PHASE DERIVATIVE MICROSCOPY OF BIOLOGICAL CELLS AND TISSUES

BY

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THESIS

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Abstract

Phase derivative microscopy (PDM), including Laplace field microscopy (LFM) and gradient field microscopy (GFM), generates high contrast images of transparent specimens. These methods utilize spatial light modulation at the Fourier plane of a bright field microscope to optically obtain the derivatives of the phase and increase the contrast of the final image. The controllable spatial modulation pattern allows obtaining both the first-order and the second-order derivative of the phase. Compared to differential interference contrast (DIC) microscopy, which is a traditional method of imaging the derivative of the phase, PDM shows higher stability because there are no mechanically moving parts involved in the operation. More importantly, unlike DIC, PDM does not use polarizing optics and, thus, it is applicable to birefringent samples. Furthermore, because it is a single-shot technique, PDM allows acquisition speed as fast as the detector allows, and therefore, it is suitable for studying live biological samples. This thesis introduces the principle of PDM, the setup, and also applications of PDM, including high contrast imaging, live cell dynamics study, and unstained tissue biopsies.
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1. Introduction

Unlabeled biological cells and tissues are mostly transparent under visible light due to the very low absorption and weak scattering. Therefore, they can be assumed as phase objects with a transmission function of the form \( t(x, y) = A \exp[i\phi(x, y)] \) [1]. Since the bright field microscopy measures the intensity of the field, the phase information, which gives the intrinsic contrast is lost at the detector and no contrast is obtained for phase objects with constant amplitude, i.e., \( |t|^2 = A^2 \). During its four-century long history, much of the microscopy development has been focused on finding modalities to achieve higher contrast [2]. Exogenous contrast, obtained by adding chemical compounds (e.g. dyes, fluorophores, or nanoparticles), has been developed essentially to turn a phase object into an amplitude object and, thus, obtain higher contrast. On the other hand, intrinsic contrast methods have been developed to use the existing information carried by the light passing through the transparent samples, without using contrast agents. These intrinsic contrast methods are valuable because of their non-invasive and label-free nature, which allows us to study the biological specimens unperturbed.

Along with Zernike’s Phase contrast (PC) microscopy [3], Nomarski’s differential interference contrast (DIC) microscopy [4] has been one of the most commonly used techniques for intrinsic contrast imaging [5-11]. DIC qualitatively provides the first-order derivative of the phase through interference of two identical but laterally shifted fields. Thus, the images obtained by DIC are very sensitive to the edges and changes in the sample. However, because of the rapid sign changes of the first-order derivatives at the edges of the samples, DIC suffers from the directional shadow artifact in the direction of the shift. Also, since DIC utilizes polarization optics, birefringent materials cannot be readily investigated.
Here, I introduce a method referred as phase derivative microscopy (PDM), based on the spatial filtering of light at the back focal plane of the objective [12-16]. This method carries the spirit of DIC by providing the first-order derivative of the phase while overcoming these two limitations of conventional DIC: the directional shadow artifact and the incapability of using birefringent samples. The former obstacle is overcome by measuring the gradient amplitude (intensity) rather than one of its components. The latter challenge is resolved by using spatial light modulation without polarizing optics to achieve interference between two shifted replicas of the image field.
2. Phase derivative microscopy principles

It is well known that lenses generate the Fourier transform of an optical field located at a focal distance away. This type of optical Fourier transform, combined with spatial filtering, has been used in many different ways to generate images with higher contrast. In detail, by building a $4f$ system using two lenses, one can gain an access to the Fourier transform of the optical field where different types of spatial filtering can take place. Phase derivative microscopy also utilizes this type of spatial filtering at the Fourier plane. In the following sections, different modalities of phase derivative microscopy that generates different type of differentials of the phase of the optical field will be described.

2.1 Laplace field microscopy (LFM)

Laplace field microscopy (LFM), as the name suggests, images the second-order derivative (Laplacian) of the phase by modulating the Fourier transform of the optical field with a parabolic amplitude mask. For an optical field, $U_s(x, y)$, the parabolic modulation with transmission function $H(k_x, k_y) = a(k_x^2 + k_y^2)$ on its Fourier transformation can be calculated by simply multiplying the modulation to the field in the spatial frequency domain, that is,

$$
\tilde{U}(k_x, k_y) = a(k_x^2 + k_y^2)\tilde{U}_s(k_x, k_y).
$$

(1)

In the spatial domain, this modulated signal generates the Laplacian of the scattered field,

$$
U(x, y) = FT\left[ a(k_x^2 + k_y^2)\tilde{U}_s(k_x, k_y) \right] = a\nabla^2 U_s(x, y).
$$

(2)
Since the detector in the imaging system takes the intensity of the field, not the field itself, for phase objects under the Born approximation, i.e. \( U_r(x, y) \approx A \exp[i \phi(x, y)] \), LFM images the square of the derivatives,

\[
I(x, y) = |A|V^2 \exp[i \phi(x, y)]^2 \\
\propto \left[ \nabla^2 \phi(x, y) \right]^2 + \left[ \left( \frac{\partial \phi(x, y)}{\partial x} \right)^2 + \left( \frac{\partial \phi(x, y)}{\partial y} \right)^2 \right].
\] (3)

Therefore, LFM captures both the second-order derivative and the first-order derivative of the phase. However, unlike DIC, LFM does not suffer from the directional shadow artifact as suggested in the square terms that enforces the symmetry.

2.2 Gradient field microscopy (GFM)

Gradient field microscopy (GFM) is another modality of PDM that images the first-order derivative of the phase of the field. There are two modes in GFM: linear-GFM that achieves a high contrast image by taking the square of a radial first-order derivative of the phase and sine-GFM that mimics DIC by generating two identical replicas of the scattered field.

2.2.1. Linear GFM

In linear-GFM, the amplitude modulation mask at the Fourier plane is a simple linear function proportional to the radial distance from the origin in the spatial frequency domain. This “cone-like” modulation, therefore, has a transmission function, \( H(k_x, k_y) = (k_x^2 + k_y^2)^{1/2} = |k| \). Thus, under the first Born approximation applied to a phase object, the intensity, \( I(r) \), at the measurement is proportional to the square of the first-order derivative of the phase,
The advantage of this linear modulation is that there is no directional shadow artifact that occurs in DIC due to the rapidly changing sign of the derivative. However, in Eq. (4), the derivative image obtained from this linear modulation assumes that the field is rotationally symmetric. Therefore, the image may be degraded for specimens without such symmetry.

2.2.2. Sine GFM

Another mode of GFM is obtained by using a sinusoidal modulation on the Fourier transform of the scattered field. Since the Fourier transform of a sine function is a difference of two identical delta functions shifted by a distance determined by the period of the sine, a multiplication by a sine function in the spatial frequency domain yields a convolution between the scattered field and two delta-functions in the spatial domain. Therefore, sine-GFM, using a sine modulation of the form

\[ H(k_x,k_y) = [1 + \sin(ak_y)] \]

generates two replicas of the scattered field. These two replicated fields then interfere at the image plane and generate a phase derivative in the direction of the shift. Since the amplitude modulation cannot modulate the field negatively, the sine modulation has to be shifted so that all the values in the function are positive. Therefore, the field at the image plane after modulation is

\[
[1 + \sin(ak_y)]U(k_x,k_y) \leftrightarrow \left[ \delta(x,y) + \frac{\delta(x,y-a) - \delta(x,y+a)}{2i} \right] * U(x,y)
\]

\[ = U(x,y) + ia \frac{\partial U(x,y)}{\partial y} \]
Applying the Born approximation for a phase object, the scattered field can be expressed as,

\[ U_s(x, y) = \exp[i\phi(x, y)], \]

and the intensity measured at the image plane is

\[ I(x, y) = \left| e^{i\phi(x, y)} - a \frac{\partial \phi(x, y)}{\partial y} e^{i\phi(x, y)} \right|^2 \approx 1 - 2a \frac{\partial \phi(x, y)}{\partial y}. \] (6)

Notice that the DC component is still carried along as a constant because of the shift in the sine modulation. This constant shift can easily be removed, however, and the pure phase derivative can be obtained. As shown in Eq. (6), sine-GFM gives the identical result as the regular DIC while providing some advantageous features that regular DIC does not have. These advantages will be discussed in Chapter 3.
3. Phase derivative microscopy setup

3.1. PDM setup

PDM (Figure 1) is built as an add-on module to the output port of an otherwise unmodified commercial bright field microscope (Olympus IX70). The white light illumination from a halogen lamp is filtered through the aperture stop to provide high spatial coherence of the illumination. The transmitted and scattered light from the sample is collected by the objective lens, and forms an image through the tube lens. At the image plane of the microscope, the PDM setup starts by forming a 4f imaging system including two lenses and an amplitude-only spatial light modulator (SLM). The liquid crystal SLM is obtained from an Epson Powerlite S5 projector with a contrast ratio of 400/1 and pixel size 13 μm. The pixel size limits the resolution in k-space, δk, which indicates the field of view at the image plane over which the modulation produced by one SLM pixel is uniform. With 150 mm focal length lens, L2, this uniformly modulated field of view in the image plane is of the order of 6.25 mm. High contrast images are obtained over the entire CCD detector area, which is a square with 13 mm sides. The Fourier transform of the image formed at the image plane is generated through lens L1 with focal length \( f_1 = 75 \) mm, which locates at a focal distance away from the image plane of the microscope. The SLM provides an amplitude filter at the Fourier plane, and lens L2 with focal length \( f_2 = 150 \) mm takes the Fourier transform of the modulated spatial frequency map back to the new image (detector, Andor iXon+ EMCCD) plane. The inset of Fig. 1 shows the three modulation patterns used at the Fourier plane in the setup to obtain 2D phase derivative (LFM), 1D radial derivative (linear-GFM), and 1D directional derivative (sine-GFM). The sine modulation used in sine-GFM is calculated to be 7.8 mm (13 μm/pixel, 600 pixels/period) and yields at the image plane the spatial shift of \( 2\delta f/a = 20 \) μm between the two separated beams. In essence, PDM module
increases the contrast by imaging the first- or second-order derivative of the phase of the sample and magnifies the image by a factor determined by the ratio $f_2/f_1$.

Figure 1. PDM setup: L1 and L2 have focal length of 75 mm and 150 mm, respectively. The spatial light modulator (SLM) with contrast ratio of 400/1 and pixel size 13 μm is obtained from Epson Powerlite S5 commercial projector. Andor iXon+ EMCCD is used for the detection. Inset: modulation filters projected on SLM for LFM, linear-GFM and sine-GFM are shown.

Unlike regular DIC, where birefringent prisms are used to generate the two interfering beams, PDM operates without any polarization optics. Thus, the PDM add-on module can be used with a regular bright field microscope, without the need for specialized optics. Furthermore, PDM has an advantage in its acquisition speed because it is a single-shot technique. No post-processing is needed for this method because the contrast enhancement is done optically. Therefore, PDM naturally provides an ability to image in real-time and the acquisition speed is limited only by the
frame rate of the detector and the refresh rate of the SLM. Also, PDM is capable of imaging through birefringent samples because it does not include polarization optics.

### 3.2. Proof of principle

To prove the principle of LFM, 3 μm polystyrene micro beads (refractive index = 1.59) immersed in oil (refractive index = 1.516) are imaged. This prepared sample exhibits low contrast under bright field illumination because of the small refractive index difference, $\Delta n$, between the beads and oil. When the parabolic amplitude filter is turned on (LFM mode), the contrast of the bead increased significantly as illustrated in Fig. 2b-c. Figure 2c shows the profiles through the two images. For a spherical sample, the phase term in Eq. (3) can be expressed as $\phi(x) = 2k_0 \left[ R^2 - x^2 \right]^{1/2}$ where $R$ is the particle radius, $x$ is the horizontal distance from the center of the bead and $k_0 = 2\pi \Delta n / \lambda$ with $\lambda$ the mean wavelength of the illumination spectrum. In order to fit the experimental profile and also include the effects of finite resolution, first the phase shift function, which essentially represents the propagating field, is convolved with a Gaussian function of root mean square width given by the diffraction limit, i.e., $\lambda / 2n_{oil} NA = 0.2 \, \mu m$. Note that the derivatives in Eq. (3) associated with this phase distribution diverge at $x = \pm R$; thus, the convolution integral in a principle value sense is performed, avoiding the singularity points. Using this new phase profile, Eq. (3) was numerically evaluated as shown in Fig. 2. The second-order derivative (curvature) of the phase takes the significant role in this case of a spherically shaped object. This profile clearly shows that PDM performs the correct operation qualitatively matching the theoretical prediction introduced in Section 3.1.
Figure 2. 3 µm polystyrene beads (n = 1.59) immersed in oil (n = 1.516) measured with bright field and LFM. (a) Bright field (BF) image of the bead. (b) LFM image of the bead. (c) Horizontal profile of each measurement (scatters) and simulated result using Eq. (3).

In order to test the ability of GFM to generate high-contrast images of transparent samples, measurements on unlabeled HeLa cells were made. HeLa cells were prepared at 30% confluency in a 35 mm glass bottom dish (MatTek, P35G-1.0-14-C, uncoated) with EMEM (ATCC, 30-2003) with 10% FBS(ATCC, 30-2020). The passaged cells then were left in the incubator for a day at 37 °C and 5% CO₂ concentration in a humidified environment, so that they can attach and flatten to the bottom of the dish. Since the switching among bright field (BF), linear-GFM and sine-GFM are done by simply changing the modulation pattern on the SLM, measurement on the
very same field of view was made possible, providing a direct comparison between these methods. Figure 3a-c show images of HeLa cells under BF, linear-GFM and sine-GFM, respectively. For the cells that are well-attached and flattened to the bottom of the dish, the contrast in BF is extremely low. Clearly, these cells become much more visible under linear-GFM with no directional artifact, and sign-GFM with $y$-directional artifact.

Figure 3. HeLa cells under (a) bright field, (b) linear-GFM, and (c) sine-GFM;40x, 0.6NA objective. (d)-(i) HeLa cells imaged with different period sine modulation. All images are taken using a 40x, 0.6 NA objective. Insets of (d)-(i) show the modulation filters used for the HeLa cell image.
As discussed in Section 3.1, one important feature of sine-GFM is that the shift amount can be controlled easily to provide the highest contrast by adjusting the period of the sine pattern. Figures 3d-i show the same HeLa cell imaged by GFM with six different sine modulations. The inset of each image represents the filter that is projected on the SLM; of course, the filter in Fig. 3d gives an image identical to that in BF since there is no modulation given at the Fourier plane. As the period of the sine modulation gets smaller (larger shift in the image plane), one can see more of the overall shape of the cells, i.e., low spatial frequency component. Conversely, for larger sine periods, small details become more visible. This result shows that sine-GFM can be optimized for objects of many different scales.

3.3. Phase derivative imaging through birefringent materials

Regular DIC uses Wollaston prisms, which prevents imaging birefringent specimens or specimens on birefringent substrates such as plastic. Therefore, for regular DIC imaging of biological cells and tissues, glass bottom dishes or glass slides must be used to avoid this birefringence effect. Different methods for birefringence-immunity in getting phase-gradient images have been suggested [17-20], and here, the birefringence-immunity of GFM system is suggested. Since GFM is built as a module that starts at the image plane of a bright field microscope, it can generate a derivative field as long as the correct image can be formed at the image plane.
Figure 4. (a)-(b) Comparison between BF and commercially available DIC on the same field of view, indicating a modest increase in contrast. (c)-(d) Comparison between BF and sine-GFM at the same field of view. Evident contrast enhancement is observed for the cells indicated by arrows.

Figure 4 shows HeLa cells imaged in plastic petri dishes using a 40x, 0.6NA objective. Figures 4a-b show the comparison between the bright field and the regular DIC imaging performance under the presence of the plastic petri dish. Notice the cells indicated by the white arrows show very low contrast in both BF and DIC, which shows the limitations of DIC when birefringent materials are used. Figures 4c-d show the comparison between BF and GFM, again for HeLa cells using a plastic petri dish. Looking at the cells indicated by the arrows, it is very clear that GFM increases the contrast of phase objects even when birefringent substrates are used. Although there is a cost of resolution that comes from smaller condenser aperture compared to
commercial DIC, GFM provides a solution for situations when DIC is not effective, like, for example, when the substrate is birefringent. This capability of using birefringent materials in DIC imaging not only expands the variety of usable materials, but also brings more cost-efficiency to DIC imaging since the plastic containers used in microscopy imaging are usually much less expensive than the glass containers.
4. Applications

4.1 Live cell imaging

Studying dynamics using certain derivatives of the spatial phase distribution rather than the quantitative phase image itself has an interesting aspect. For dynamic specimens, the data analysis is based on the dispersion relation that connects the temporal and spatial frequencies of the fluctuations of the sample. This relation is obtained directly from the differential equations that govern the motions (e.g., the diffusion equation in the case of Brownian particle transport). This means that, for studying dynamics, one does not need to know the phase shift quantitatively; rather, one of its derivatives is sufficient. Therefore PDM will allow for novel investigations of dynamic systems, while bringing great simplifications in the optical setup. In this section, some studies on dynamic samples are introduced to illustrate the advantage of using PDM.

4.1.1 Time-lapse imaging of a HeLa cell culture using LFM

An important feature of PDM is that it can acquire images as fast as the camera allows. Thus, it is suitable for studying highly dynamic specimens such as living cells. HeLa cells were prepared with 40% confluency in a 35 mm glass bottom dish containing culture medium F-12K (Kaighn’s modification of Ham’s F-12 with L-glutamine). After passaging, the dish was left in the incubator for 3 hours as the cells were settling down. As shown in Fig. 5a-b, LFM successfully increases the contrast of a flat HeLa cell and shows both overall cell structure and the subcellular structures. Furthermore, Fig. 5c shows time-resolved imaging of HeLa cells in culture taken using LFM for 35 minutes as the cell rounds up. It is remarkable that the increase in contrast is
conserved over the entire period of measurement. This is because of the very high stability of the PDM system, which does not have any mechanically moving parts.

![Figure 5](image.png)

Figure 5 (a) Bright field image of a HeLa cell. (b) LFM image of the same cell as in (a), but a few minutes later. (c) Dynamic measurement of a HeLa cell using LFM.

### 4.1.2 Red blood cell membrane dynamics study using sine-GFM

Compared to linear-GFM or LFM, the light power lost through the SLM is significantly lower for sine-GFM and, as a result, it allows measurements with shorter exposure time and higher acquisition rate. In fact, as discussed in Chapter 3, sine-GFM can acquire images as fast as the camera acquisition speed since there is no need for additional image processing. Therefore, sine-GFM is suitable for studying dynamic samples such as biological cells. Since sine-GFM measures the first-order derivative of the phase, it is very sensitive to fluctuations where there is a rapid change of the field. In particular, GFM can detect nanoscale motions of red blood cell (RBC) membranes. These fluctuations have been studied actively in the past few years for both
their interesting dynamics at the basic science level [21-26], and their potential for diagnosing disease at the single cell level [27].

Figure 6. Dynamic measurement of red blood cell (RBC) membrane fluctuations. (a) One frame from a time-lapse measurement of a blood smear on a glass slide imaged by sine-GFM. (b) 2D (space-time) power spectrum associated with the time-lapse displacement. (c) Spatial power spectrum that shows the sine function applied by the SLM. (d) MSD vs. k after sine removal, for normal RBCs (errors indicated standard deviation for N = 20 cells). $k^2$ and $k^4$ power laws are indicated as a green and red line, respectively. (e) MSD vs. k for osmotically swollen spherocyte (errors indicated standard deviation for N = 20 cells). $k^2$ power law is indicated as a green line. (f) Comparison between the average MSDs for the normal and swollen RBCs.

In previous studies, the dynamics of RBC membrane fluctuation have been studied in terms of the relationship between the spatial frequency, k, and the mean-square displacement (MSD) of the RBC membrane, $\Delta u^2(k)$ [28].
\[ \Delta u^2(k) = \frac{k_B T}{\kappa k^4 + \sigma k^2}. \] (7)

In Eq. (7), \( k_B \) is the Boltzmann constant, \( T \) is the absolute temperature of the RBC, \( \kappa \) is the membrane bending modulus, and \( \sigma \) is the apparent tension coefficient. Later, it was found that the physical origin of the tension mode is the coupling between compression and bending modes [25]. From the equation, it is expected that the MSD of the membrane depends on \( k^2 \) at low spatial frequencies and on \( k^4 \) at high spatial frequencies.

Here, sine-GFM is used to measure the spatial power spectrum, \( \Delta u^2(k) \). The spatial power spectrum of the sine-GFM image is, to a good approximation, \( \sin(ak_y) \left| U(k_x, k_y) \right|^2 \), as obtained by taking the modulus squared of Eq. (5) and ignoring the sine squared term. Thus, the sinusoidal function, which is identical to the modulation at the Fourier plane, has to be removed to obtain the MSD. Figure 6 shows this procedure and the result of RBC membrane dynamics under sine-GFM. Blood smear on a glass slide was imaged using a 100x/1.4NA oil immersion objective along with the GFM module and the acquisition rate was eight frames per second. Figure 6a shows one frame of the time-lapse stack of RBC sine-GFM images. In order to get a spatiotemporal power spectrum, the 3D power spectrum of the time-resolved images is taken and resliced to show the spatial frequency along the modulation direction (\( k_y \)) and temporal frequency, \( \omega \). This \( \omega-k_y \) domain image, Fig. 6b, contains full information about the spatiotemporal fluctuations of RBC membranes. By taking the average over \( \omega \), the MSD multiplied by the sine function is obtained (Fig. 6c). Since this sine function is due to the SLM filter, this modulation on the MSD can be numerically removed via a simple division. Figure 6d shows the resulting MSD, averaged over 20 measurements, for normal discocyte RBCs, where
the expected $k^{-2}$ and $k^{-4}$ power laws are observed. In Fig. 6e, it shows the MSD, averaged over 20 measurements, for osmotically swollen spherocyte RBCs, prepared by adding water to the blood smear. Figure 6f shows a comparison between the dynamics of discocytes and spherocytes. Interestingly, the $k^{-2}$ power law was dominant, even at high spatial frequencies, in the case of spherocytes. This result suggests that the bending mode is subdominant throughout the entire spatial domain. This result is consistent with recent studies performed by quantitative phase imaging, where the spatial correlations associated with the membrane fluctuations appeared narrower for swollen cells.

Remarkably, the results show that useful information can be retrieved from nanoscale fluctuations using only a derivative of the phase, without the quantitative phase itself. This is quite a general result, i.e., similar information as in Figs. 6d-e can be obtained using a commercial DIC microscope. This type of measurement is much more easily implemented than a quantitative phase imaging method (see [1] for a review of such methods). However, the price paid for not using full (quantitative) phase information is the lack of quantitative statements that one can make about the mechanical parameters of the membrane, e.g., $\kappa$ and $\sigma$. In other words, using the gradient images to calculate the power spectra, there is no method to normalize the curves (e.g., Fig. 6e). Thus, the curve cannot be fitted to extract the quantitative parameters. If quantitative information is the goal, one should use instead quantitative phase imaging, as shown in [1, 22-27].
4.2. Label-free diagnosis of human biopsies

4.2.1. HGPIN condition

High-grade prostatic intraepithelial neoplasia (HGPIN) is a condition that mimics prostate cancer with glands showing genetic and immunohistochemical changes associated with prostate cancer and tissue biopsies showing the presence of prominent nucleoli and Roman bridges in glands [29, 30]. HGPIN is a lesion that is sometimes confused with prostate cancer in biopsies because it has atypical cells, large prominent nucleoli but, in contrast with cancer, it has basal cells in the periphery of the glandular structures. Because it mimics the morphology of prostate cancer biopsies, there is a need for investigation into HGPIN biopsies in order to correctly diagnose that they are in fact not malignant. Currently, cytokeratin 34BE12 and p63 immunohistochemistry markers are used to exclude the diagnosis of carcinoma by identifying basal cells [30]. Immunohistochemistry however delays diagnosis, usually by one day and frequently more.

4.2.2. HGPIN detection using GFM

The histologic presence of basal cells distinguishes HGPIN from prostate cancer. Since these cells are small in size, it is expected that they show rapid change in optical path length at the boundary and that they can be detected by GFM. With this idea, multiple unstained biopsies with HGPIN condition are imaged using GFM with a high magnification, high numerical aperture objective.
Figure 7a shows an image of a HGPIN biopsy taken under sine-GFM with 100x/1.4NA oil immersion objective on an Olympus IX70 microscope. Since this high magnification objective cannot image the whole biopsy at once, this image is produced by stitching 154 (14 X 11) images. Even though one core has to be imaged in many separate tiles, the fast acquisition speed of GFM allows high throughput in the measurement (10 minutes/biopsy assuming no error in stitching). In Fig. 7b, a quantitative phase image of the same tissue taken using spatial light interference microscopy (SLIM, [31]) is shown. This image is also a stitch of 80 (10 X 8) images taken using a 40x/0.75NA microscope objective. For each section (one field of view), it took 4 second to image the quantitative phase and for the whole tissue area, it took a total of 9 minutes including the focusing time. Figure 8 shows 12 different HGPIN biopsies imaged by the same GFM setup.
Figure 8. Images of 12 different HGPIN biopsies taken under GFM with 100x/1.4NA oil-immersion objective. Each image is produced by stitching over 100 separate images.

In order to show the high contrast at the edges more closely, the top row of Fig. 9 shows the GFM image of specific areas in HGPIN biopsies. Notice that the patchy basal cells are clearly seen between the epithelium and stroma area. Although they do not have any specific optical marker, it is possible to notice these cells from their boundaries, which shows high value in GFM, and also from their shape. To ensure this qualitative detection of basal cells done by eye based on the shape of the cells, the images are compared to the images of the same area taken using SLIM (the bottom row of Fig. 9), which is a quantitative phase imaging technique [31]. SLIM measures the quantitative refractive indices of a biopsy and uses it as a marker for different structures. It has been shown in Wang et al. [32] that the basal cells have low refractive
indices in the quantitative phase image taken with SLIM and can be detected easily.

Figure 9. GFM (top) and SLIM (bottom) images of HGPIN biopsies showing the areas with basal cells. Images in the same row show the exact same region for comparison between GFM and SLIM. Basal cells are marked yellow in the GFM images and red in the SLIM images for clarity.

Although there is no quantitative information in the GFM images, unlike in SLIM images, GFM shows very high sensitivity to edges, which gives the relevance in studying small and rapidly changing structures such as basal cells. Also, it has been shown that the qualitative detection of basal cells using GFM matches well with the result from SLIM. Therefore, GFM can be used for detection of the HGPIN condition in a fast (single-shot) and label-free manner.
5. Conclusion

Phase derivative microscopy, as introduced, is an add-on module to a commercial bright field microscopy system and utilizes the intrinsic contrast of transparent specimens to obtain high contrast images of transparent samples. The basic principle of PDM is based on Fourier filtering of an optical field, which has already been proved to be effective in many different studies. This method, PDM, carries the essence of differential interference microscopy as it increases the contrast by imaging the derivative of the phase of the optical field passing through the sample of interest, while adding some more beneficial features, such as fast acquisition rate, high mechanical stability, adjustability from using a spatial light modulator, cost efficiency, and capability of using birefringent materials. Furthermore, for certain modalities, PDM successfully removes the directional shadow artifact of DIC, which has been one of the largest drawbacks of DIC imaging. With all these advantageous features of PDM, in this thesis, two of many possible applications of PDM are shown: live cell imaging and human biopsy diagnosis. As shown in these applications, PDM has a high potential to be used in both basic science and clinical applications.
Appendix A. Structure reconstruction from sine-GFM images

A1. Structure reconstruction from two perpendicular sine-GFM images

In case of GFM, the phase information is still obtained in the intensity image as a form of a derivative. Therefore, by integrating over the shear direction, the phase information can be retrieved. One concern in this technique is that the integration constant cannot be uniquely defined for each line-integral. Therefore, at least two directions of shear need to be used to uniquely determine the phase of the structure. GFM, with no moving mechanical parts, can easily obtain differential images in different shear directions by applying sine modulations in different directions. Yin et al. [33], have presented a method to reconstruct the phase from DIC images with multiple shear directions by utilizing the minimization of the L_2 error and by introducing two different regularization factors.

First the gradient in direction $\theta$ can be represented as a convolution of a differential kernel, $d$, and the structure $f$,

$$g_\theta = \nabla_{\theta} f = d_{\theta} * f.$$  \hspace{1cm} (8)

Then the L_2 error is

$$E(f) = \int d\theta \left[ \left( \sum_i (d_{\theta_i} * f - g_i) \right)^2 + \omega_{sm} (a * f)^2 + \omega_{sp} f^2 \right].$$  \hspace{1cm} (9)

In Eq. (9), $\omega_{sm}$ is the regularization factor for local smoothing, $a$ is the local smoothing kernel, and $\omega_{sp}$ is the sparse structure regularization factor. To minimize this L_2 error, the derivative of Eq. (9) with respect to $f$ is set to zero,

$$0 = \frac{\partial E(f)}{\partial f} = 2\omega_{sm} a * a * f + 2\omega_{sp} f - 2 \sum_i d_{\theta_i} * (d_{\theta_i} * f - g_i).$$  \hspace{1cm} (10)
and taking the Fourier transform, the original structure in the Fourier domain can be obtained through simple matrix arithmetic of the Fourier transforms of the known factors,

\[
F = -\left(\sum_i D_{\theta i} \cdot G_i \right) / \left(\omega_{mp} A^2 + \omega_{sp} - \sum_i D_{\theta i}^2\right).
\] (11)

In this project, two different shear directions \(\theta = 0^\circ\) and \(\theta = 90^\circ\) are used, therefore, the summation in Eq. (11) is only for two elements. Once \(F\) is obtained from Eq. (11), the phase of the original specimen can be obtained by taking a two-dimensional inverse Fourier transform.

**A2. Results**

Using the inverse problem and reconstruction model from Section A1, in this section, I will present some results of the structure reconstruction.

**A2.1. Simulations**

Since the measurement cannot be made for the optical field, numerical simulation is done to verify the inverse problem in the field. Using quantitative phase images obtained from the spatial light interference microscopy (SLIM) system [31], the optical field is simulated by taking the complex exponential of the phase information. Then the GFM system is simulated by taking the Fourier transform of the simulated field, multiplying by the sine modulation and then taking another Fourier transform of the modulated field. Furthermore, complex random noise is added to the field and the intensity of this simulation is obtained to simulate the GFM measurement. Figure 10b shows the result of this simulation with the sine modulation filter in the inset. In order to show the inverse problem and reconstruction of the object, the inverse operator is calculated using the sine filter that is used to generate the GFM image and then applied to the simulated
field. By taking the argument of this reconstructed field, the original phase is reconstructed, which is shown in Fig. 10c.

Figure 10. (a) QPI image of two 3 μm beads. (b) Simulated GFM image obtained from (a) and the inset shows the sine modulation that is used in this simulation. (c) Reconstructed phase image by solving the inverse problem in the optical field.

Another simulation is performed on biological cells (U2OS) and the result is shown in Fig. 11. In this simulation, sine modulation in the horizontal direction is applied as opposed to the vertical sine modulation in Fig. 10.

Reconstruction using the intensity data is also performed on the simulated images using the result in Eq. (11). Knowing the sine modulation applied to generate the GFM image, the derivative kernel $D$ is automatically determined. Therefore, all the parameters in Eq. (11) are known to the problem, and it allows the reconstruction as shown in Fig. 12.
Figure 12. (a) Original QPI image of an HT29 cell. (b) Simulated GFM image with horizontal shear. (c) Simulated GFM image with vertical shear. (d) Reconstructed phase image from the combination of two GFM images. (e) Reconstructed phase image from horizontal shear GFM image. (f) Reconstructed phase image from vertical shear GFM image.

One thing to note in Fig. 12 is that using two different GFM images using two different shear directions gets rid of all the streak artifacts coming from the inconsistent integral constants. In Figs. 12(e-f), the artifacts are very prominent when only one direction of shear is used.

Also, to demonstrate the effect of the regularization factor (sparse structure regularization factor), different values of the regularization factor are applied to the reconstruction and shown in Fig. 13. Once a value larger than $1 \times 10^{-5}$ was used, however, the reconstruction was not successful anymore.

Figure 13. HT29 cell reconstruction with different regularization factors.
Overall, to the simulated data, the reconstruction method worked well to retrieve the original structure of the sample.

**A2.2. Experimental data**

Finally, with the verification done in Section A2.1, the same method has been applied to the experimental data obtained from the actual GFM system. Samples used in this part are blood smears, especially the red blood cells. The result is shown in Fig. 14 along with the measured GFM image and also the reconstructions from only one shear direction.

![Figure 14. Structure reconstruction on measured GFM images. (a) and (b) show the measurement and (c) and (d) show the reconstructions from one shear direction. (e) shows the reconstruction from the combination of two shear directions, which shows the clear discocyte structure of a normal RBC.](image-url)
References


