Diffraction phase and fluorescence microscopy

YongKeun Park, Gabriel Popescu, Kamran Badizadegan*, Ramachandra R. Dasari, and Michael S. Feld

G. R. Harrison Spectroscopy Laboratory, Massachusetts Institute of Technology, Cambridge, MA 02139
gpopescu@mit.edu

**) Department of Pathology, Harvard Medical School and Massachusetts General Hospital, Boston, MA 02114*

Abstract: We have developed diffraction phase and fluorescence (DPF) microscopy as a new technique for simultaneous quantitative phase imaging and epi-fluorescence investigation of live cells. The DPF instrument consists of an interference microscope, which is incorporated into a conventional inverted fluorescence microscope. The quantitative phase images are characterized by sub-nanometer optical path-length stability over periods from milliseconds to a cell lifetime. The potential of the technique for quantifying rapid nanoscale motions in live cells is demonstrated by experiments on red blood cells, while the composite phase-fluorescence imaging mode is exemplified with mitotic kidney cells.

OCIS codes: (180.0180) Microscopy; (170.2520) Fluorescence microscopy; Hilbert phase microscopy, Diffraction phase microscopy.

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1. Introduction

During recent years optical phase-based and fluorescence techniques have advanced considerably [1]. While optical path-length shifts though cells provide structural information, fluorescence imaging of particular molecules of interest reveals functional details about the biological system. In this paper we present diffraction phase and fluorescence (DPF) microscopy as a novel multimodal imaging technique, which provides simultaneous quantitative phase and fluorescence imaging of live cells. By combining the nanoscale structure and dynamics acquired from the optical phase measurement with the molecular insight offered by specific fluorophores, DPF should find important applications in cell biology.

Phase contrast (PC) and differential interference contrast (DIC) microscopy have been used extensively to infer morphometric features of cells without the need for exogenous contrast agents [1]. However, both PC and DIC are qualitative in terms of optical path-length measurement, i.e. the relationship between the irradiance and phase of the image field is generally nonlinear.

Quantifying the optical phase shifts associated with biological structures gives access to information about morphology and dynamics at the nanometer scale. Over the past decade, the development of quantitative phase imaging techniques has received increased scientific interest. Several point-measurement techniques have been applied for investigating the structure and dynamics of live cells [2-8]. However, imaging large field of view samples requires raster scanning, which is typically a time-consuming procedure. Full-filed phase measurement techniques, on the other hand, provide simultaneous information from a large number of points on the sample. Several such methods have been proposed in the literature [9-16]. In our laboratory, we used an actively stabilized interferometer to obtain phase maps of live cells [17]. We have also developed Fourier phase microscopy (FPM) to provide quantitative phase images of biological samples with remarkable stability over extended periods of time [18, 19]. Hilbert phase microscopy (HPM) renders a quantitative phase image from a single interferogram recording and, thus, is amenable for investigating rapid phenomena in transparent structures [20]. Its potential for quantifying the shape and dynamics of red blood cells has been demonstrated [21]. Most recently, diffraction phase microscopy (DPM) has been proposed in our laboratory to combine the single shot benefit of HPM with the common path geometry associated with FPM [22]. Therefore, DPM allows for fast imaging rates without compromising phase stability.

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However, this type of measurement lacks specificity, i.e. the optical path-length detected is not characteristic to a particular molecular structure. On the other hand, recent advances of the green fluorescent protein (GFP) technology allow an intrinsically fluorescent molecule to be genetically fused to a protein of interest in live cell populations, which offers the benefit of specificity. In the following, we present for the first time, to our knowledge, an investigation that combines optical path-length maps of live cells with images of specific structures tagged by fluorophores.

2. Diffraction phase and fluorescence microscope

The DPF experimental setup is depicted in Fig. 1. An inverted microscope (IX71, Olympus Inc.) is equipped for standard epi-fluorescence, using a UV lamp and an excitation-emission filter pair, F1-F2. An Ar^{2+} laser (λ =514μm) is used as an illumination source for transmission phase imaging. Through its video output port, the microscope produces the image of the sample at the image plane IP1 with magnification M=40. The lens system L1-L2 is used to collimate the unscattered field (spatial DC component) and further magnify the image by a factor f2/ f1=3, at the plane IP2. An amplitude grating G is placed at IP2, which generates multiple diffraction orders containing full spatial information about the sample image. The goal is to isolate the 0^{th} and 1^{st} orders and to create a common-path Mach-Zender interferometer, with the 0^{th} order as the reference beam and the 1^{st} order as the sample beam. To accomplish this, a standard 4-f spatial filtering lens system L3-L4 is used. This system selects only the 0^{th} and 1^{st} order and generates the final interferogram at the CCD plane. The 0^{th} order beam is low-pass filtered using a pinhole placed at the Fourier plane L3 so that it becomes a plane wave after passing through lens L4. The spatial filter allows passing the entire frequency information of the 1^{st} order beam and blocks the high frequency information of the 0^{th} beam. Compared to conventional Mach-Zender interferometers, the two beams propagate through the same optical component, which significantly reduces the longitudinal phase noise, without the need for active stabilization. The fluorescence light also passes through the grating that generates two diffraction spots in the Fourier plane of lens L3. However, due to its spatial incoherence, the fluorescence spots are much larger than the pinhole, which blocks the 0^{th} order almost entirely. Therefore, the fluorescence image information is carried to the CCD by the 1^{st} order of diffraction. A commercial digital camera (D50, Nikon Inc) is used to capture both the interferogram and the fluorescence image. The CCD has a resolution of 3000x2000 pixels and each pixel is 7.8x7.8 μm in size. From the interferogram recorded, the quantitative phase image is extracted via a spatial Hilbert transform, as described in Ref. [20]. The grating period is 30 μm, which is smaller than the diffraction spot of the microscope at the grating plane (47 μm). Thus the optical resolution of the microscope is preserved.

In order to asses the stability of the instrument against the phase noise, we acquired sets of phase images and computed the standard deviation associated with regions in the field of view containing no sample. The phase images were acquired at 300 frames/second. The measured fluctuations were averaged over two frames in time and over 5x5 pixels in space. The histogram of the optical path-length standard deviations measured for an area of 50x50 pixels was computed. Remarkably, the histogram had a mean value of 0.23nm, and a width of 0.024 nm which demonstrates the sub-nanometer path-length stability of the instrument. This noise level is due to the mechanical vibrations present in the system, slight instability of the laser output, as well as photon noise that arises in the detection process. Our experiments showed that the mechanical stability of the interferometer is the dominant noise component and that there is room for improvement before the ultimate shot noise level is obtained. The significant mechanical stability showed here is due entirely to the common path interferometric geometry and does not involve any modality of active stabilization.

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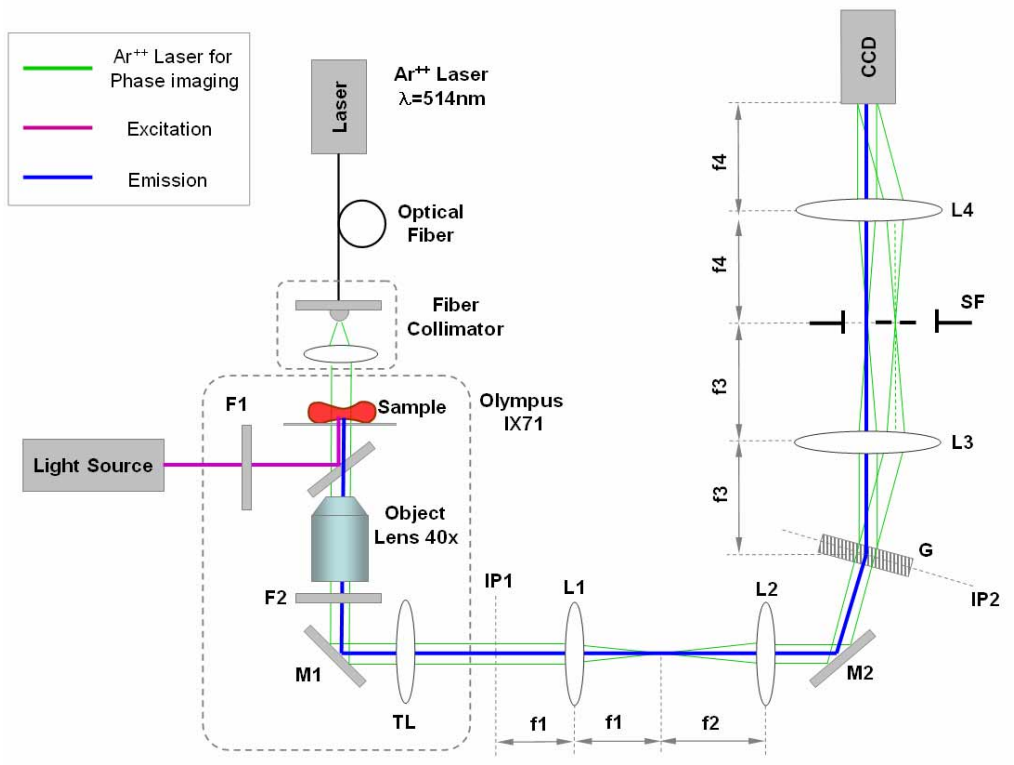

Fig. 1. DPF setup. F1, 2, filters; M1, 2, mirrors; L1-4 lenses (f1-4, respective focal lengths); G, grating; SF, spatial filter; IP1, 2, image planes; SF, spatial filter.

3. DPF microscopy of live cells

Due to its single shot nature, the DPF technique can be applied to investigating dynamic phenomena in live cells over temporal intervals that span from the millisecond scale or less to an entire cell cycle. We demonstrate this versatility with experiments of red blood cell (RBC) membrane fluctuations, which take place at the millisecond scale, and by imaging white cell activity in blood smears, which develops over periods of minutes. Droplets of whole blood were placed between cover slips without further preparation. Figure 2(a) depicts an example of such quantitative phase image of an RBC. The corresponding movie shows the thermal fluctuations of the RBC membrane. This phenomenon of RBC "flickering" has been observed many years ago and intense efforts have been focused on understanding this dynamic process [23]. Using DPF to quantify these membrane motions can provide a valuable tool for non-contact assessment of membrane mechanical properties.

Figure 2(b) shows a quantitative phase image of a whole blood smear containing an RBC and an eosinophil as identified by its characteristic bi-lobbed nucleus and prominent cytoplasmic granules. The corresponding movie (1 frame/ s) captures a spectacular phenomenon where the granulocyte "attacks" the RBC and eventually breaks its membrane, leading to lysis of the red blood cell. (Dynamics of RBC lysis was previously quantified at the millisecond scale using Hilbert phase microscopy [21]). These experiments demonstrate the ability of the instrument to quantify dynamic phenomena at arbitrary time scales.

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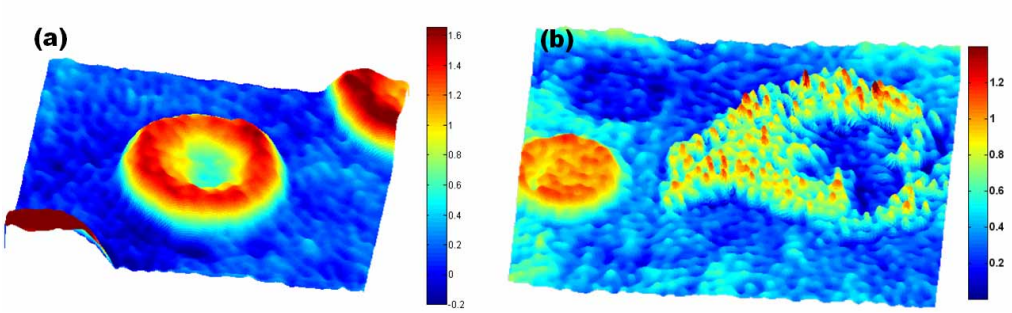

Fig. 2. (a). (4 Mb) MOVIE: Thermal fluctuations of red blood cell membrane. (b) (4 Mb) MOVIE: Eosinophil chemotaxis and attack on red blood cell. Color bars represent optical phase in radians.

In order to illustrate the combined phase-fluorescence imaging capability, we performed experiments of kidney (mesangial) cells in culture. The cells were imaged directly in culture dishes, surrounded by culture medium. Prior to imaging, the cells were treated with Hoest solution for 60 minutes at 38°C and 5% CO_2. This fluorescent dye binds to the DNA molecules and is commonly used to reveal the cell nuclei. Figure 3 shows an example of our composite investigation.

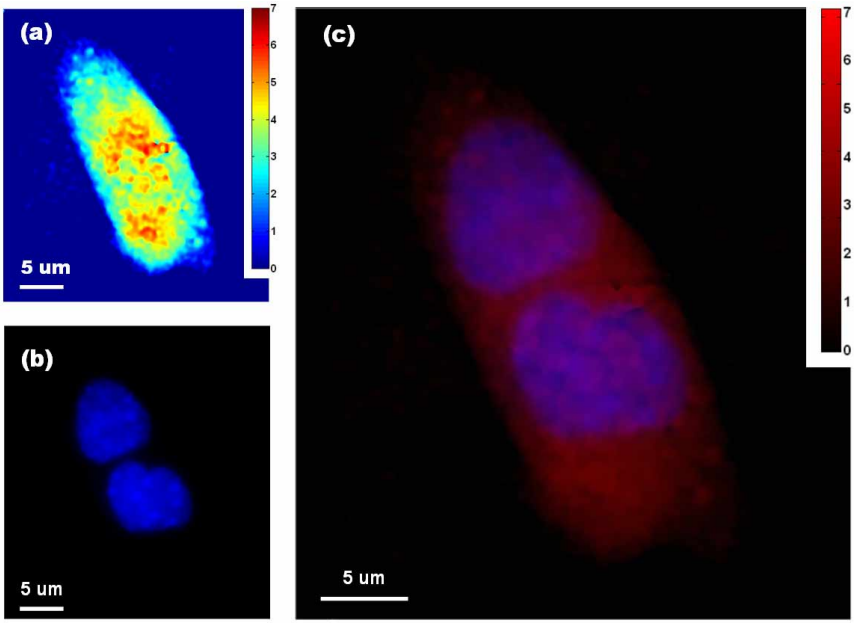

Fig. 3. (a). Quantitative phase image of a kidney cell. The colorbar indicates optical phase shift in radians. (b) Fluorescence image of the same DNA-stained cell. (c) Overlaid images from (a) and (b): red- DPM image and blue- epi-fluorescence image. Colorbar indicates phase in radians.

The quantitative phase image of a single cell is shown in Fig. 3(a). Figure 3(b) shows the fluorescence image of the same cell, where it becomes apparent that the cell is in the process of mitosis, indicated by the two separated nuclei. Figure 3(c) shows the composite image. By taking advantage of the difference in the spatial coherence of the two fields, the fluorescence and phase imaging light pass through the same optics, without the need for separation by using, for instance, dichroic mirrors. While the diffraction grating provides a stable geometry

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for interferometry, it introduces light losses which may affect fluorescence imaging especially of weak fluorophores. However, this aspect can be ameliorated by using a sinusoidal amplitude grating that maximizes the diffraction in the +1 and -1 orders. These two beams can be used for interference, which will introduce light loss in the fluorescence channel by only a factor of two larger than in the absence of grating.

4. Summary

In summary, we presented a novel instrument that combines quantitative phase and epi-fluorescence microscopy of live cells. The phase measurement itself was improved with respect to our previous reports to offer better control on the spatial sampling by the CCD, which was accomplished by adding the telescopic lens system L1-L2. Using the Olympus IX71 microscope enhanced the stability of the system, which allows investigating biological samples at various time scales. Due to the common path geometry used, the optical path-length stability has a remarkable value of 0.23 nm, which is superior to our previous reports. The single-shot feature of the phase measurement grants the flexibility of investigating both very fast (millisecond scale) and slow (up to days) phenomena in cells. We believe that DPF represents a significant advance towards simultaneous quantitative and molecular imaging of live cells.

One potentially unique application of this multimodal investigation is to study the effects of various membrane binding molecules to the mechanical properties of cell membranes. Red blood cells are a convenient model for studying membrane fluctuations and quantitative phase microscopy is a suitable method for quantifying such motions [24]. Using the DPF instrument for studying both the membrane dynamics and cell cytoskeleton integrity (by GFP-spectrin fusion) may provide a valuable tool for better understanding of cytoskeleton- lipid bilayer interaction.

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