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Abstract. Quantitative three-dimensional imaging of cells can provide important information about their morphology as well as their dynamics, which will be useful in studying their behavior under various conditions. There are several microscopic techniques to image unstained, semi-transparent specimens, by converting the phase information into intensity information. But most of the quantitative phase contrast imaging techniques is realized either by using interference of the object wavefront with a known reference beam or using phase shifting interferometry. A two-beam interferometric method is challenging to implement especially with low coherent sources and it also requires a fine adjustment of beams to achieve high contrast fringes. In this letter, the development of a single beam phase retrieval microscopy technique for quantitative phase contrast imaging of cells using multiple intensity samplings of a volume speckle field in the axial direction is described. Single beam illumination with multiple intensity samplings provides fast convergence and a unique solution of the object wavefront. Three-dimensional thickness profiles of different cells such as red blood cells and onion skin cells were reconstructed using this technique with an axial resolution of the order of several nanometers. [DOI: 10.1117/1.3589090]

Keywords: phase contrast imaging; three dimensional microscopy; phase retrieval; angular spectrum propagation; speckle field.

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Phase contrast microscopy of cells can provide important information on their dynamics and morphological changes. Several methods are used in optical microscopy for imaging unstained specimens by converting the phase information to amplitude variations. Most common among these are Zernike and Nomarski methods.[1,2] Interference techniques such as digital holography can also be used for phase contrast imaging microscopy, yielding the quantitative phase values rather than just images.[3–12] Digital holographic microscopy has the added advantage of numerical focusing. But most of these methods require interference between the object wavefront and a reference wavefront for extraction of quantitative phase information. The use of two separate coherent beams and their coherent interference may be challenging for some applications, including 1. use of low coherence sources and/or short wavelengths such as UV and x-rays; 2. noise due to mechanical vibrations during the interference recording; 3. miniaturized instrumentation, etc. Here the use of a single beam phase retrieval technique for quantitative phase contrast imaging microscopy of cells using multiple intensity samplings of a volume speckle field in the axial direction is described. The low frequency diffraction pattern resulting from the interaction of a coherent wavefront with the object under investigation is converted into a high frequency but detectable intensity pattern (speckle pattern) using a random binary mask or a diffuser plate. This volume speckle field is sampled at several axial planes and is used iteratively in a phase retrieval algorithm which implements the propagation of the wavefront between the sampling planes using a scalar diffraction integral. Multiple intensity samples provide fast convergence and a unique solution. Three-dimensional thickness profiles of different cells such as red blood cells and onion skin cells were reconstructed using this technique. An axial resolution of the order of several nanometers was obtained.

The experimental realization of the technique is shown in Fig. 1. In the experimental setup a low power He–Ne laser (max. optical power is 1 mW) working at 611.8 nm is utilized. Commercially available low-cost laser diodes can also be used. Collimated wavefront from the laser illuminates the object under investigation. The diffracted low frequency wavefront is then converted into a high frequency but detectable intensity pattern using a diffuser placed after the microscope objective.[13,14]

The scattered light (diffracted wavefront) from the object contains whole field information about the cell. In conventional interferometric microscopy, this object wavefront is combined with a reference wavefront to form an interferogram. This interferogram can be used to retrieve the phase as in the case of digital holography. In our proposed approach, instead of recording the interference pattern, the Fresnel diffraction pattern from the object, is directly recorded thereby eliminating the need for the reference beam. By evaluating the changes occurring to the diffraction field at various axial positions, the phase of the object wavefront can be recovered.[13–16] But most of the cells produce diffraction fields which do not vary appreciably with axial position. So the evaluation of the change in the diffraction field becomes difficult. To remedy this problem, the diffraction intensity pattern needs to be converted into a higher frequency yet detectable intensity pattern. For coherent illumination, this can be achieved by introducing a diffuser or a random mask (amplitude or phase) in the path of the object beam.[13–16] This converts the diffraction field into a volume speckle field. Speckles do have appreciable intensity variation both in the transverse as well as axial directions suitable for evaluating the diffraction intensity change and to reconstruct the phase.

The reconstruction process starts by assuming a phase which is combined with the square root of the intensity pattern (amplitude) at the first sampling to yield the complex amplitude at this plane.[13] Since complex amplitude is available, it can be propagated to the next sampling plane using scalar diffraction integral. In the present case and angular spectrum propagation (ASP) integral is used for this purpose.[17] The advantage of using an ASP

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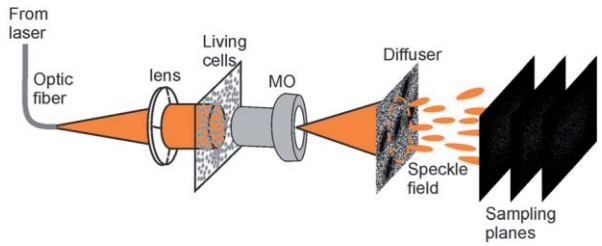

Fig. 1 Experimental setup for single beam quantitative phase contrast imaging microscopy.

integral is that it does not require any paraxial approximation and hence can be used for short distance propagation. The phase obtained from the propagation is extracted and is combined with the amplitude at the second recording plane and is again propagated to the next sampling plane. This is continued until the last sampling plane is reached. The convergence is checked by comparing the intensity obtained by propagation with the sampled intensity. If they cannot be correlated within a required threshold value, the entire propagation process is repeated until the desired correlation is achieved. In order to reduce the number of intensity samplings, the algorithm is implemented iteratively. After reaching the last sampling plane, the wavefront is propagated back to the first sampling plane by the same process. The initially assumed phase is used only once at the beginning of the reconstruction process. The use of multiple samplings and iterative use of the ASP integral provides a fast convergent, and unique and accurate solution of the wavefront. Once the correct complex amplitude at any sampling plane is obtained, it can be propagated to any other plane including the image plane.

First set of experiments were done on human red blood cells. A cover slip was placed over the smears made on glass slides. A 100× microscopic objective with 0.8 NA was used for magnification. A ground glass diffuser (with a mask with aperture size 2.8 mm × 2.8 mm) placed at 14 cm distance from the objective lens converted the low frequency diffraction field into the speckle field. A CCD sensor with 8-bit dynamic range and 1024 × 1024 pixels with pixel pitch of 4.65 μm was used for sampling the resulting speckle pattern. It was mounted on a motorized translation stage. The first sampling plane was 40 mm from the diffuser and a total of 30 intensity samplings axially separated by 1 mm were recorded. This setup can record up to 3 intensity patterns separated by 1 mm in a second. All the diffracted intensity patterns were not utilized for the reconstructions. Same samplings measurements were performed without the object (cells) to record the surrounding medium, which was then used for phase comparisons. The reconstructed complex amplitude at the first sampling plane is propagated to the image plane which is situated to its left and the phase is extracted. This is then subtracted from the obtained phase without the object (cells) to yield the phase contrast image. Here comparison of the phases with and without the cell directly converts the optical path length data into thickness information. Also, it compensates the aberrations present in the system. This approach is different from the studies conducted earlier where a digital compensation of the aberrations were performed.[13,14] Figures 2(a) and 2(b) show the reconstructed intensity and phase at the image plane using 15 intensity samples axially separated by 2 mm and with 10 iterations of the reconstruction algorithm.

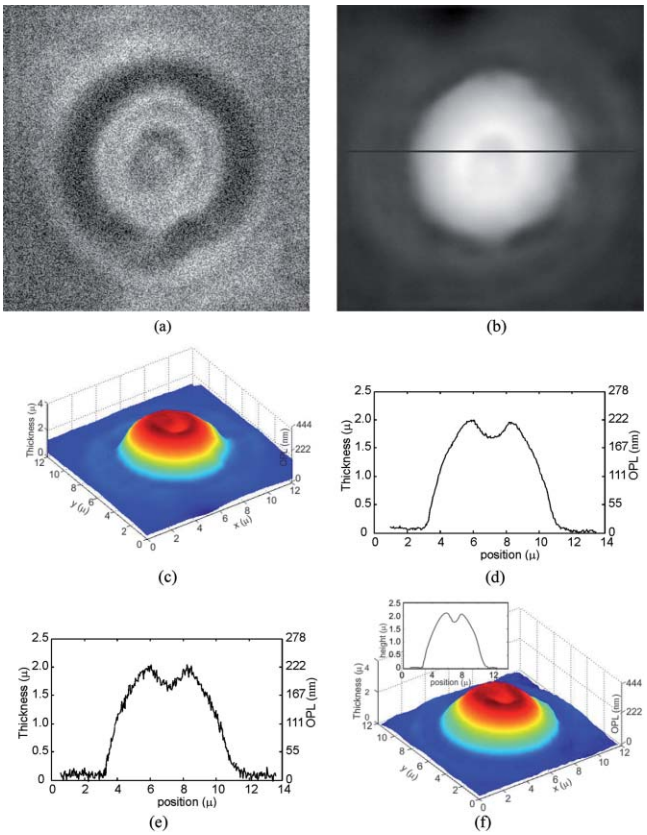

Fig. 2 Experimental results obtained with RBCs. (a)–(e) Present results using a single beam reconstruction. (a) Reconstructed RBC intensity at image plane, (b) obtained phase difference using 15 intensity samples, (c) corresponding thickness profile, (d) cross-sectional profile of RBC shown in (c), (e) cross-sectional profile of RBC obtained using 5 intensity samples, (f) thickness profile of a different RBC obtained with DHM.The cross-sectional profile is shown in the inset. The thickness profile obtained with DHM is very similar to the one obtained with single beam reconstruction.

The whole reconstruction process took about 70 s in a PC with core i7 processor and 8GB RAM. The thickness profile of the cell is computed from the obtained phase difference by assuming a constant refractive index $n_{\text{cell}} = 1.42$ for the red blood cells (RBC) and $n_{\text{medium}} = 1.33$ for the surrounding medium (plasma).[18] The relationship $h = (\Delta\phi\,\lambda/2\pi)/(n_{\text{cell}} - n_{\text{medium}})$ provides the cell thickness as shown in Fig. 2(c), where $\Delta\phi$ is the computed phase difference and λ is the vacuum wavelength of the source. The RBC cross section plotted along the line in Fig. 2(b) is shown in Fig. 2(d). This thickness profile is similar to the one obtained with other quantitative techniques.[11,12,19] The accuracy in the thickness measurement of the technique was computed from an area in the phase map where there is no cell. A smaller variation from the mean value in this region indicates higher accuracy in the axial measurement. The standard deviation of the thickness profile in this area provides the longitudinal accuracy and it was measured at 18.2 nm. Also, reconstructions were investigated with a lower number of intensity samples. It was found that the axial resolution decreases to 52 nm when 5 intensity samples separated by 5 mm were used for reconstruction [Fig. 2(e)]. Below 4 intensity samples, it was not possible to have proper reconstructions. The thickness profile of a different

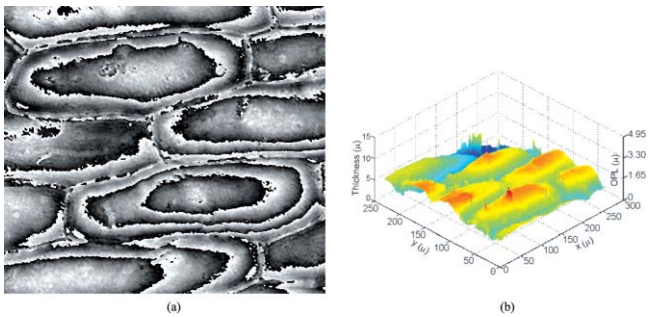

Fig. 3 Experimental results obtained with onion skin cells. (a) Obtained phase difference and (b) three-dimensional thickness and optical path length change of the cells.

red blood cell is obtained with a DHM for comparison. The thickness profiles obtained with single beam reconstruction [see Fig. 2(d)] is similar to the one obtained with a digital holographic microscope (DHM) as shown in Fig. 2(f). The cross-sectional cell profile using DHM is shown in the inset of Fig. 2(f). The cell reconstructions using DHM and single beam are similar but not identical because the RBCs used in each experiment are different. The computed accuracy in thickness measurement using DHM was less than 6 nm. A phase contrast image with single beam technique was used to compute the volume, area, and the diameter of the cell. The cell area is computed by thresholding the thickness information by the mean thickness of the region where no cell exists. Multiplying this by the thickness provides the volume of the cell.[19] The computed volume, area, and diameter were 94.3 femtoliters, 48.7 μm^2, and 7.86 μm respectively. These values are comparable with the literature values obtained with other quantitative microscopic techniques.[19] Figures 3(a) and 3(b) show the results obtained in the case of onion skin cells. Here, a 10× microscopic objective with NA = 0.25 was used. The rest of the experimental parameters was the same as that for RBC. In these experiments, $n_{\text{cell}} = 1.34$ and $n_{\text{medium}} = 1$ was used for the computation of cell thickness profile. Also, simulations were carried out using computer generated microscopic phase objects to check the accuracy of the reconstructions using this technique. It was found that the method provides very accurate reconstructions with less than 1% error for the simulated objects.

The results presented show that quantitative phase contrast imaging of cells can be done with a single beam phase retrieval technique without the use of a reference beam or using digital holography or phase shifting interferometry. The technique has the capability of numerical focusing as in digital holographic microscopy. Higher numbers of sampling planes will provide better reconstruction (unique solution) and faster convergence. But large numbers of sampling planes means more distance from the diffuser as sampling planes separated by small distances will not yield appreciable intensity variation in the axial direction. This results in the loss of higher spatial frequencies and hence lower reconstruction resolution. However, the iterative use of the ASP reconstruction algorithm remedies this problem. The main limitation in dynamic studies using this technique is the time for data acquisition, which is about 6 to 10 s presently. This makes the technique in the present form capable of studying very low frequency dynamics.

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