protein, most of the cytoplasm, etc.)
bright pink. The staining is necessary because of its good specificity, and more importantly, its
good exhibition of the structure information where the cells and connective tissues can be
identified. However, the staining, though it might be specific with immunological detection,
tends to be delicate and vulnerable to the fixation which forms cross-links both within and
between proteins. In other words, a fixative which preserves protein structure and/or
confirmation is required, which might not be easily accessible.

Towards this end, various label-free techniques have been developed based on both the
inelastic (spectroscopic) and elastic (scattering) interaction between light and tissues. Thus,
significant progress has been made in near-infrared spectroscopic imaging of tissues [143-156].
On the other hand, light scattering methods operate on the assumption that subtle tissue
morphological modifications induced by cancer onset and development are accompanied by
changes in the scattering properties and, thus, offer a non-invasive window into pathology [106,
115, 122, 157-162]. Despite these promising efforts, light scattering-based techniques currently
have limited use in the clinic. A great challenge is posed by the insufficient knowledge of the
tissue optical properties. An ideal measurement will provide the tissue scattering properties over
broad spatial scales, which, to my knowledge, remains to be achieved.

In an effort to overcome these limitations, intense efforts have been devoted in recent
years toward developing quantitative phase imaging (QPI) methods, where optical path length
information across a specimen is quantitatively retrieved (for a review see [2]). QPI is a label-
free approach that has the remarkable ability to render morphological information from
completely transparent structures with nanoscale path-length sensitivity [23, 25, 163]. It has been
shown that the knowledge of the amplitude and phase associated with an optical field transmitted through tissues captures the entire information regarding light-tissue interaction, including scattering properties [27, 28, 164]. Yet, the potential of QPI for label-free pathology has not been explored.

Here I employ SLIM [63] to image entire unstained prostate and breast biopsies and perform a side by side comparison with stained pathological slides. I demonstrate in a direct manner that based on the refractive index distribution, SLIM can reveal cellular and subcellular structures in completely transparent tissue slices. The phase shift, as a measure of the dry mass (the content that is not water, e.g. proteins) [78, 79], actually renders structure, texture and content information. The refractive index distribution of tissue is an intrinsic marker of disease and, thus, holds great diagnostic value.

7.1 Tissue Imaging Using SLIM

SLIM’s principle of operation is described in Chapter 2. Briefly, SLIM combines Zernike’s phase contrast microscopy [1] with Gabor’s holography [29] and yields quantitative optical path-length maps associated with transparent specimens, including live cells and unstained tissue biopsies. Due to the broadband illumination light [165] and the common-path interferometric geometry, SLIM is highly sensitive to path-length changes, down to the sub-nanometer scale [166].

I implemented SLIM with a programmable scanning stage, which allows for imaging large areas of tissue, up to centimeter scale, by creating a montage of micron-resolution images. The number of individual images in the montage depends on the size of the biopsy and varies from several hundred to several thousand. The transverse resolution is limited only by the numerical aperture of the objective and varies in the experiments from 0.4 μm for a 40×, 0.75NA
objective to 1 μm for a 10×, 0.3NA objective. The spatial path-length sensitivity of the SLIM images, i.e. the sensitivity to path-length changes from point to point in the field of view, is remarkably low, approximately 0.3 nm [63]. Since the maximum path-length values are up to the wavelength of light, 530 nm, the signal-to-noise ratio across the image is of the order of 1,000.

![Figure 7.1 | Camera picture of unstained and stained slides.](image)

From left to right: unstained slide, H&E stained slide, K903 stained slide and P504S/AMACR stained slide. Each slide is 1 inch by 3 inch.

The specimen preparation is detailed in Appendix H. Briefly, prostate tissue from a patient was fixed with paraffin and sectioned in 4 μm thick slices. Four successive slices were imaged as follows. One unstained slice was de-parafined and placed in xylene solution for SLIM imaging. The other three slices were stained with H&E; immunohistochemically stained using antibodies against cytokeratin 34 beta E12 (high molecular weight CK903) and alpha
methylacyl-CoA-racemase (AMACR), also known as p504s respectively; and imaged with the same microscope via the bright field channel equipped with a color camera (see Fig. 7.1 for picture of stained and unstained slices).

**Figure 7.2 | Multimodal imaging of prostate tissue slices. Objective: 10X/0.3.** The field of view is 2.0 cm × 2.4 cm. The size of the blowout area (in red circle) is 630 μm × 340 μm. (a). Bright field image of unstained slice (montage of 4,131 images). (b). Bright field image of H&E stained slice (montage of 828 images). (c) SLIM phase map of the unstained slice (montage of 4,131 images); color bar indicates optical path length in nm. Insets show the respective enlarged are indicated as red ellipse.

Figure 7.2a shows the bright field (i.e. common intensity) image of an unstained prostate biopsy. Clearly, the image contrast is very limited, which indicates the long standing motivation for the use of staining in clinical pathology. The H&E stained slice is shown in Fig. 7.2b. The contrast is greatly enhanced as the tissue structures show various shades of color, from dark purple to bright pink. Figure 7.2c shows the optical path-length map rendered by SLIM, which represents a mosaic of 4,131 individual images. Since the tissue thickness is known throughout the specimen, the SLIM image quantitatively captures the spatial fluctuations of the refractive
index, which fully determines the elastic interaction with the optical field, i.e. its light scattering properties [127]. The refractive index is proportional to the tissue dry mass concentration [25], which provides complementary information with respect to the dye affinity revealed in common histopathology (Figs. 7.2b-c).

### 7.2 Refractive Index Signatures at the Cellular Scale

Both SLIM and stained tissue images were obtained using a 10× (NA=0.3) objective, which captures multiscale information down to subcellular structures. Figure 7.3 illustrates the ability of SLIM to reveal particular cell types based on their refractive index signatures. Due to their discoid shape and high refractive index, red blood cells are easily identifiable in the SLIM images (Figs. 7.3a-b). Lymphocytes were found to exhibit high refractive index in SLIM images (Fig. 7.3c) and dark staining in H&E (Fig. 7.3d), utilizing immunohistochemical stain, namely leucocyte common antigen (CD45) (Fig. 7.3e). In a different area of the tissue I found a particular type of cell that seems unlike the rest: while their refractive index is distinctly high, they are sparsely distributed within the tissue (Fig. 7.3f). In H&E, they appear as black dots (Fig. 7.3g). Due to their negative immuno-staining for epithelial, myoepithelial, and lymphocytes, these particular cells were identified as stromal (Fig. 1.5). Thus, SLIM reveals intrinsic optical signatures of cellular and subcellular structures in unstained tissue biopsies. This capability is exploited below in problems of clinical relevance: breast and prostate tissue diagnosis.
Figure 7.3 | SLIM imaging signatures. (a-b) Red blood cells with SLIM (a) and H&E (b). Red blood cells can be identified by their unique shape. Scale bar: 20 μm. (c-e) Lymphocytes with SLIM (c), H&E stain (d) and CD45 stain (e). Lymphocytes were confirmed with CD45 staining. Scale bar: 100 μm. (f-g) Stromal cells with SLIM (f) and H&E stain (g). Scale bar: 100 μm. Color bar indicates optical path length in nm.
7.3 Detection of Micro-Calcifications in Breast Biopsies

Further, I found interesting optical markers associated with calcifications in the breast. Mammogram is an important screening tool for detecting breast cancer [167]. The presence of abnormal calcifications, i.e. calcium phosphate and calcium oxalate [168], warrants a further
work-up. Distinguishing between calcium oxalate and calcium phosphate is clinically important. Specifically, it is uncommon for calcium oxalate crystals to be associated with breast malignancy [169, 170], though they can be associated with papillary intraductal carcinoma [171]. Calcium oxalate crystals account for 12% of mammographically localized calcifications that typically prompt a biopsy procedure [172]. Calcium oxalate is more difficult to detect radiologically and these crystals are easily missed in the biopsies because they do not stain with H&E [173]. These crystals are birefringent and, thus, can be observed in polarized light [174]. However, if the index suspicion is not high, the pathologist typically does not use polarization microscopy and calcium oxalate can be missed. The apparent absence of calcification in tissue biopsies reported by the pathologist has significant clinical impact, including repeated mammograms and additional, unnecessary surgical intervention. Therefore, a consistent means for detecting calcium oxalate is desirable as it significantly decreases medical costs and patient anxiety.

Figure 7.4 illustrates how SLIM may fulfill this challenging task. In Fig. 7.4b, the dark H&E staining was identified by pathologists as calcium phosphate. This structure is revealed in the SLIM image as having inhomogeneous refractive index, with a different texture from the surrounding tissue. More importantly, the calcium oxalate crystals are hardly visible in H&E (Fig. 7.4d); the faint color hues are due to the birefringence of this type of crystal. Clearly, calcium oxalate exhibits a strong refractive index signature, as evidenced by the SLIM image. Therefore, SLIM’s ability to detect calcium oxalate in unstained breast biopsies may drastically reduce the “false negative” rate following positive mammography.
7.4 Label-Free Cancer Detection

I further studied biopsies from prostate cancer patients. Eleven biopsies from 9 patients were imaged with both SLIM and H&E, as illustrated in Figs. 7.5a and 7.5b, respectively (for details, see Appendix H). For each biopsy, the pathologist identified regions of normal and malignant tissue. From the SLIM image, I computed the map of phase shift variance, \( \langle \Delta \phi(r)^2 \rangle \), where the angular brackets denote spatial average (calculated over \( 32 \times 32 \, \mu\text{m}^2 \)). Figure 7.5c illustrates the map of the scattering mean free path, calculated from the variance as \( l_s = L / \langle \Delta \phi(r)^2 \rangle \) [127].

The average values obtained are close to those reported in the literature [175, 176]. The spatially resolved scattering map shows very good correlation with cancerous and benign areas. These findings confirm in a direct way the importance of tissue light scattering as a means for cancer diagnosis [117, 158-162, 177-183]. It can be easily seen that the regions of high variance, or short scattering mean free path, correspond to the darker staining in H&E, which is associated with cancer. Similar trends can be found in the anisotropic factor \( g \) (Fig. 7.5d). The measurements indicated that the disease affects the tissue architecture in such a way as to render it more inhomogeneous.

As long as the quantitative phase map is obtained, it can be computationally colored for different purposes. Because H&E is the most popular staining for histology, I intended to computationally color the \( l_s \) map such that the unstained images will resemble the stained ones.
Figure 7.5 | Multimodal imaging a prostate tissue biopsy with malignancy; field of view 1.48 cm X 1.44 cm. (a) SLIM unstained slice, color bar indicates optical path length in rad; the red lines marked the specific cancerous areas (1-3) and the green lines benign areas (4-6), as identified by the certified pathologist. (b) H&E stained slice with the same areas marked. (c) Scattering mean free path ($l_s$) map of the tissue slice with the same areas marked. Color bar indicates $l_s$ in μm. (d) Anistropic factor $g$ map. (e-g). Histogram of the SLIM images, $l_s$ map and $g$ map. The number in the image corresponds to the respective areas in (a).
Figure 7.6 | Statistical parameters for 49 cancerous areas and 51 benign areas from 11 biopsies.
Out of all eleven cases, seven cases are rated Gleason grade 6/10, two cases are rated Gleason grade 7/10, one case is Gleason grade 9/10 and one case is benign. (a) Standard deviation vs. mean. (b) Skewness vs. mean. (c) Kurtosis vs. mean. (d) Mode vs. mean. (e) Standard deviation/mean vs. standard deviation. (f) Mode vs. standard deviation/mean.

In order to quantitatively analyze the information contained in the refractive index for the cancer and benign regions, the statistical parameters of 1st-4th order are computed via the respective histograms. Figures 7.5e-f exemplify the histograms associated with regions in the SLIM, l_s and g maps, respectively. Based on these distributions, I calculated the mean, standard deviation, mode, skewness, and kurtosis for each of the 49 cancer and 51 benign areas from eleven biopsies (see Fig. I.1 and Table I.1 in Appendix I). Out of all eleven cases, seven cases are rated Gleason grade 6/10, two cases are rated Gleason grade 7/10, one case is Gleason grade 9/10 and one case is benign. Thus, a multi-dimensional data space, in which we searched for the best separation, was generated between the two groups of data points. Figures 7.6a-f show various representations, i.e. projections onto various planes within the multidimensional data space. Clearly, all representations show significant separation between the two groups. I found
that the mode vs. mean provides 100% separation (specificity) between the data points, as shown in Fig. 7.6d. The classifier can be defined according to different statistical criteria. False positive and true negative can be obtained accordingly. To decrease the dimension, common techniques such as principle component analysis (PCA) and Bayes estimator can be explored.

Figure 7.7 | Statistic parameters for breast cancer vs. benign for 82 cancer areas and 52 benign areas from 13 biopsies. (a) Standard deviation vs. mean. (b) Skewness vs. mean. (c) Kurtosis vs. mean. (d) Mode vs. mean.

Further, the cancer and benign breast tissues are compared in Fig. 7.7. For breast tissue, simple statistical parameters obtained from histogram, such as mean, standard deviation, skewness, kurtosis and mode work to certain extend, but not as good as for prostate biopsies. It is necessary to study the spatial correlation and morphological parameters for breast cancer diagnosis.
7.5 Study of Other Diseases

Besides 44 prostate slides from 11 prostate biopsies (each biopsy has unstained, H&E, K903 and P504S four slides), I have scanned another 52 slides from 26 breast biopsies (each biopsy has unstained and H&E slides), including two cases for calcium phosphate and seven cases for calcium oxalate.

Further, six slides from three tonsil biopsies are scanned for actinomyces. One example is shown in Fig. 7.8, where the actinomyces are marked in H&E by the pathologist. The actinomyces turn out to be low refractive index and low spatially variant, which clearly show very high scattering mean free path ($l_s$ map). Apparently SLIM unstained slice and its computationally colored $l_s$ map can pick up the abnormalities and provides diagnostic value. Additional examples can be found in Appendix I, Figs. I.2 and I.3.

Another 24 slides from 12 small intestine biopsies are scanned and processed for the study of amyloid. Figure 7.9 shows an example, where both variance map and the $l_s$ map show similarities to the H&E stained images. More examples can be found in Fig. I.4.
Figure 7.8 | SLIM multimodal imaging for actinomyces tonsil. Left: SLIM unstained slice, color bar indicates optical path length in rad; Center: $I_s$ map; Right: H&E stained slice. The actinomyces are marked by the pathologist in H&E. The yellow arrows point to the same area (actinomyces) in the blowup.
Figure 7.9 | SLIM multimodal imaging for amyloid in small intestine biopsies. The whole field of view is 1.94 cm X 2.07 cm. (a) SLIM image, color bar indicate nm. SLIM is stitched from 3468 tile images. (b) H&E image. It is stitched from 736 tile images. (c) Variance of SLIM image with radius 32 pixels. (d) Ls map.

7.6 Discussion

Our method targets *in vitro* optical property measurements of tissues. The exact optical properties might differ from those *in vivo*; e.g., optical properties might change after the lesion,
after the fixation (frozen of paraffinic), after the staining and so on. However, those *in vitro* slices are exactly used in everyday diagnosis of pathologists. As long as the handling of the tissue is consistent, the absolute difference between *in vivo* and *in vitro* does not matter. The relative trends within each category which clearly indicate normal or malignant are the final goal for diagnosis.

I showed that, based on the refractive index distribution, SLIM can reveal cellular and subcellular structures in transparent tissue slices. In breast biopsies, the refractive index map correctly identifies microscopic sites of calcifications, which are informative in breast cancer diagnosis and prognosis. The spatial fluctuations of refractive index as captured by the histogram mode, mean, standard deviation, skewness, and kurtosis, strongly correlate with the malignant regions in prostate biopsies. Remarkably, the 2D representation of mode vs. the fluctuation contrast (i.e. standard deviation divided by the mean) separates prostate cancer from normal with 100% confidence as tested on 100 tissue regions from 11 different biopsies.

These promising results warrant further studies devoted to using SLIM as a label-free method for cancer detection in biopsies. The prospect of a highly automatic procedure, together with the low cost and high speed associated with the absence of staining, may make a significant impact in pathology at a global scale. Pathologists are in need of a tool to pre-screen slides for areas of concern. Such a tool is already available for pap smears, with improvement in detection rates of individual malignant cells dispersed among thousands of benign. It is not difficult to imagine that a high throughput instrument that measures tissue refractive index would be incorporated into daily activity of pathologists. We can envision a scenario where several unstained slices of tissue will be screened by this technology until something indicative of malignancy or positive margin of resection is detected. This tool would point out which slices of
tissue should be submitted for histology. Further, not all diagnostic and prognostic problems are in the area of established cancer. The method will be especially useful in liver, lung and colon diseases where incipient fibrosis is an issue.

Finally, it is likely that this type of imaging will impact further the field of optical diagnosis by providing direct access to scattering properties of tissues. Thus, a database of SLIM images associated with various types of tissues, both healthy and diseased, will allow light scattering investigators to look up the scattering mean free path and anisotropy factors of tissues and ultimately predict outcomes of particular experiments. It is my intent to create a library of optical properties (e.g. refractive index statistical parameters, $l_s$, $g$ and so on) of different biopsies for both normal and malignant tissues (e.g. breast, prostate, lung, colon and so on). The results show that a simple catalogue such as $l_s$ is not sufficient to differ malignancy from normal, but a combination of all measured parameters will.

Different imaging modalities, such as MRI, CT, ultrasound and OCT, though with different operation principles and targeting different objects, exemplify how the physics and engineering will make an impact on both diagnosis and research. Of these modalities, SLIM is a new imaging technique providing sub-nanometer quantification of optical thickness of unstained biopsy slices. The ability to collect structure and content information without external staining could have a significant impact on histology like H&E.
CHAPTER 8: POLARIZATION-SENSITIVE PHASE MICROSCOPY

Polarization is the fundamental property of electromagnetic fields that describes the orientation of the oscillating electric field vector [184]. Recently, the theory of polarization has been generalized to include statistical behavior of optical fields [185, 186]. Polarization-based techniques of investigation essentially probe the anisotropy in the induced charge displacement within a given sample and, thus, have the capability to sense molecular level organization. In the early 1940s, R. Clark Jones developed a “field-based” 2x2 matrix formalism to describe the anisotropic response of a material in terms of its complex (i.e. phase and amplitude) behavior [187, 188]. However, because polarization experiments have been largely limited to intensity measurements, the Stokes-Muller formalism, an intensity-based framework, has been commonly used instead (see Ref. [189] for a review).

Polarization-based techniques have received significant interest in biomedicine [190-194]. In particular, polarization-sensitive microscopy can reveal inner structures of cells without the need for exogenous contrast agents [195]. Quantifying the optical phase delays associated with live cells also gives access to intrinsic information about morphology and dynamics. Thus, quantitative phase imaging (QPI) has become an increasingly active field in recent years [2].

In this chapter a microscopy technique is presented that is both quantitative in phase and polarization-sensitive. This method, referred to as Jones phase microscopy (JPM), extracts, for the first time to my knowledge, the full Jones matrix in each point within the field of view associated with a transparent sample. JPM uses a modified version of the Hilbert phase microscope (HPM) [12], but it can be extended to other QPI methods such as diffraction phase microscopy, digital holography and SLIM. HPM is similar to the Lieth-Upatnieks analog holography [196] and off-axis digital holography [197, 198], in the sense that it uses spatial
modulation to encode the phase information of the object. However, in HPM the measurement is performed in the image plane of a microscope rather than a Fresnel (i.e. out-of-focus) plane, which offers important advantages in the case of thin samples, as follows. First, the signal sampling, phase reconstruction and unwrapping are more rigorously performed in the image plane than in the Fresnel zone, where high-frequency interference patterns and phase discontinuities may occur. Second, in the image plane of a thin and transparent sample, such as live cells, the intensity is evenly distributed, which utilizes efficiently the limited dynamic range of the CCD. Finally, this approach does not require the Fresnel transform numerical calculation, as the measurement is already in the image plane.

The experimental setup is shown in Fig. 8.1. HeNe laser radiation is coupled into a 50/50 fiber coupler and the two output beams are used as the arms of a Mach-Zender interferometer. On the object arm, I use an inverted microscope (Meiji 5200) equipped with an 80× (NA=0.9) objective to image the sample. This image is further relayed to the CCD (512 EMCCD, Princeton Instruments) via the L1-L2 lens system, with magnification 2.5x. The reference beam is collimated, magnified by the same L1-L2 system, and makes a small angle with the object beam, such that interference fringes are generated at the CCD (512x512 pixels per frame). The intensity recorded has the following form:

\[ I(x) = I_R + I_S(x) + 2[I_R I_S(x)]^{1/2} \cos[qx + \phi(x)], \]  

(8.1)

where \( I_R \) and \( I_S \) are the reference and the sample irradiance distributions, \( q \) is the spatial frequency of the fringes and \( \phi \) is the spatially varying phase associated with the object. These fringes are typically sampled by 5-6 pixels, which fulfills the sampling requirement for preserving the optical resolution of the microscope [196]. HPM uses the basic principle of reconstructing a complex analytic signal from its associated real part. The complex analytic...
signal formalism was first introduced in optics by Gabor [29]. In the spatial domain, this type of reconstruction became practical with the advancement of computer algorithms for fast Fourier transformation, as demonstrated by Takeda et al. [199] and later applied to microscopy by Cuche et al. [200]. Thus, as also described in Refs. [12, 201], the quantitative phase image associated with the sample is reconstructed using a 2D spatial Hilbert transform. A detailed sampling analysis can be found in Appendix J.

In order to control the polarization of the beams on each arm, I used two polarizers \( P_O \) and \( P_R \), which transform the elliptical polarizations of the fields from the optical fiber into linear polarization along controllable directions. In the laboratory system of reference, the Jones matrix of an arbitrary sample is defined as

\[
J = \begin{bmatrix}
J_{xx} & J_{xy} \\
J_{yx} & J_{yy}
\end{bmatrix},
\]

(8.2)

where the matrix elements are complex. In order to interrogate the sample, I prepare two incident electric vectors, oriented at +45° and -45° with respect to the reference axis,

\[
E_{+45} = C_1 \begin{bmatrix} 1 \\ 1 \end{bmatrix},
\]

(8.3a)

\[
E_{-45} = C_2 \begin{bmatrix} 1 \\ -1 \end{bmatrix},
\]

(8.3b)

with \( C_{1,2} \) real constants. I perform 4 sets of measurements, each containing phase and amplitude images \( (Y_{ij} \text{ complex, } i, j=1, 2) \), corresponding to the analyzer \( P_A \) oriented parallel and perpendicular with respect to the two directions,

\[
\begin{bmatrix}
Y_{11} \\
Y_{12}
\end{bmatrix} = C_1 \begin{bmatrix} J_{xx} + J_{xy} \\ J_{xx} + J_{yy} \end{bmatrix},
\]

(8.4a)
\[
\begin{bmatrix}
Y_{21} \\
Y_{22}
\end{bmatrix} = C_2 \begin{bmatrix}
J_{xx} - J_{xy} \\
J_{yx} - J_{yy}
\end{bmatrix}
\tag{8.4b}
\]

Figure 8.1 | Schematic plot of Jones phase microscopy. He-Ne laser radiation is coupled into 50/50 fiber coupler and the two output beams are used as the arms of a Mach-Zender interferometer. On the object arm the fiber output was collimated and sequentially polarized before incident into the sample. The scattered and unscattered light from the sample was collected by the microscope objective. The scattered light forms an intermediate image near the collimator \( C_1 \) and then is relayed onto the CCD by a 4F system comprised of Fourier lens pair \( L_1 \) and \( L_2 \). The polarizer analyzer \( P_A \) is placed between the two Fourier lens pairs. The reference beam is collimated by \( C_2 \) and relayed by the same 4F system, makes a small angle with the object beam, and interfere with the unscattered light collected by the objective such that interference fringes are generated at the CCD plane. To get high contrast interference pattern, a neutral density filter (NDF) and polarizer \( P_R \) are added to the reference path. The polarization map shows the polarization orientation of the four measurements. For \( Y_{11} \), \( P_O \) is at 45° and \( P_A \) is at 0°; for \( Y_{22} \), \( P_O \) is at 45° and \( P_A \) is at 90°; for \( Y_{21} \), \( P_O \) is at -45° and \( P_A \) is at 0°; for \( Y_{22} \), \( P_O \) is at -45° and \( P_A \) is at 90°.

We can rewrite Eqs. 8.4a-b in a compact form, by stacking them into a single 4x4 matrix form,
\[
\begin{bmatrix}
Y_{11} \\
Y_{21} \\
Y_{12} \\
Y_{22}
\end{bmatrix} =
\begin{bmatrix}
C_1 & C_1 & 0 & 0 \\
C_2 & -C_2 & 0 & 0 \\
0 & 0 & C_1 & C_1 \\
0 & 0 & C_2 & -C_2
\end{bmatrix}
\begin{bmatrix}
J_{xx} \\
J_{xy} \\
J_{yx} \\
J_{yy}
\end{bmatrix}.
\]

The inset of Fig. 8.1 describes the four polarization combinations. The 4 complex elements of the Jones matrix are obtained by inverting the 4x4 matrix \( C \) in Eq. 8.5. The constants \( C_1 \) and \( C_2 \) were retrieved by performing the measurement with no sample, i.e. with \( J \) as the identity 2x2 matrix. This procedure is robust and works for an arbitrary transparent and anisotropic sample, for which the condition \( \det(C) \neq 0 \) always holds.

An important step in the measurement is to find a meaningful relationship between different phase measurements, as it is well known that only phase differences are experimentally accessible (via field cross-correlations). Thus, the phase maps we measure for \( Y_{ij} \) actually may have different reference values, which can result in an additive phase constant between the different maps. In order to solve this ambiguity, additional measurements are necessary, depending on the sample. For many biological samples of interest this is not an issue if we can use an area where no anisotropic structure exists (e.g. area with no cells on a cover slide can be used as phase reference).

I demonstrated the principle of operation of JPM with measurements of the Jones matrix associated with a controllable spatial light modulator (SLM). The transmission SLM is made of a twisted nematic liquid crystal, which is controlled via the red channel of an RGB video input. A vertical polarizer is placed in front of the SLM and aligned with its principal axis, such that the expected Jones matrix contains non-zero terms in the right column only. The result of this measurement is shown in Fig. 8.2. It can be seen that the amplitude maps on the left column are
close to the expected zero level, to within the noise level. In addition, the phase maps on this column are very noisy, as expected, because they are associated with fields of very low amplitudes, where phase is not well defined. On the right column of the SLM Jones matrix, both amplitudes and the phase maps are reconstructed with high signal-to-noise and show the expected pattern inputted via the RGB signal. In order to find the phase relationship between $J_{xy}$ and $J_{yy}$, I performed an additional intensity measurement with the analyzer placed at 45° such that the two terms are coupled.

I proved the ability of JPM to extract Jones matrices associated with biological samples by imaging live neurons in culture. Primary hippocampal rat neurons were established through a previously published process [58]. Figure 8.3 shows the Jones matrix maps of a single neuron. As can be seen, the amplitude of the diagonal terms show very little contrast, attesting that the cell is transparent. By contrast, the diagonal phase maps reveal the structure of the neuron with soma (cell body) and several processes (i.e. axon or dendrites) clearly visible. The difference between these two phase images is very small, proving that the dichroism is negligible in this
case. The off diagonal elements show zero amplitudes and structureless phase distribution, which indicate lack of measurable polarization effects.

![Figure 8.3](image)

**Figure 8.3** | Amplitude and phase maps of Jones matrix for live neuron. For each subplot, left is the amplitude map and right is the phase map. The dimension of each map corresponds to 22 μm. (a) $J_{xx}$, (b) $J_{xy}$, (c) $J_{yx}$, (d) $J_{yy}$.

In summary, JPM is a new direct technique to extract the Jones matrix of a transparent and anisotropic sample in a spatially resolved manner. The experiment described here is carried out in transmission geometry. However, the procedure is equally applicable for a reflective type illumination. The authors are currently working to improve the sensitivity of the technique to polarization changes and to make JPM amenable for dynamic studies of transparent and anisotropic systems.

I have demonstrated direct retrieval of Jones matrix associated with transparent and anisotropic samples using laser interferometry. For the laser system the background noise is about 7 nm, compared to 0.3 nm as demonstrated by SLIM. Because SLIM greatly improved the spatial topography resolution, much higher polarization sensitivity can be achieved. It will be even more exciting if we are able to get polarization-sensitive SLIT in the near future.
CHAPTER 9: SUMMARY AND OUTLOOK

9.1 Summary

Many questions in biology could be answered if lens based optical microscopy featured the resolution of electron microscopy, or if the electron microscope operated under physiological conditions. SLIM actually improved the axial sensitivity of a lens based optical microscope from micron range to sub-nanometer range, both in space and in time. It is a novel type of microscopy that renders quantitative nanoscale information from structures and motions in live cells without physical contact or staining.

Due to the limited coherence of the illumination light, SLIM has the ability to perform depth sectioning through cells, which so far has been limited to (fluorescence) confocal microscopy. The results demonstrate that rich, previously unobservable information can be captured in the spatially-resolved cell refractive index fluctuations. SLIM provides a window into the fundamental processes of the cell, such as polymerization kinetics in the cytoskeleton and organelle transport, essential to understanding the function of both healthy and diseased cells.

Further clinical studies show that refractive index can be used as a marker for diseases, where prostate tissues with cancers and breast tissues with calcifications are examined. Because of its implementation with existing phase contrast microscopes, SLIM could have a significant impact in cell biology studies and clinical research where it can cover broad spatio-temporal scales.
9.2 Outlook

SLIM, as a specific optical imaging modality, belongs to the larger field of biomedical imaging. The development of imaging has been deemed as one of the major advances in science and technology. After all, most people still believe that “seeing is believing.” Oftentimes, I believe that a picture is worth a thousand words.

All imaging modalities can be classified into two categories: wave based far-field imaging, such as optical imaging, SEM, ultrasound, MRI, etc., and near field or contact imaging, including AFM, surface profiler, near field optics, etc. Far field detection is always based on the wave equation due to the fact that only a wave can propagate a long distance. The interaction between the wave and the sample, no matter the nature of the wave, is characterized by the speed of the wave. For electromagnetic waves, it comes down to the product of electric susceptibility and magnetic susceptibility, which is a complex second order tensor within the linear regime.

Now let us focus on optical waves. A key question is: How much information can we get from light? SLIM is dedicated to the measurement of the phase associated with the field. It is straightforward to measure the amplitude, as shown in Chapter 5. Due to the nature of the light, polarization provides another degree of freedom of the sample under examination, as discussed in Chapter 8. I also discussed the scattering measurement in Chapter 5. Scattering is a wavelength dependent optical process. Because SLIM uses broadband illumination, it is possible to directly investigate the interaction between radiation and matter as a function of wavelength, i.e. spectroscopy.

The next important step for technology development, as far as the physics goes, is spectroscopy. The spectral information is contained in the rings around the scatterers. One possible way to do spectroscopy is to attach certain scatterers (such as small polystyrene beads)
to the cells under investigation and analyze the ring effect, which contains the spectral information. A more controllable approach is to combine SLIM with FTIR; i.e., instead of the liquid crystal phase modulator, use a piezo-controlled ring mirror that can shift the phase continuously and over a relatively large distance (e.g. about 100λ, 50 μm). Similar to the relationship between time domain optical coherence tomography (OCT) and frequency domain OCT, the recovered autocorrelation function in turn will give us the spectra distribution at individual pixels, or spatially resolved spectroscopy. The device can be fabricated using microfabrication techniques on a wafer.

All the aforementioned technologies open the avenue to many applications for both basic studies in biology and material sciences and clinical applications. As for the basic studies, I will not be surprised to see more study of cell cycle dependent growth, cell behavior under stress and cell modeling with continuum mechanics.

During the cell growth study, I have measured cell cycle dependent growth on mammalian cells and showed that G2 phase exhibits the highest growth rate and an exponential trend. At the same time, the trend for cell growth in G1 and S is not as clear. I also observed profound growth at mitosis, the cause of which is not clear to us and is a topic for future research. It will be very interesting to study the cell growth during other phases, such as G1, S and M, with proper cell growth modeling.

It will be of great interest to combine micro-devices with the technology presented here to study cell dynamics; cells could be trapped in a microfluidic device while being observed with a QPI method. One interesting application is to study how cells grow and behave under stress (i.e. mechanical forces). It has been recently reported by several groups that mechanical forces play a vital role in human stem cell differentiation. It is also possible to apply different shear
forces by controlling the flow in a microchannel and study how cells behave under such conditions. Any finding from such experiments will undoubtedly be very interesting.

The mechanical models from solid mechanics, to my understanding, are very general and can be further extended to live cells; i.e., one could use continuum mechanics to model the cell membrane, etc. Based on the unprecedented image quality we obtained from SLIM, Laplacian phase microscopy (LPM) can be used for tracking vesicles and organelles in living cells, which further reports on the viscoelastic properties of the cell.

Another very important future direction, as discussed in Chapter 7, is the clinical applications. As a proof of principle study, the spatial fluctuations of refractive index—as captured by the histogram mode, mean, standard deviation, skewness, and kurtosis—strongly correlate with the malignant regions in prostate biopsies. These promising results will stimulate further studies devoted to using SLIM as a label-free method for cancer detection in biopsies. The next step is to apply our methodology to a range of other tissue biopsies such as breast, lung, colon, etc. In unstained breast biopsies, the refractive index map correctly identifies sites of calcification, which are informative in breast cancer diagnosis and prognosis.

Since only histograms are used for current work, the distribution of refractive index and morphology of the tissue are yet to be studied. Extensive image processing needs to be explored such as correlation, edge and region detection, image segmentation, object recognition, etc. This study will require collaboration with people in the related fields.

The prospect of a highly automated procedure, together with the low cost and high speed associated with no staining, may make a significant impact in pathology at a global scale. I anticipate that this type of imaging will impact the field of biophotonics further by providing direct access to the imaging and scattering properties of tissues. Thus, I envision that a database
of SLIM images associated with various types of tissues, both healthy and diseased, will allow investigators to look up tissue optical properties and ultimately predict outcomes of particular experiments.

SLIM is based on broadband visible light, but it would not be surprising to see a similar system working in X-ray or infrared due to the same electromagnetic wave nature. Many theorems developed here can also be extended to other wave forms such as ultrasound; e.g., the 3D reconstruction could be based on the detection of the sound wave amplitude and phase. SLIM is focused on the linear interaction between the light and the sample, which is also the fundamental reaction. The phase of field for higher order response, such as SHG, THG, etc., can be investigated and may lead to new findings.
LIST OF PUBLICATIONS

Under review
2. Z. Wang, K. Tangella, A. Balla, and G. Popescu, "Tissue refractive index as marker for disease," (under review)

In press

In print:
APPENDIX A: DESIGN DETAILS OF SLIM MODULE

SLIM setup is based on 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 10 nm), 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 4× 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 (120 W 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 #4108121005000) 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 (12 V, 100 W with collector, ZEISS catalog #4230000000000), a bulb (12 V, 100 W 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 #4207819910000). 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 150 mm doublet (Thorlabs, AC508-150-A1-ML) and a focal length 200 mm doublet (Thorlabs, AC508-150-A1-ML). Fourier lens L1 (doublet with focal length 300 mm, Thorlabs, AC508-300-A1-ML) and Fourier lens L2 (doublet with focal length 500 mm, Thorlabs, AC508-500-A1-ML) forms another 4f system. The LCPM (array
size $7.68 \times 7.68 \text{ mm}^2$, Boulder Nonlinear, XY Phase series, Model P512-0635) is placed at the back focal plane of L1 and thus overlay with the back focal plan 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 6.45 × 6.45 μm$^2$, ZEISS catalog #4265099901000). The details of the optical layout is shown in Table A.1.

Table A.1 Detailed Lens Design Parameters for SLIM Module

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Figure A.1 | Front panel of house for Labview LCPM control and synchronization.
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, the 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 (see Fig. A.1). Matlab and ImageJ are used for phase image processing and visualization.
APPENDIX B: FABRICATION OF NANOTUBES

Figure B.1 shows the fabrication process of the nanotubes. Three epitaxial structures of Al$_{0.75}$Ga$_{0.25}$As/In$_{0.3}$Ga$_{0.7}$As/GaAs were grown by metalorganic chemical vapor deposition (MOCVD) on (100) GaAs substrate to fabricate strain-induced rolled-up nanotubes. A Thomas Swan atmospheric pressure MOCVD reactor was used for growth and TMGa, TMAI, TMIn, and AsH$_3$ were precursors for Ga, Al, In and As, respectively. The growth temperature for In$_{0.3}$Ga$_{0.7}$As and GaAs was 625 °C. The 500 nm-thick Al$_{0.75}$Ga$_{0.25}$As serves as a sacrificial layer and each 6nm-thick In$_{0.3}$Ga$_{0.7}$As and GaAs of the strained bilayer shows rolling behavior when it is released from the sacrificial layer.

Image reversal photolithographic patterning technique with AZ5214 photoresist was used for lift-off with the 4x50 μm rectangle-shaped mask. After negative patterning, Cr and Au with the nominal thickness of 3 nm each were deposited in sequence under the pressure of 5x10$^{-7}$ torr. CHA electron-beam evaporation system was used for the controlled thin metal film and in situ quartz crystals were used to monitor deposition rates and final thicknesses.

![Figure B.1](image)

Figure B.1 | Fabrication process of nanotubes. (a) Three epitaxial layer structures. (b) Negative patterning. (c) Deposition of Cr and Au. (d) Lift-off. (e) Transferring pattern with the wet chemical etching solution [H$_2$SO$_4$: H$_2$O$_2$: H$_2$O=1:8:80], followed by diluted HF etching [HF:H$_2$O=1:1] to release the strained bilayer. (f) The formation of metal-nanotubes.

After lift-off process, metal patterns act as the mask against wet etching solution. Wet chemical etching (H$_2$SO$_4$: H$_2$O$_2$: H$_2$O=1:8:80) was used to transfer pattern down to the sacrificial layer. Once the sacrificial layer was exposed, wet chemical etching with diluted HF
[49%HF:H₂O=1:1] was carried out to remove the Al₀.₇₅Ga₀.₂₅As layer and release the bilayer from the substrate. As the sacrificial is etched away, the strained bilayers start rolling up with metal thin films and then form the metal nanotubes. These tubes on GaAs substrate were transferred onto glass substrate in methanol solution using a sonicator. Images of the fabricated nanotubes can be found in Fig. B.2 and Fig. B.3.

**Figure B.2 | Nanotube visualization with SEM.** (a) SEM image of metal nanotubes array on GaAs substrate. (b-c) Zoomed-in SEM image of metal nanotubes.

**Figure B.3 | Nanotube visualization with optical microscopy.** (a) The optical image of randomly placed metal nanotubes after transferred onto the glass substrate. (b) Zoom-in optical image of the single tube.
APPENDIX C: PRIMARY CELL CULTURES AND IMMUNOCYTOCHEMISTRY

a) Hippocampal neurons

Primary hippocampal neuron cultures were established through the previously reported protocol [58]. The CA1-CA3 region of hippocampi from postnatal (P1-P2) Long-Evans BluGill rats were removed, enzymatically digested (25.5 U/mL papain, 30 min Worthington Biochemical Corp., Lakewood, NJ), then rinsed, dissociated, and centrifuged (1400 rpm) in supplemented Hibernate-A. Cell pellets were resuspended in Neurobasal-A, counted on a hemacytometer, and plated at 100-125 cells/mm² into glass-bottomed fluorodishes (FD-35, World Precision Instruments, Sarasota, FL). This serum-free media greatly inhibits mitotic cell proliferation; however, in our hands we observe fluorodishes promoting a modest retention of mitotic cells, which we attribute to the fluorodish. The glass surface demonstrates a robust hydrophobic interaction with low protein containing aqueous solutions. Both Hibernate-A (Brain Bits, Springfield, IL) and Neurobasal-A (Invitrogen) were free of phenol red and were supplemented with 0.5 mM L-glutamine (Invitrogen), B-27 (Invitrogen), 100 U/mL penicillin and 0.1 mg/mL streptomycin (Sigma). Cells were housed in a humidified incubator with 5% CO₂ at 37 °C until used; imaging was performed at room temperature unless otherwise specified.

b) Mixed hypothalamic glial cultures for imaging microglia

Mixed glial cultures were established through bilaterally dissecting the ventral hypothalamus from postnatal (P1-P2) Long-Evans BluGill rats. Dissection, enzymatic digestion, tissue dissociation and centrifugation steps were carried out in Hibernate-A, supplemented as defined above. Tissue was digested with papain (25.5 U/mL, Worthington) for 35-40 min at 37 °C. Following papain treatment, the hypothalamic tissue was rinsed (1 mL) prior to trituration wherein the tissue was mechanically dissociated in Hibernate-A (2 mL) using a fire-polished Pasteur pipette. Undissociated tissue was permitted to settle and the supernatant transferred to a new 15 mL vial and centrifuged at 1400 rpm for 5 min. Hibernate supernatant was aspirated off of the pellet and cells were resuspended in culture media: Neurobasal-A media without phenol red (Invitrogen), supplemented with 1% fetal bovine serum, B-27 (Invitrogen), 100 U/mL penicillin and 0.1 mg mL⁻¹ streptomycin (Sigma), and 50 ng/mL recombinant human brain derived neurotrophic factor (BDNF, PeproTech). Using a hemacytometer and the trypan blue exclusion criterion, viable cells were counted, then diluted in culture media, and plated at 380
cells/mm² into fluorodishes previously coated with laminin (25 μg/mL) for 1-2 hours, followed by poly-D-lysine coating for 10-15 min (100 μg/mL). Microglial activation and proliferation is achieved in these studies by nutrient starvation; cell cultures were not fed for 9 days while housed in humidified air with 5% CO₂, maintained at 37 °C. Prior to imaging reactive microglia, culture media was exchanged with Hibernate-A containing 50 mM KCl at room temperature (~25 °C) for 2 hours.

c) Immunocytochemistry of cultured primary neurons

Immunocytochemical labeling of neuronal cultures was performed based on the previously published protocol [58]. Cultures were gently rinsed twice with 2 mL of pre-warmed (37 °C) 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS) followed by a 30 min incubation of 4% paraformaldehyde in PBS on a rotating platform shaker (Gyrotory shaker, model# G76, New Brunswick Scientific). The fixed cells were then permeabilized with 0.25% Triton in PBS for 5-10 min. To block non-specific antibody binding, cultures were incubated with 5% normal goat serum (NGS) or 10% bovine serum albumin in PBS for 30 min. Cells were then labeled by incubating the fixed cultures in primary and secondary antibodies diluted into 2.5% NGS in PBS. Primary antibodies used include: anti-microtubule-associated protein 2 (MAP2) 1° antibody (host, rabbit: Cat# AB5622 Chemicon), monoclonal anti-α2,8-polysialic acid (PSA) 1° antibody #735 (provided by Rita Gerardy-Schahn, Medizinische Hochschule, Hannover, Germany), anti-synapsin mouse monoclonal antibody (Synaptic Systems, cat# 106001). Secondary antibodies were goat-anti-rabbit Alexa 350 and goat-anti-mouse Alexa 488 (Invitrogen). 4',6-diamidino-2-phenylindole (DAPI) was used last and incubated with samples for 5 min at room temperature. Following cell labeling, the fixed cultures were rinsed with PBS and imaged immediately in PBS.
APPENDIX D: METHODS FOR CELL GROWTH

a) *Cell culture and manipulation*

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 2 mM thymidine. After 24 hrs, cells were washed thrice with fresh medium, grown for 12 hrs, and incubated with 2 mM 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).

b) *Live cell imaging*

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 (Fig. D.1). 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. Time-lapse SLIM images were acquired with Zeiss EC Plan-Neofluar 10×/0.3 PH1 M27 (ZEISS catalog #4203419911000) and the corresponding fluorescence images were recorded using a Zeiss EC Plan-Neofluar 40×/0.75 PH2 (ZEISS catalog # 4203619910000). Excitation light for the fluorescence measurements was provided by X-Cite 120XL package (120W HBO/Halide fluorescence illumination, ZEISS catalog #4108092050000) and a FITC filter set (ZEISS catalog # 4236060000000). Every 5 min the sample was scanned in an 8 × 8 tile pattern to achieve a total field of view 3.2 × 2.4 mm². The exposure time is 8 ms for each image at full lamp power (3200 K, or 10.7 V) and the total
scanning time is 1 minute and 4 seconds. The transmission shutter is off before and after each scanning.

Figure D.1 | Perfusion chamber and pump settings. (a) Closed perfusion (source: ZEISS). (b) Front panel of Labview pump control.
For the cell cycle study, cells were transferred to MatTek dish (35 mm dishes, No.1.5 glass thickness and 10 mm 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 (7 mL) and covered with a cover glass (diameter 42 mm) 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 continuous 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). Excitation light for the fluorescence measurements was provided by a X-Cite 120XL package (120 W HBO/Halide fluorescence illumination, ZEISS catalog # 4108092050000) and a FITC filter set (ZEISS catalog # 4236060000000). Every 15 min, 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 (3200 K, or 10.7 V) 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 mm², 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 (120 W 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 60 ms at a time. A careful adjusted reflection illumination field aperture assures only minimum light leakage exists on the neighboring cells during mosaic scanning.
c) Segmentation and Data Analysis

For automatic segmentation binary masks were prepared using threshold, erosion and dilation operations followed by a watershed algorithm; the software was implemented in MATLAB (MathWorks). The results from the automatic segmentation were used to measure mean parameters and cell number. However, due to the U20S cells complicated morphology, motility and tendency to aggregate, accurate automatic tracking of single cells proved difficult. Though this problem may be overcome by a more robust segmentation software, I resorted to manual segmentation to prove the utility of this method. Manual segmentation was performed using the ROI manager available in ImageJ. The total dry mass of individual cells is then calculated as described below. Prior to calculating the mass, negative phase values were set to zero to minimize effects from the halo artifact. The raw data is fitted using spline interpolation which is subsequently low pass filtered.

d) From phase to dry mass

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, \( \gamma=0.2 \text{ mL/g} \) is the refractive increment of protein, which corresponds to an average of reported values [79], and \( \phi(x, y) \) is the measured phase. The total dry mass is then calculated by integrating over the region of interest in the dry mass density map. Note that, even though in reality the refractive increment may vary slightly from cell type to cell type, this will only change the absolute value of the mass; i.e., it will not change the shape of the growth curves, which are of the greatest interest here. To get a more accurate measurement of the true dry mass, the projected maximum of 3 z-slices centered around the middle of each cell was used to calculated the dry mass density map. To automatically detect the center position in each z-stack the mean phase of each z-slice was calculated and the slice with the maximum mean value was chosen as the center slice [202]. For the U2OS cells a running average of the raw data is calculated, with a window size of 75 minutes. It can be seen in the fixed cell measurements for both systems that the SLIM system is stable enough to perform sensitive growth experiments.

e) Cell cycle dependent measurements

The cell cycle-dependent growth measurements are accomplished by utilizing multimodal imaging, i.e. combining simultaneous fluorescence and SLIM imaging. Thus, it is possible to study single cell growth during each phase of the cell cycle separately, in an asynchronous
culture. I imaged YFP-PCNA (Yellow Fluorescent Protein -- Proliferating Cell Nuclear Antigen) transfected human osteosarcoma (U2OS) cells, which monitors PCNA activity via the fluorescence channel. This activity is greatest during the DNA synthesis of the cell cycle and is observed in the localization of the fluorescence signal, which, thus, reveals the S-phase of the cell cycle. This information, along with the initiation and completion of mitosis, gives a clear indication of the cell cycle progression. Figure 3.14 illustrates how this procedure allows the assessment of cell growth during the different phases of a complete cell cycle; thus, in an unsynchronized population, using the PCNA marker, the cells are grouped according to their cycle phase, essentially achieving a posteriori synchronization (Fig. 3.16), which, to my knowledge, can only be done with a combination of SLIM and fluorescence measurement. For the ensemble averages shown in Fig. 3.16 the growth curve for each cell was interpolated to fit the average time spent in that part of the cell cycle by all the cells included in the ensemble. It must be emphasized that a major advantage of using an optical microscopy method to measure cell growth is the ability to visually determine the nature of the mass changes. For example, since the measurement of the cell shape and projected area, as well as the detection of debris, can be carried out simultaneously in the region of the cell, this inclusion can be corrected numerically.
APPENDIX E: MATHEMATICAL DEFINITIONS

Definition of 2D Fourier transform:

\[ G(k_x, k_y) = FT \{ g \} = \int_{-\infty}^{\infty} \int_{-\infty}^{\infty} g(x, y) e^{-j(k_x x + k_y y)} dx dy. \]  

(E.1)

Inverse Fourier transform:

\[ g(x, y) = FT^{-1} \{ G \} = \frac{1}{4\pi^2} \int_{-\infty}^{\infty} \int_{-\infty}^{\infty} G(k_x, k_y) e^{j2\pi(k_x x + k_y y)} dk_x dk_y. \]  

(E.2)

Plane wave: \( Ae^{j\vec{k} \cdot \vec{r}} \).

2D Fourier transform of point spread function \( P(x, y; k) \)

\[ \tilde{P}(k_x, k_y; k) = \int_{-\infty}^{\infty} \int_{-\infty}^{\infty} P(x, y; k) e^{-j(k_x x + k_y y)} dx dy. \]  

(E.3)

Now we try to seek its relationship with the pupil function:

\[ P(x, y; k) = \frac{e^{j kf_z}}{j \lambda f_z^2} \int_{-\infty}^{\infty} \int_{-\infty}^{\infty} \tilde{P}_u(u, v) e^{-j\frac{2\pi}{\lambda} f_z x - j\frac{2\pi}{\lambda} f_z y} dudv \]  

\[ = \frac{e^{j kf_z}}{j 2\pi k} \int_{-\infty}^{\infty} \int_{-\infty}^{\infty} \tilde{P}_u(ks_x, ks_y) e^{-j k x - j k y} dk_x dk_y. \]  

(E.4)

Thus we have

\[ \tilde{P}_u(k_x, k_y) = \frac{j k e^{-j kf_z}}{2\pi} \int_{-\infty}^{\infty} \int_{-\infty}^{\infty} P(x, y; k) e^{j k x + j k y} dx dy. \]  

(E.5)

\[ \tilde{P}_u(-k_x, -k_y) = \frac{j k e^{-j kf_z}}{2\pi} \int_{-\infty}^{\infty} \int_{-\infty}^{\infty} P(x, y; k) e^{-j k x - j k y} dx dy \]  

\[ = \frac{j k e^{-j kf_z}}{2\pi} \tilde{P}(k_x, k_y; k). \]  

(E.6)
APPENDIX F: DECONVOLUTION ALGORITHM

Here we provide a detailed mathematical description of SLIM 3D reconstruction based on the DAMAS [92] iterative deconvolution.

For a transparent sample such as a live cell, the 3D complex field measured U is the result of the convolution between the scattering potential of the specimen and the point spread function (PSF) of the microscope,

\[ U(r) = \chi(r) \odot P(r), \]  

where \( \chi(r) = n^2(r) - 1 \) the susceptibility and \( \odot \) the 3D spatial deconvolution. \( \tilde{U}(k), \tilde{\chi}(k) \) and \( \tilde{P}(k) \) represent the FFT of \( U(r), \chi(r) \) and \( P(r) \). In order to reduce the number of iterations needed for convergence, a regularized division of PSF and \( U \) by the FFT of the PSF in the spectral domain is performed, which gives the modified deconvolution problem

\[ U_w(r) = \chi(r) \odot P_w(r). \]  

A non-negative solution is then sought by iteration. The aforementioned algorithm can be expressed as follows:

a. Compute the forward FFT of \( U \) and \( P \);

b. For each frequency \( k \), compute \( \tilde{U}_w(k) = \frac{\tilde{P}^*(k)\tilde{U}(k)}{\tilde{P}^*(k)\tilde{P}(k)+\gamma} \) and \( \tilde{P}_w(k) = \frac{\tilde{P}^*(k)\tilde{P}(k)}{\tilde{P}^*(k)\tilde{P}(k)+\gamma} \);

c. Compute the inverse FFT of \( \tilde{P}_w(k) \) to obtain \( P_w \);

d. Set \( a = \sum_{x,y,z} P_w \);

e. Set solution \( \chi(r) = 0 \);

f. Iterate
   (1) \( \tilde{\chi}(k) \) = forward FFT of \( [\chi] \);
   (2) Let \( \tilde{R}(k) = \tilde{P}_w(k)\tilde{\chi}(k) \);
   (3) \( R(r) = \) inverse FFT of \( [\tilde{R}(k)] \);
   (4) \( \chi(r) \leftarrow \chi(r) + [U_w(r) - R(r)] / a \) for each \( r \);
   (5) Replace each negative value of \( \chi(r) \) by 0.
APPENDIX G: EXTENSION OF SCATTERING PHASE THEOREM

Discussion of $l_s$
The connection between phase histogram and scattering parameter $l_s$ is discussed here. It turns out for both Gaussian and Lorentz distribution, the scattering coefficient $l_s$ will take exactly the same form. I show every distribution can be approximated as Gaussian at the mean phase point, which is the reason that, for small phases, I obtained the same expression for $l_s$ with different assumptions.

In the previous derivation, the DC part of the field was shown as

$$\hat{U}(0) = \int e^{i\phi(r)} d\vec{r}. \quad (G.1)$$

Since $\phi(\vec{r}) = \phi_0 + \delta\phi(\vec{r})$ and $\langle \delta\phi(\vec{r}) \rangle = 0$, the expansion of Eq. G.1 to the second order shows

$$\hat{U}(0) = e^{i\phi_0} \left(1 - \frac{1}{2} \left\langle [\delta\phi(\vec{r})]^2 \right\rangle \right). \quad (G.2)$$

From Lambert-Beer's law, we have

$$e^{-L/l_s} = \left|1 - \frac{1}{2} \left\langle [\delta\phi(\vec{r})]^2 \right\rangle \right|^2 = 1 - \left\langle [\delta\phi(\vec{r})]^2 \right\rangle. \quad (G.3)$$

From Eq. G.3, the $l_s$ is retrieved as

$$l_s = \frac{L}{\left\langle [\delta\phi(\vec{r})]^2 \right\rangle}. \quad (G.4)$$

Recall that in the above process, the major approximation is in Eq. G.2, where we actually expand the exponential phase function as $e^{i\delta\phi(r)} = 1 + i\delta\phi(r) - 0.5\delta\phi^2(r)$. Since $\langle \delta\phi(\vec{r}) \rangle = 0$, only the quadratic term left. What we expand is actually the function $e^{ix}$ at 0, where $x = \delta\phi(r)$.

Assume that within the integration area, at each position $\vec{r}=(x,y)$, we have a phase associated as $\phi(\vec{r})$. The distribution along the position can be done in a histogram, where the spatial information is lost. If a similar procedure is applied to all the phase values in the area of Eq. G.1, the integration can be rewritten as
\[ \hat{U}(0) = \int_{-\infty}^{\infty} e^{i\phi} P(\phi) d\phi, \]  

where \( P(\phi) \) is the probability distribution of the histogram, which is obtained by normalizing the histogram. For a normal distribution of phase shifts, where the probability density function is a Gaussian function of the form \( \exp\left[-\phi^2 / 2\sigma^2\right] / \sqrt{2\pi\sigma} \), Eq. G.5 can be calculated as

\[ \hat{U}(0) = \frac{1}{\sqrt{2\pi\sigma}} \int_{-\infty}^{\infty} e^{i\phi} \exp\left[-\phi^2 / 2\sigma^2\right] d\phi = e^{-\sigma^2/2}, \]  

where Poisson integration \( \int_{-\infty}^{\infty} e^{-a^2x^2} dx = \sqrt{\pi / |a|} \) is used. Combining Eq. G.4 and Eq. G.6, we have

\[ I_s = \frac{L}{\sigma^2}. \]  

In other words, if \( \sigma^2 = \langle \delta^2(\vec{r}) \rangle \), Eq. G.4 is recovered without any approximation. Here the mean phase shift is assumed 0 in Eq. G.6. The same results can be obtained if the mean phase is not 0, which will add a leading phase term \( \exp(i\phi_0) \). This term plays no role when intensity ratio is considered later.

As shown previously, it is not necessary to assume Gaussian distribution from the start to get Eq. G.4. For any smooth curve \( f(x) \), if the first order derivative is 0, i.e. \( f'(x) = 0 \), then the curve can be approximated by a parabola, including Gaussian function \( \exp(-x^2) \). The central part can also be approximated by a Gaussian as long as the parameter is small. In our case, the phase shift is small (e.g. in tissue slices), and Gaussian distribution is in fact a good approximation for the histogram around the mean.

Now if we push this idea a little further, more different distributions can be used. We first look at the Lorentz line shape.

Lorentz distribution is studied for the gas spectra line shape. Its shape can be approximated by a Lorentz function as long as all molecules behave in the same way, e.g. pressure broadening, natural lifetime broadening, transit time broadening, etc. As a comparison, Gaussian line shape is a result that all molecules behave differently (distribution), such as Doppler broadening and power broadening. If homogeneous broadening is assumed, the distribution will be Lorentz.

If the half maximum width of Lorentz shape is \( 2\alpha \), the distribution is

\[ P(\phi) = \frac{\alpha / \pi}{\phi^2 + \alpha^2}. \]  

Combining Eq. G.5 and Eq. G.7, we have
\[ \tilde{U}(0) = \int_{-\infty}^{\infty} e^{i\phi} \frac{\alpha}{\phi^2 + \alpha^2} d\phi. \] \hspace{1cm} (G.8)

Since \( \Phi \) is second order in the denominator, the integration exists. On the real axis, there is no isolated singularity. We first extend the integration to the whole complex plane and take the integration route as shown in Fig. G.1.

![Figure G.1 | Integration route taken in the complex plane.](image)

Because the integration at \( R -> \infty \) is 0 along the upper circle and there is only one pole at \( \Phi = \alpha i \), Eq. G.8 can be written as

\[ \tilde{U}(0) = 2\pi i \times \text{Res} \left[ \frac{\alpha e^{i\phi}}{\alpha^2 + z^2}, ai \right] = e^{-\alpha}, \] \hspace{1cm} (G.9)

where \( \text{Res} \) means residue. Here, if \( \alpha = \sigma^2 = \langle \delta \phi^2 \rangle \), we recover the same results for Gaussian distribution. Similarly, if the mean of the phase is not 0, only a phase shift will be introduced and all conclusion holds if intensity is considered.

The distribution of the histogram actually contains very important information of the tissue scattering, provided the spatial distribution is quasi-homogeneous (i.e. the integration area is large enough that will resemble the whole tissue around it). More detailed mechanism analysis, i.e. why the histogram will take a certain shape, might shade the light for the future directions. The dynamics of the histogram changes, e.g. the membrane histogram dynamics, might also provide insight for the molecular transport within the membrane and even within the cell.

**Discussion of g**

Assuming the phase function is \( P(q) \), the anisotropic factor will be \( g = \langle \cos \theta \rangle \). Note that
\[
\cos \theta = 1 - 2 \sin^2 \frac{\theta}{2} \quad \text{(G.10)}
\]

and

\[
q = 2k_0 \sin \frac{\theta}{2}, \quad \text{(G.11)}
\]

then we have

\[
\cos \theta = 1 - \frac{q^2}{2k_0^2}. \quad \text{(G.12)}
\]

The anisotropic factor is thus

\[
g = \int \left(1 - \frac{q^2}{2k_0^2}\right) P(q) 2\pi q dq = 1 - \frac{1}{2k_0^2} \int q^2 P(q) 2\pi q dq, \quad \text{(G.13)}
\]

where we assume the probability should be \(P(q) 2\pi q\). The reason for this will be clear later.

The measured phase map is assumed to be \(f(x, y)\), and thus the scattering will be

\[
\tilde{f}(k_x, k_y) = \iint f(x, y) e^{-i(k_x x + k_y y)} \, dx \, dy. \quad \text{(G.14)}
\]

In order to connect with \(q\), the polar coordinate is used instead. Assume

\[
\begin{cases} 
x = r \cos \phi \\
y = r \sin \phi
\end{cases} \quad \text{(G.15)}
\]

and we have Jacobi as \(|J| = \frac{\partial (x, y)}{\partial (r, \phi)} = \left| \begin{array}{cc} \frac{\partial x}{\partial r} & \frac{\partial y}{\partial r} \\ \frac{\partial x}{\partial \phi} & \frac{\partial y}{\partial \phi} \end{array} \right| = \cos \phi \sin \phi \begin{vmatrix} \cos \phi & \sin \phi \\ -r \sin \phi & r \cos \phi \end{vmatrix} = r; \text{ therefore,}
\]

\[
\tilde{f}(k_x, k_y) = \int_0^\infty \int_0^{2\pi} f(r \cos \phi, r \sin \phi) e^{-i(k_x r \cos \phi + k_y r \sin \phi)} r dr d\phi. \quad \text{(G.16)}
\]

Now we change the coordinate in the \(k\) space to polar coordinate too, i.e.

\[
\begin{cases} 
k_x = q \cos \alpha \\
k_y = q \sin \alpha
\end{cases} \quad \text{(G.17)}
\]

and assume the function \(f\) does not depend on the angle \(\phi\); then we have
\[ \tilde{f}(q) = \int_0^\infty f(r)rdr \int_0^{2\pi} e^{-iqr\cos(\varphi)}d\varphi. \]  
\hspace{1cm} (G.18)

Since zero order Bessel function \( J_0(a) = \frac{1}{2\pi} \int_0^{2\pi} e^{-ia\cos(\varphi)}d\varphi \), Eq. G.18 can be rewritten as

\[ \tilde{f}(q) = 2\pi \int_0^\infty f(r)rJ_0(rq)dr. \]  
\hspace{1cm} (G.19)

Eq. G.19 is called Hankel transform (sometimes also called Fourier-Bessel transform), which has inverse relationship as

\[ f(r) = 2\pi \int_0^\infty \tilde{f}(q)qJ_0(rq)dq. \]  
\hspace{1cm} (G.20)

The phase function is thus defined as

\[ P(q) = \frac{|\tilde{f}(q)|^2}{\int_0^{2\pi} |\tilde{f}(q)|^2 2\pi qdq}. \]  
\hspace{1cm} (G.21)

Now we refer back to Eq. G.13, which can be re-written as

\[ g = 1 - \frac{1}{2k_0^2} \int_0^\infty |\tilde{f}(q)|^2 qdq. \]  
\hspace{1cm} (G.22)

Now if we take one derivative of Eq. G.19, we have

\[ \frac{df(r)}{dr} = 2\pi \int_0^\infty (-q\tilde{f}(q))qJ_1(rq)dq, \]  
\hspace{1cm} (G.23)

where we use the Bessel function properties \( \frac{d}{dz}J_v(z) = \frac{v}{z}J_v(z) - J_{v+1}(z) \). The inverse transform will be

\[ -q\tilde{f}(q) = 2\pi \int_0^\infty \frac{df(r)}{dr}rJ_1(rq)dr. \]  
\hspace{1cm} (G.24)

Using the Parseval’s theorem for the Hankel transform,

\[ \int_0^\infty |\tilde{f}(q)|^2 qdq = \int_0^\infty \left| \frac{df(r)}{dr} \right|^2 rdr. \]  
\hspace{1cm} (G.25)

Thus Eq. G.20 can be rewritten as
If the object is a phase object, i.e. $f(r) = E_0 e^{i\phi(r)}$, then

$$g = 1 - \frac{\int_0^\infty \left| \frac{df(r)}{dr} \right|^2 rdr}{2k_0^2 \int_0^\infty |f(r)|^2 rdr}. \quad \text{(G.27)}$$

Equation G.27 agrees with Eq. 6.14, because for rotational symmetric function, $d\phi(r)/dr = \nabla \phi(r)$. It can be further extrapolated to the unsymmetrical case of $\nabla \phi(r)$ in Eq. 6.14.
APPENDIX H: METHODS FOR TISSUE FIXATION, STAINING AND DATA PROCESSING

Tissue preparation

All tissues were handled according to safety regulations by the Institutional Review Board at University of Illinois and Provena Covenant Medical Center. The tissue used in this procedure was embedded in paraffin and cut on a Leica RM2255 microtome at 4 μm thickness. Seven sequential sections were cut for each specimen block to allow for multimodal imaging. The sections were placed in a water bath at 38 °C. Each section was collected on a separate glass slide making seven sets of slides per specimen. Five of these sets consisted of positively charged glass slides to prevent the tissue from washing off during immunohistochemical staining procedures. The two sets of untreated slides were heat fixed in a 70 °C slide drying oven for 15 minutes. The slides were then deparaffinized using a routine protocol on a Sakura DRS-601 staining instrument consisting of two changes of Xylene for a total of 3 minutes, two changes of flex 100 alcohol for a total of 3 minutes, and two changes in flex 95 alcohol again for a total of 3 minutes. The slides were then rinsed in distilled water. One set of slides was then dehydrated using the opposite protocol so that the slides finished in two changes of xylene. These slides were then coverslipped after the dehydration procedure on a Tissue-tek coverslipping instrument using KP-Tape with xylene used as the mounting media. The other set of slides continued through a routine hematoxylin and eosin staining procedure using a Surgipath brand stain system on the same Sakura staining instrument that was used for the deparaffinization process. These slides were then dehydrated and coverslipped using the same process as the unstained slides.

The immunohistochemical slides were heat fixed in the same 70 °C section drying oven for 30 minutes. These slides were then deparaffinized and stained on a Ventana Benchmark XT staining instrument using preset protocols defined by Ventana with the following immunohistochemical stains: high molecular weight keratin clone 34betaE12 (also known as cytokeratin 903), leukocyte common antigen clone RP2/18 (also known as CD 45), and P504S clone 13H4 (also known as AMACR) as well as the appropriate previously known positive and species-specific negative controls. After the immunohistochemical staining procedure, the slides were then washed with Dawn liquid dish soap and distilled water to remove the Ventana brand liquid coverslipping reagent, and then dehydrated and coverslipped using the same procedure as the two untreated sets of slides used for the unstained and hematoxylin/eosin stained slides.
**Image acquisition and processing**

For all the stained images, 10× objective by ZEISS is used, with lamp power 3200 K, camera exposure setting 0.4 μs and tile size 872.51 μm by 655.22 μm. For SLIM images, 10× Ph1 objective by ZEISS is used, with lamp power 3200 K, camera exposure 8 ms and tile size 388.28 μm by 290.92 μm. The average scanning time for each stained image is 1.4 s, and for each SLIM image is 1.0 s.

A Java-based software plug-in based on ImageJ [203] was developed to create a montage of the entire biopsy by stitching high-resolution images. As an example, in Fig. 7.2 the H&E image size is 2.0 × 2.4 cm² and is stitched from 828 color images obtained by a 10× objective. Due to 2.25× additional magnification in the SLIM system, 4,131 images were used to generate the stitched SLIM images. Then the SLIM image were scaled to the same magnification of H&E, rotated, registered and cropped to get the accurate overlay as shown. Usually the image size is several gigabytes for both H&E and SLIM stitched images, which has to be scaled to about 1 to 2 GB for practical handling, storage and processing. All processing has been performed on a server equipped with eight CPUs and 20 GB RAM.

For all the stained images, 10× objective by ZEISS is used, lamp power 3200 K, condenser NA 0.24, camera exposure setting 0.4 μs. For SLIM images, 10× Ph1 objective by ZEISS is used, lamp power 3200 K, camera exposure setting 8 ms.
APPENDIX I: TISSUE IMAGES

Figures I.1-I.5 show tissue images used in the main text. Table I.1 summarizes the prostate biopsies used for the study, listing the detailed biopsy information and acquisition status for each sample.

Figure I.1 | Eleven cases used for the prostate cancer study. First column: SLIM images, color bar in nm; second column: H&E stained images; third column: $l_i$ map, color bar in $\mu$m; fourth column: K903 stained images; fifth column: P504S/AMACR stained images. Out of all the eleven cases, seven cases are rated Gleason grade 6/10, two cases are rated Gleason grade 7/10, one case is Gleason grade 9/10 and one case is benign. See Table I.1 for detailed biopsy information.
Figure I.2 | SLIM multimodal imaging for actinomyces tonsil. Left: SLIM unstained slice, color bar indicates optical path length in rad; Center: Ls map; Right: H&E stained slice. The actinomyces are marked by the pathologist in H&E. The yellow arrows point to the same area (actinomyces) in the blowup.
Figure I.3 | SLIM multimodal imaging for actinomyces tonsil. Left: SLIM unstained slice, color bar indicates optical path length in rad; Center: Ls map; Right: H&E stained slice. The actinomyces are marked by the pathologist in H&E. The yellow arrows point to the same area (actinomyces) in the blowup.
Figure I.4  | Five cases of SLIM multimodal imaging for amyloid in small intestine biopsies. From left to right: SLIM images, color bar indicate nm; H&E images; Variance of SLIM image with radius 32 pixels; $I_\text{s}$ map.
Figure I.5 | Stromal cells identification. Objective is 10X/0.3. (a) SLIM image; color bar indicates optical path length in nm. (b) H&E stained image. The high refractive index cells in a show as black. (c) P63 stained image. P63 is a recently characterized p53-homolog that is consistently expressed by basal/somatic stem cells of stratified epithelia, myoepithelial cells of the breast and salivary glands, and proliferative compartment of gastric mucosa. The cells are negative and thus not myoepithelial cells. (d) CD45 stained image. CD45 antibody reacts with both alloantigens and all isoforms of the CD45 leukocyte common antigen (LCA), and is used as a reporter of lymphocytes in prostate tissues. The cells are negative and thus not lymphocytes. (e) pan-CK stained image. Pan-CK is intended for laboratory use to identify epithelial cells using light microscopy. The cells are negative and thus not of epithelial origin.
Table I.1 Prostate biopsies (see also Fig. I.1 for stitched images)

<table>
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<th>Sample</th>
<th>Solution (Contrast)</th>
<th>Objective</th>
<th>Binning</th>
<th>Size (Column×Row)</th>
<th>Gaussian Blur R</th>
<th>Mosaic Zoom</th>
<th>Exposure Time (ms) (3200K)</th>
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<td>Xylene</td>
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<td>No</td>
<td>36 × 48 (14.0 × 14.0)</td>
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<td>0.2</td>
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<td>0.9</td>
<td>0.4</td>
</tr>
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<td>0.4</td>
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APPENDIX J: SAMPLING FOR JONES PHASE MICROSCOPY

The CCD image acquisition is analogous to the digital conversion process. In order to decide the sampling frequency, we need to know the highest frequency of the interferogram. If the scattered wave is \( a(x,y) \), and the reference beam is \( A \exp(-j2\pi f_0 y) \), then the intensity distribution across the recording plane is [196, 199]:

\[
I(x, y) = |A|^2 + |a(x, y)|^2 + A^* a(x, y) \exp(j2\pi f_0 y) + A a^* \exp(-j2\pi f_0 y).
\]

The bandwidth of \( a(x,y) \) is limited by the aperture of the objective, which is assumed as \( B \) for coherent illumination as shown in Fig. J.1. The bandwidth of the term \( |a(x, y)|^2 \) is thus \( 2B \). According to Fig. J.1, the highest frequency presents in the interferogram will be \( f_0 + B \). Thus Nyquist sampling frequency is \( 2(f_0 + B) \). In other words, the sampling period will be \( 1/2(f_0 + B) \) and the fringe period is \( 1/f_0 \), which implies \( 2 + 2B/f_0 \) pixels are required to sample one fringe period. In order to avoid aliasing in the interferogram, it is evident that the fringe frequency \( f_0 \) should be larger than \( 3B \). Thus \( 8/3 \) pixels are needed to sample one fringe period.

Figure J.1 | Spectra analyses. (a) Spectra of the object. (b) Spectra of the recorded intensity image. \( G_a \) is the spectrum of \( a(x,y) \), \( G \) is the spectrum of \( I(x,y) \), \( G_1 \) is the spectrum of \( |a(x, y)|^2 \), \( G_2 \) is the spectrum of \( A^* a(x, y) \exp(j2\pi f_0 y) \), and \( G_3 \) is the spectrum of \( A a^* \exp(-j2\pi f_0 y) \).

In experiments I found that a slightly higher sampling frequency, e.g. 5 or 6 pixels per fringe, usually gives better results.

In contrast to off-axis digital holography, since they record the image out of the focal plane, the spectrum of \( a(x,y) \) is not strictly bandwidth limited, resulting aliasing in the sampling process.
REFERENCES


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