Phase imaging flow cytometry using a focus-stack collecting microscope

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This letter introduces a fluidics-based focus-stack collecting microscope. A microfluidic device transports cells through the focal plane of a microscope, resulting in an efficient method to collect focus stacks of large collections of single cells. Images from the focus stacks are used to reconstruct the quantitative phase of cells with the transport-of-intensity-equation method. Using the phase imaging flow cytometer, we measure three-dimensional shape variations of red blood and leukemia cells. © 2012 Optical Society of America

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Individual cells are often challenging objects to image because they lack sufficient contrast in bright-field microscopes. Consequently, stains and fluorescent dyes are frequently used to enhance contrast but can often disrupt the health of living cells. An alternative is to directly image the refractive index contrast of the cell with its background to produce a phase image. Phase contrast and differential interference contrast microscopes have proven to be extremely powerful imaging platforms for cytometry. However, these techniques have artifacts and provide only qualitative phase information.

Recently, there have been several reports of quantitative phase imaging using diffraction phase imaging [1], digital holographic microscopy [2], and transport-of-intensity (TIE)-based imaging [3]. The TIE-based technique has unique advantages such as the following: it is a noninterferometric technique, it is computationally simple, it works with partially coherent sources, and it does not require phase unwrapping. It uses a sequence of images captured at different focal depths to calculate the derivative of intensity along the optical axis and relates it to the phase by a second-order partial differential equation [4]. To acquire the focus stack, either the camera or the object is mechanically translated, which limits the applicability of the technique to static objects. Recent reports using volume holography [5] and chromatic aberrations [6] indicate the importance of developing new strategies that do not require mechanical motion to acquire a focus stack.

In this letter, we introduce a phase imaging microscope that acquires focus stacks of individual cells using fluid flow to translate the object through the focal plane. Fluid flow is an ideal method to manipulate nonadherent cells, such as blood cells, and has proven to be extremely valuable in flow cytometry. The schematic of the proposed phase imaging flow cytometer (PIFC) is shown in Fig. 1(a). To record the focus stack, we mounted a microfluidic device at an angle θ with respect to the x axis as shown in the figure. A top view of the schematic is shown in Fig. 1(b). Because of the tilt, fluid flow translates the sample along the z axis as well as the x axis, resulting in a spatially dependent defocus across the channel. As the cell flows through the channel, a high speed camera captures multiple frames, constituting a focus stack. Figures 1(c) and 1(d) show the corresponding focus stack for a red blood cell and a leukemia cell, respectively. For a given frame rate and flow velocity, the change in focus (Δz) between images is governed by the tilt angle (θ) as described by the following equation:

$$\Delta z = \Delta x \tan \theta = \frac{(\Delta p_n)(p_x)}{M} \tan \theta,$$  (1)

where Δx is the projection onto the x axis of the distance traveled by the cell between capturing two images, Δp_n is the shift in number of pixels of the sample position in the images, p_x represents the pixel size of the camera in the x direction, and M is the magnification of the imaging system.

The change in the recorded intensity of the image of the phase object along the optical axis is well described by the transport-of-intensity equation:

$$\nabla_z \cdot \left[ I_0(x, y) \nabla \phi(x, y) \right] = -k \frac{\partial I(x, y, z)}{\partial z} \bigg|_{z=0}.$$  (2)

where k, \( \nabla_z \), \( I_0(x, y) \), \( \phi(x, y) \), and \( \frac{\partial}{\partial z} \) denote wavenumber, gradient operator \( \nabla_z \equiv \partial_x \mathbf{x} + \partial_y \mathbf{y} \) (x, y being basis vectors), in-focus image intensity, phase to be retrieved, and the derivative of intensity along the optical axis evaluated at \( z = 0 \), respectively. Representing \( I_0(x, y) \) as \( \nabla_z \psi(x, y) \), the preceding equation can be rewritten as a standard Poisson equation:

$$\nabla_z^2 \psi(x, y) = -k \frac{\partial I(x, y, z)}{\partial z} \bigg|_{z=0}.$$  (3)

We used a fast Poisson solver and two intensity images (one at focus and one defocused to calculate \( \frac{\partial I(x, y, z)}{\partial z} \bigg|_{z=0} \) using the forward difference approach) for solving the preceding equation to obtain \( \psi(x, y) \). To further estimate \( \phi(x, y) \), we solved the following Poisson equation:

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\[ \nabla^2 \phi(x, y) = \nabla \cdot \left( \frac{\nabla \psi(x, y)}{I_0(x, y)} \right). \]  

Figure 2 shows quantitative phase images of a healthy red blood cell (RBC) and a leukemia cell (HL60) flown through the microfluidic device at a velocity of 40 mm/sec. Figures 2(a) and 2(d) show bright-field images of the cells when they are in focus. The axial derivative of the intensity of the cells is calculated using the in-focus image and a defocused image, with the amount of defocus (\( \Delta z \)) being 2.7 \( \mu \)m. Quantitative phase images of the cells, calculated using Eqs. 3 and 4, are shown as color-mapped images in Figs. 2(b) and 2(e), respectively. Our experimental three-dimensional (3D) shape measurement results match the predicted shape of RBCs in flow [7], which have the upstream end flattened by the pressure gradient and the downstream end bulged. We observed that the minimum thickness of the flap-tail and maximum thickness of the bulged front end regions were 400 nm and 4.7 \( \mu \)m, respectively. These values are obtained by converting the phase image of the cell shown in Fig. 2(b) into height map according to the following equation:

\[ h(x, y) = \frac{\phi(x, y)}{k \Delta n}, \]

where \( \Delta n \) is the difference in refractive indices of RBC and water, taken as 1.418 [8] and 1.336, respectively.

In these experiments, we used a blue LED (central wavelength of 455 nm) as the light source, and the combination of a Nikon 50x micro-objective lens, a tube lens, and a CMOS camera (Mikrotron MC1362) as the imaging system. The microfluidic device is fabricated by soft lithography in polydimethylsiloxane. The dimensions of the channels are 5 \( \mu \)m deep and 20 \( \mu \)m wide, which are marked in Fig. 1(a) as \( t \) and \( h \), respectively.

In a quiescent fluid or on a slide, healthy RBCs have a biconcave discoid shape, whereas in a flow with a parabolic velocity distribution, RBCs have been found to take a parachutelike shape [7] and a slipperlike shape [9]. There has been great interest in studying the exact shape that RBCs take in flow and the possible reasons behind it [10,11]. Moreover, change in RBCs deformability indicates a pathological condition and can be used to diagnose disease (if measurements are made on large numbers of cells), such as diabetes mellitus, spherocytosis, sickle cell anemia, and malaria [12,13]. Imaging flow cytometry is a powerful technology for characterizing cell morphology but has previously been limited to two-dimensional image analysis [14,15]. The PIFC has the potential to add 3D shape characterization to the power of imaging flow cytometry.

Using the PIFC, we measured the quantitative phase of human red blood cells in flow subject to isotonic (0.137 M NaCl concentration) and hypotonic solutions (0.064 M NaCl concentration). We then used the measured phase values of the cells to generate a scatterplot that clusters cells based on the symmetry of their mass distribution. Figure 3 shows a scatterplot of 800 RBCs, whose images are recorded within a duration of 5 s at a rate of 5000
frames per second, with 10 μs exposure time. RBCs in isotonic solutions take parachutelike or slipperlike shapes in flow. In contrast, RBCs in a hypotonic solution become spherized, and we found that even in flow, these cells deformed much less than the healthy isotonic cells and consequently maintained a symmetric distribution of their mass.

To calculate the mass distribution of cells, morphological operations of the Image Processing Tool Box of MATLAB are applied to the estimated phase maps. The cells are segmented from the background and the major-axis length is determined and divided into two equal portions: a front half and a rear half. The sum of the phase values of the front half and rear half, which are denoted as $\Sigma_{\text{front}}$ and $\Sigma_{\text{rear}}$, respectively, of each cell are plotted in Fig. 3(a). It is evident from the figure that the spherized cells have almost equal distribution of mass in the front and rear, whereas normal cells have more mass in the front than in the rear and lie primarily above the 45° line. A few representative phase reconstructions of normal RBCs in the marked gated regions of Fig. 3(a) are shown in Figs. 3(b), 3(c), 3(d), and 3(e), and for that of spherized RBCs in Figs. 3(f), 3(g), 3(h), and 3(i), respectively. The mean and standard deviation of $\Sigma_{\text{front}}$ for normal RBCs are 1.55 and 0.65, and for spherized RBCs are 1.02 and 0.16, respectively. The fact that healthy RBCs in flow take a variety of shapes and orientations is reflected by the larger measured standard deviation. In comparison, RBCs in hypotonic solution lie close to the 45° line and have only a small variation.

To conclude, we introduced a focus-stack collecting microscope that effectively combines advantages of imaging flow cytometry and quantitative phase microscopy. Using the PIFC, we reported an experimental demonstration of label-free high-throughput quantitative phase imaging of human red blood cells. To our knowledge, it is the first time quantitative phase imaging has been performed on live cells in flow. We believe that the PIFC has the potential to be useful as a clinical diagnostic tool, as well as a high-throughput method for characterizing the 3D shape of nonadherent cells.

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References