Invited Article: Digital refocusing in quantitative phase imaging for flowing red blood cells

Han Sang Park, Silvia Ceballos, Will J. Eldridge, and Adam Wax
Department of Biomedical Engineering, Duke University, Durham, North Carolina 27708, USA

Abstract
Quantitative phase imaging (QPI) offers high optical path length sensitivity, probing nanoscale features of live cells, but it is typically limited to imaging just few static cells at a time. To enable utility as a biomedical diagnostic modality, higher throughput is needed. To meet this need, methods for imaging cells in flow using QPI are in development. An important need for this application is to enable accurate quantitative analysis. However, this can be complicated when cells shift focal planes during flow. QPI permits digital refocusing since the complex optical field is measured. Here we analyze QPI images of moving red blood cells with an emphasis on choosing a quantitative criterion for digitally refocusing cell images. Of particular interest is the influence of optical absorption which can skew refocusing algorithms. Examples of refocusing of holographic images of flowing red blood cells using different approaches are presented and analyzed.

INTRODUCTION
Quantitative phase imaging (QPI) has been developed as a means to visualize the structure, dynamics, and function of biological cells without exogenous contrast agents. The approach achieves nanometer depth sensitivity at millisecond time scales, producing phase images of optically transparent cells. Several research groups have used QPI for cellular analysis, establishing feasibility, but to date, there has been no successful implementation of a QPI system suitable for application as a cell based diagnostic.

One criterion that is needed for cell based diagnostic applications is high throughput, which seems to be achievable by imaging flowing cells in a microfluidic device. QPI has been previously applied to imaging in microfluidics, ranging from monitoring microfluidic mixing to sperm selection. One experiment has shown cell imaging through turbid microfluidic channels using digital holography with an emphasis on reducing the effect of turbidity. This approach demonstrated relatively fast imaging at 8 frames per second (fps) but did not show high throughput imaging since the analysis of one cell required 5.5 min of processing time. A more recent device from this group showed a common path interferometer implemented using a diffractive element. The approach allows QPI in a microfluidic chip, suggesting a robust platform that could enable high throughput, but experiments with this platform have not extended to the analysis of biomedically relevant cells. Another compelling approach is the use of transport of intensity equations to
reconstruct the phase profile. A recent study used this approach to image flowing cells. The method is non-interferometric and uses images from multiple focal planes to reconstruct the phase via the Poisson equation. The results showed cell images with limited spatial and phase resolution and required 10 exposures for each cell; thus, throughput can still be regarded as limited.

Another effort used diffraction tomography to reconstruct the phase profile of a flowing cell. In this experiment, 500 angular spectra were captured over 100 ms using off-axis digital holography and line illumination. The data were inverted to produce a phase profile. While specific processing times are not given, the study was limited to a handful of cells (N = 60) and the need for 500 exposures/cell means that even at 5000 fps, only 10 cells/s could be examined. A similar effort from another group exploited the tumbling of cells in a channel to obtain tomographic projections. This approach was able to measure up to 200 exposures for each cell as it tumbled at rates of 150 cells/min with the reconstruction taking up to 20 s/cell.

As a final example, time stretch methods for QPI have been used to obtain images of flowing cells. In this approach, the frequency content of a short optical pulse is spatially dispersed to encode each lateral position at a specific wavelength. The detected pulse is then stretched in time using a nonlinear fiber and amplified. The output is detected by using a fast digitizer to reconstruct the lateral profile. This method does offer high throughput, imaging cells at a flow rate of 1.3 m/s. However, the lateral resolution, achieved by spectral encoding, is limited (2.5 μm) and the complex scheme [multiple sources, amplifiers, and high speed analog-to-digital converter (ADC)] does not lend itself to a high throughput cell based diagnostic.

Analysis of red blood cells (RBCs) has been a natural target for QPI since they are homogenous in their healthy state and a uniform refractive index can be assumed, greatly simplifying analysis. The utility of QPI for RBC studies was shown in a quantitative analysis of malaria infected RBCs. This study presented changes in refractive index and morphology due to infection, focusing on membrane flexibility as a means to discriminate cells. This approach used a tomographic imaging method which requires a large number of measurements for each cell and significant computation time, ultimately limiting application to just a few cells at a time. Studies from our group examined membrane fluctuations in RBCs using time series of QPI images. It was found that healthy RBCs showed greater magnitude fluctuations (50 nm RMS) compared to both round (25 nm RMS) and crescent sickle cells (10 nm RMS), suggesting that QPI is useful for studying this disease. Recent studies have focused on practical aspects of blood cell analysis. The first pursued real time RBC testing and used automated analysis via graphics processing unit to extract morphological descriptors. This work did not show high throughput or analysis of flowing cells but illustrated the power of creating parametric descriptors of RBCs. The second study used QPI to record morphology and fluctuations of RBCs, finding that banked blood exhibited a 20% reduction in temporal membrane fluctuations over a six week period. In an effort to increase throughput, our group has used QPI data for identifying malaria infection, using machine learning to achieve high accuracy and spectroscopic information to detect consumption of hemoglobin.
In our previous work using QPI to analyze microscopic objects, we have shown that accurate refocusing is essential to enable a quantitative comparison. Digital autofocusing is accomplished in QPI by using the Fresnel propagation to correct the complex field. In QPI, selecting the optimal distance for phase reconstruction is typically based on image sharpness which is usually assessed from the amplitude image. For pure phase objects, the optimal focal plane corresponds to the amplitude image with the least contrast. Multiple algorithms have been proven to work well for particular samples. These include minimum variance (MV), minimum gradient (MG), Gini Index (GI), and Tamura Coefficient (TC). The robustness of these algorithms can depend on the sample under examination.

In this paper, we present an analysis of QPI refocusing with an emphasis on the accurate analysis of RBCs in flow. Experimental data are acquired for tumbling RBCs using a typical off-axis QPI scheme and simple, linear microfluidic channels. The need for a robust criterion for determining focal depth is discussed as a necessary criterion for enabling quantitative analysis. The impact of the high absorbance of RBCs at certain wavelengths is examined as a limiting factor in digital refocusing, with the performance of several refocusing algorithms analyzed and compared. Finally, the quantitative analysis of flowing RBCs is presented.

**METHODS AND MATERIALS**

**Quantitative phase imaging**

Figure 1 shows the quantitative phase imaging system that was used to image red blood cells in flow. The digital holography system is equipped with a supercontinuum laser source (Fianium SC-400–4) that is spectrally filtered to select a 1.12 nm full-width at half-maximum spectral bandwidth from the broadband light with a center wavelength that is tuned to 680 nm.

Using a customized, tunable spectral filter, a wavelength band is selected and the filtered light is coupled into a single-mode fiber for transmission to the interferometer. The fiber output is collimated by using an objective lens and passed through a linear polarizer before entering the Mach-Zehnder interferometer. In the interferometer, the illumination light is separated into the sample (S) and reference (R) arms using a beam splitter (BS) which are then path-matched within the coherence length of the filtered light with mirror-based retroreflectors (RRs) on translation stages. An off-axis interferogram is generated by mixing the light which has interacted with the sample with the reference field which is offset in the propagation angle. Matched microscope objectives (Zeiss Plan-NeoFLUAR 40 × 0.75 NA) are used in each arm, creating an image of the sample with an effective magnification of ~107×. The CMOS camera (Photron FastCam SA-4, 1024 × 1024 px, 10-bit data capture) detects the off-axis interferogram.

As described in previous studies, the interferograms are digitally processed to produce quantitative phase images, $\Delta \phi(x, y)$, using the following steps: the acquired interferograms are Fourier transformed and then spatially filtered around the carrier frequency in the Fourier domain to isolate one of the complex conjugates. The filtered two-dimensional spatial frequency information is demodulated and then inverse Fourier transformed to produce a complex image of the sample wavefront, containing both amplitude and phase information.
Finally, the phase is isolated by taking the angle of the complex spatial information. Any low-order background phase noise caused by temporal drift between the two interferometer arms is removed by fitting the background to 5th-order polynomials. A background hologram of the chamber is also captured separately and subtracted from the corresponding sample images.

Changes in the optical path length (OPL) due to RBCs in the sample arm are calculated as

\[ \Delta OPL(x, y, t) = \Delta n(x, y, t) \cdot h(x, y, t) = \Delta \phi(x, y, t) \cdot \frac{\lambda}{2\pi}, \]  

(1)

where \( h(x, y, t) \) is the height map of the object and \( \Delta n(x, y, t) \) is the refractive index map. The red blood cells are automatically segmented from each field of view (FOV) by applying both optical path length and area thresholds. Within each FOV, all objects with \( \Delta OPL > 10 \) nm are identified as potential RBCs. Upper and lower area thresholds are used to exclude objects that are significantly bigger or smaller than known RBC size.\(^{27}\)

**Blood sample preparation**

A whole blood sample was collected from a healthy, non-pregnant donor with informed written consent following the protocols approved by the Duke University Institutional Review Board (IRB). The red blood cells were isolated from the plasma using the following purification methods: 10 μl of whole blood was diluted in 1 ml of Dulbecco’s phosphate-buffered saline containing calcium chloride and magnesium chloride (DPBS+/+, Gibco). Then, the blood suspension was centrifuged at 6000 rpm for 5 min at room temperature. After centrifugation, the RBC pellet at the bottom was isolated by removing the supernatant. This process was repeated 2 times to ensure complete isolation of RBCs. The isolated RBC pellets were diluted with 3 mL of a medium composed of 2.51 g of bovine serum albumin in lyophilized powder form (Sigma-Aldrich) diluted in 10.12 g of DPBS+/+. Using a refractometer (Bellingham + Stanley), the refractive index of the medium was measured to be 1.372 at 23 °C. The diluted solution was loaded on a syringe pump system (1 mL BD syringe and New Era syringe pump) operating at 1.5 μl/min for the experiment.

**Development of flow chambers**

In order to image flowing RBCs, chambers were fabricated using SU-8 photoresists. Coverslips (VWR #1.5, 22 × 40 mm) were spin coated with SU8 (Microchem) in a headway spin coater. Then, the substrates were exposed to a pattern mask using a UV lamp on a Karl Suss MA6 mask aligner. After the development process, the height of the photoresists was measured to be 8.60 μm using a profilometer with 5 × 5 mm of open space. Then, the developed chamber was bonded to a glass slide with both the inlet and outlet using a customized manual press leveraging bolted joint design\(^{28}\) to apply controlled pressure using a torque wrench.

**Digital refocusing and optical volume**

In our study, sequential images of RBCs moving through the microfluidic channels are refocused at each frame using several algorithms from other works, including minimum
variance (MV),\textsuperscript{22} minimum gradient (MG),\textsuperscript{22} Gini Index (GI), and Tamura Coefficient (TC)\textsuperscript{23,24}. Initially, all RBCs in flow are digitally refocused to an average distance determined using MV of amplitude for the first 5 frames to place them closer to the plane of focus rather than simply relying on the rough estimate of manual focusing.

Equation (2) gives the variance calculation that determines optimal refocusing distance using MV by considering the minimum deviation of the gray level amplitude distribution $g(x, y)$ from the mean gray value ($\bar{g}$) for an image with dimensions $N_x \times N_y$:

$$\text{Variance} = \frac{1}{N_xN_y} \sum_{x,y} [g(x,y) - \bar{g}]^2. \hspace{1cm} (2)$$

MG employs variations in neighboring pixel values within the amplitude image to determine the refocus distance. Mathematically this translates to calculating the summation of the first derivative of the amplitude image as shown in Eq. (3),\textsuperscript{22}

$$\text{Gradient} = \sum_{x=1}^{N_x-1} \sum_{y=1}^{N_y-1} \sqrt{[g(x,y) - g(x-1,y)]^2 + [g(x,y) - g(x,y-1)]^2}. \hspace{1cm} (3)$$

Tamura coefficient and Gini index algorithms are based on edge sparsity using the gradient of the complex optical wavefront\textsuperscript{23,24} as shown in Eqs. (4) and (5), respectively. These two functions are expected to reach a maximum at the correct focus distance,

$$\text{Tamura Coefficient}(\mathbf{c}) = \sigma(\mathbf{c})/\mathbf{c}, \hspace{1cm} (4)$$

where $\mathbf{c}$ is the data vector (gradient of complex field) of size $N$ and $\sigma$ is the standard deviation.

$$\text{Gini Index}(\mathbf{c}) = \frac{2}{N} \sum_{k=1}^{N} \frac{k}{N} \hat{a}[k] - 1 - \frac{1}{N}, \hspace{1cm} (5)$$

where $a[k]$ ($k = 1, ..., N$) are the sorted entries of $\mathbf{c}$ in ascending order and $\hat{a}[k] = \frac{a[k]}{\langle \mathbf{c} \rangle}$ are the sorted entries normalized by the mean $\langle \mathbf{c} \rangle$.

As introduced in Ref. 20, we will use optical volume (OV) as a benchmark for quantitative comparisons of the different algorithms and the effects of defocus. Optical volume is an invariant metric that is preserved as long as there is no exchange between the cell and the medium throughout the flow. The OV metric is preferred for this analysis because unlike dry mass,\textsuperscript{29} it does not depend on the refraction increment ($\alpha$), which can be seen to vary in the literature. Furthermore, although not an issue for homogenous RBCs, the refraction increment can be difficult to calculate for other cell types which are more heterogeneous. For comparison, OV measurements can be easily converted to dry mass as needed, but both
metrics are expected to remain constant as a cell changes orientation. Thus, the OV metric can be used to evaluate whether each refocusing algorithm has retained the true information about each RBC. The $\Delta OPL$ [Eq. (1)] distribution is integrated to obtain OV as

$$OV(t) = \iint_{x,y} \Delta OPL(x, y, t) dxdy = \iint_{x,y} \Delta n(x, y, t) \cdot h(x, y, t) dxdy. \quad (6)$$

**RESULTS**

During the flow through the chamber, an RBC can tumble and change its physical orientation (videos 1 and 2 of the supplementary material). In Fig. 2(a), examples of different orientations of the cells are shown including those parallel ($t = 0$ s and 6.95 s, horizontal) and perpendicular ($t = 1.99$ s, vertical) to the surface of the chamber. The optical volumes obtained from the images of these cells digitally propagated by different distances from $-15 \, \mu m$ to $5 \, \mu m$ are shown in Fig. 2(b).

The rate of optical volume change over the propagation distance is not consistent throughout the different orientations. The OV of a vertically oriented cell (cell at $t = 1.99$ s) changes faster than that for horizontal cells (cells at $t = 0$ or 6.95 s), with respect to the refocusing distance. Therefore, the range of OVs of RBCs at different orientations varies over the propagation distances. Also, the range of OVs for this particular tumbling cell is at a minimum for the propagation distance $z = -3.28\, \mu m$. We again note that it is expected that the physical volume of the cell is stable throughout the flow regardless of the orientation of the RBC.

Figure 3(a) shows the focusing distances calculated using different algorithms (MV, MG, GI, and TC) to optimize the focus for the RBC from Fig. 2(a) as it flows through the micro-channel. In order to assess the metrics used to calculate the best plane of focus, the corresponding OV of the RBC propagated by the calculated focusing distances is also shown in Fig. 3(b). As stated earlier, OV is an invariant metric in the flow experiment that should not change without an exchange between the content of the cell and the medium. In addition, the propagation distance at which the range of OVs of different orientations of the cell is minimum ($-3.28 \, \mu m$) as well as the corresponding OV propagated by this distance throughout the flow is shown in Fig. 3.

As can be seen in Fig. 3(b), the OV values do not change much, while the orientation of the cell is steady, such as between $t = 2.65$ s and 6.95 s. The average OV changes were 2.22%, 2.06%, 2.33%, 2.47%, and 2.37% using the MV, MG, GI, TC, and minimum range of OV, respectively, demonstrating a fairly consistent distribution. By contrast, the OV calculated using these different metrics changes significantly as the cell changes its orientation to vertical during the flow. The changes in optical volume from horizontal orientation ($t = 0$ s) to vertical orientation ($t = 1.99$ s) when the optical field is propagated at different distances using the MV, MG, GI, and TC are 11.75%, 7.24%, 5.31%, and 5.31%, respectively, a much

**SUPPLEMENTARY MATERIAL**

See supplementary material for videos 1 and 2.
broader range. However, the OV change between the cells for these two orientations when the focusing distance was determined using the minimum range of OV is 2.05%, which is consistent with the variations seen for the cell with steady orientation.

In order to assess the different metrics, 50 tumbling RBCs were imaged at 500 fps using QPI. For each cell, 30 frames that are evenly distributed in time were segmented and analyzed. The changes in OV calculated using refocusing algorithms are greatly different between the horizontally and vertically oriented RBCs. Two frames are selected to represent these two different orientations throughout the flow, determined using maximum and minimum area, respectively (Fig. 4, inset). Because the 2D apparent area of the cell decreases as it tumbles from horizontal to vertical orientation, cell frames with horizontal and vertical orientation can be identified using maximum and minimum area, respectively. Figure 4 shows the optical volume change between the two orientations for the five different refocusing metrics described above.

The average optical volume changes between the cells at horizontal and vertical orientation propagated using minimum range of OV, MV, MG, GI, and TC are 1.39 ± 1.09%, 15.21 ± 11.42%, 9.31 ± 7.61%, 7.48 ± 7.25%, and 9.94 ± 6.02%, respectively.

DISCUSSION

In our experiment, refocusing algorithms are assessed for flowing RBCs using optical volume. As the RBCs flow through the micro-chamber, they can change their orientation and tumble, as shown in Fig. 2(a). However, OV is an invariant parameter that only depends on the refractive index and physical height of the cell and therefore should remain constant during rigid-body tumbling and even flow-induced deformation. As demonstrated in our previous paper,20 OV of a cell can also be influenced by digital propagation of the optical field when refocusing the image, as shown in Fig. 2(b). The relationship between OV change and the digital field propagation distance depends on the orientation of the cell. As demonstrated in Fig. 2(b), the OV of a vertically oriented cell (t = 1.99 s) will change more rapidly over a given propagation distance relative to that of a horizontally oriented cell (t = 0 s or 6.95 s). Consequently, defocus due to inaccurate estimation of the focal plane can significantly influence the OV measurements of these vertically oriented cells. This in turn will limit the validity of quantitative measurements of the cells during flow experiments using QPI.

A key finding of this work is that there is a propagation distance at which the OV of the RBCs is seen to converge for the different orientations, which forms the basis of the minimum range OV refocusing distance. Consider a normal healthy RBC with dimensions of 7.5–8.7 μm in diameter and 1.7–2.2 μm in thickness27 that is flowing parallel to the surfaces of the glass slides. It is reasonable to assume that its focal plane coincides with the middle of the chamber channel height where there is minimum friction from the top and bottom surfaces. It is also reasonable to assume that when the cell orients perpendicular to the glass surfaces and assumes a vertical position in the micro-chamber (height of 8.60 μm), the mid-height of the RBC is consistent with the plane of focus while the cell is in horizontal orientation, at the middle of the channel (Fig. 5).
Therefore, when the RBC is tumbling and changing its orientation during flow, the plane of focus can be assumed to change slowly or even remain constant at or near the middle of the chamber. While other refocusing algorithms can sometimes produce refocusing distances of 5 or 10 μm, an outcome that disagrees with the physical dimension of the channel, using the minimum change in OV over propagation, coincides with a plane of focus at or near the middle of the chamber.

In order to assess the focusing distance using minimum range of OV, the change in OV is calculated using different algorithms, producing analyses like that shown in Fig. 3. For some orientations, poor agreement in computed focusing distances is seen between the four algorithms. However, the discrepancy in OV while the cell is flowing relatively flat to the surfaces, for example, as shown from t = 2.65 s to 6.95 s in Fig. 2, is minimal. As can be seen in Fig. 2(b), when the RBCs are horizontally oriented, the change in optical volume with respect to the focusing distance is not as rapid as those of the vertically oriented RBCs. Therefore, the difference in performance of these algorithms is minimal when they are used for RBCs with stationary, quasi-planar orientation.

By contrast, the change in OV between cells in horizontal to vertical orientation is seen to be much larger when refocusing using the various algorithms, suggesting that the propagation distance used in the reconstruction of the phase images is not accurate. Overall, the differences in OV seen between the two orientations are above 5% and as large as 12% when using the various algorithms. This shows that despite of all the advances that have been made using digital refocusing to enable a quantitative comparison of QPI data, a more dynamic solution is required to image flowing cells.

In QPI of RBCs, the error in the calculation of the plane of focus using these algorithms is most likely due to absorption of light which influences the amplitude distribution. The algorithms assume that the sample is a pure phase object without absorption, whereas RBCs have strong natural absorbers, hemoglobin molecules. As can be seen in Fig. 6, the absorption maps of the cells in different orientations can be heterogeneous depending on the orientation of the cells and concentration of hemoglobin within. Therefore, when the algorithms try to find a plane of focus where the amplitude image has minimal variation, they are optimizing on an inaccurate criterion as can be evidenced by the large OV changes seen with improper focusing in Fig. 3.

Figure 7 shows the amplitude maps for a given RBC at the refocusing distance determined by the various algorithms. Using minimum range of OV as a criterion produces an amplitude map which is not as homogeneous as those determined by the other algorithms. However, the amplitude distribution of the RBC in focus should have some heterogeneity that describes the distribution of hemoglobin across the natural discoid shape of the RBC. Thus, when an algorithm seeks to find a plane of focus where the amplitude image has minimal variation, it is optimizing on an inaccurate criterion as can be evidenced by the large OV changes seen with improper focusing in Fig. 3.

Based on our experimental and computational investigation, calculation of the physically meaningful OV seems to be a good criterion to determine when digital refocusing algorithms
break down. By using the focusing distance calculated with the minimum range of OV throughout the flow, the OV metric is stable during the tumbling process regardless of the orientation, as shown in Fig. 3(b). When all the methods are compared using 50 different RBCs flowing through the chamber, the average OV change from horizontal to vertical orientation is much lower using the minimum range of OV compared to other methods (Fig. 4).

However, using minimum range of OV to determine the plane of focus is not a dynamic solution to the current problem. To accurately use this method, the QPI system would need to image the sample at different orientations to find the minimum range of OV across orientations. Yet dynamic imaging of cells in flow may require analysis from just few imaging frames of a cell in a single orientation. One solution is to note that the height of the chamber is consistent, regardless of cell orientation. It may be possible to constrain the channel height to prevent orientation changes so that the plane of focus remains consistent although it is not clear if such a constrained channel geometry can enable high throughput. In addition, it is difficult to observe induced OV changes due to material exchange with the environment due to external factors such as osmotic pressure and mechanical stress. In order to develop QPI into a clinically relevant cell diagnostic method by integrating it with a microfluidic device for high throughput, refocusing problems for absorbing cells at all observed orientations must be addressed.

CONCLUSION

In this paper, RBCs flowing through a micro-fluidic channel are imaged using a QPI system. Problems with applying refocusing algorithms to absorbing samples are identified. An alternative focusing criterion based on using minimum range of OV is proposed and shown to calculate accurate optical volume measurements of an RBC regardless of its orientation. The proposed solution is limited to multiple constraints such as the dimension of the micro-chamber and the need to obtain multiple images at different orientations; however, this newly found information may be particularly useful in developing clinically viable QPI-based cytometers with increased throughput by employing micro-fluidic channels.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

Grant support from NSF (No. CBET 1604562) and NIH (No. 1R21-ES029791) and financial funding from the Duke University are gratefully acknowledged. We also thank the members of the BIOS lab for helpful discussions.

References

1. Shaked NT, Rinehart MT, and Wax A, “Dual-interference-channel quantitative-phase microscopy of live cell dynamics,” Opt. Lett. 34(6), 767–769 (2009). [PubMed: 19282926]
2. Park G et al., “Visualization and label-free quantification of microfluidic mixing using quantitative phase imaging,” Appl. Opt. 56(22), 6341–6347 (2017). [PubMed: 29047833]
3. Eravuchira PJ, Mirsky SK, Barnea I, Levi M, Balberg M, and Shaked NT, “Individual sperm selection by microfluidics integrated with interferometric phase microscopy,” Methods 136, 152–159 (2018). [PubMed: 28958952]

4. Bianco V, Paturzo M, Finizio A, Calabuig A, Javidi B, and Ferraro P, “Clear microfluidics imaging through flowing blood by digital holography,” IEEE J. Sel. Top. Quantum Electron. 20(3), 89–95 (2014).

5. Paturzo M et al., “Microscopy imaging and quantitative phase contrast mapping in turbid microfluidic channels by digital holography,” Lab Chip 12(17), 3073–3076 (2012). [PubMed: 23740323]

6. Bianco V et al., “Endowing a plain fluidic chip with micro-optics: A holographic microscope slide,” Light: Sci. Appl. 6(9), e17055 (2017).

7. Gorghi SS and Schonbrun E, “Phase imaging flow cytometry using a focus-stack collecting microscope,” Opt. Lett. 37(4), 707–709 (2012). [PubMed: 22344155]

8. Sung Y et al., “Three-dimensional holographic refractive-index measurement of continuously flowing cells in a microfluidic channel,” Phys. Rev. Appl. 1(1), 014002 (2014).

9. Merola F et al., “Tomographic flow cytometry by digital holography,” Light: Sci. Appl. 6(4), e16241 (2017).

10. Mahjoubfar A, Chen C, Niazi KR, Rabizadeh S, and Jalali B, “Label-free high-throughput cell screening in flow,” Biomed. Opt. Express 4(9), 1618–1625 (2013). [PubMed: 24094682]

11. Lau AK, Shum HC, Wong KK, and Tsia KK, “Optofluidic time-stretch imaging—an emerging tool for high-throughput imaging flow cytometry,” Lab Chip 16(10), 1743–1756 (2016). [PubMed: 27099993]

12. Chen CL et al., “Deep learning in label-free cell classification,” Sci. Rep. 6, 21471 (2016). [PubMed: 26975219]

13. Park Y et al., “Refractive index maps and membrane dynamics of human red blood cells parasitized by plasmodium falciparum,” Proc. Natl. Acad. Sci. U. S. A. 105(37), 13730–13735 (2008). [PubMed: 18772382]

14. Choi W et al., “Tomographic phase microscopy,” Nat. Methods 4, 717–719 (2007). [PubMed: 17694065]

15. Shaked NT, Satterwhite LL, Telen MJ, Truskey GA, and Wax A, “Quantitative microscopy and nanoscopy of sickle red blood cells performed by wide field digital interferometry,” J. Biomed. Opt. 16(3), 030506–1–030506–3 (2011). [PubMed: 21456860]

16. Pham H, Bhaduri B, Tangela K, Best-Popescu C, and Popescu G, “Real time blood testing using quantitative phase imaging,” PLoS One 8(2), e55676 (2013).

17. Bhaduri B, Kandel M, Brugnara C, Tangella K, and Popescu G, “Optical assay of erythrocyte function in banked blood,” Sci. Rep. 4, 6211 (2014). [PubMed: 25189281]

18. Park HS, Rinehart MT, Walzer KA, Chi J-TA, and Wax A, “Automated detection of P. falciparum using machine learning algorithms with quantitative phase images of unstained cells,” PLoS One 11(9), e0163045 (2016).

19. Rinehart MT, Park HS, Walzer KA, Chi J-TA, and Wax A, “Hemoglobin consumption by P. falciparum in individual erythrocytes imaged via quantitative phase spectroscopy,” Sci. Rep. 6, 24461 (2016). [PubMed: 27087557]

20. Rinehart MT, Park HS, and Wax A, “Influence of defocus on quantitative analysis of microscopic objects and individual cells with digital holography,” Biomed. Opt. Express 6(6), 2067–2075 (2015). [PubMed: 26114029]

21. Liebling M and Unser M, “Autofocus for digital Fresnel holograms by use of a Fresnelet-sparsity criterion,” J. Opt. Soc. Am. A 21(12), 2424–2430 (2004).

22. Langehanenberg P, Kemper B, Dirksen D, and von Bally G, “Autofocusing in digital holographic phase contrast microscopy on pure phase objects for live cell imaging,” Appl. Opt. 47(19), D176–D182 (2008). [PubMed: 18594573]

23. Tamamitsu M, Zhang Y, Wang H, Wu Y, and Ozcan A, “Comparison of Gini index and Tamura coefficient for holographic autofocusing based on the edge sparsity of the complex optical wavefront,” 1–9 (2017); e-print arXiv: 1708.08055.
24. Zhang Y, Wang H, Wu Y, Tamamitsu M, and Ozcan A, “Edge sparsity criterion for robust holographic autofocusing,” Opt. Lett. 42(19), 3824–3827 (2017). [PubMed: 28957139]
25. Rinehart M, Zhu Y, and Wax A, “Quantitative phase spectroscopy,” Biomed. Opt. Express 3(5), 958–965 (2012). [PubMed: 22567588]
26. Liebling M, Blu T, and Unser M, “Complex-wave retrieval from a single off-axis hologram,” J. Opt. Soc. Am. A 21(3), 367–377 (2004).
27. Diez-Silva M, Dao M, Han J, Lim CT, and Suresh S, “Shape and biomechanical characteristics of human red blood cells in health and disease,” MRS Bull. 35(5), 382–388 (2010). [PubMed: 21151848]
28. Serra S, Schneider A, Malecki K, Huq S, and Brenner W, “A simple bonding process of SU-8 to glass to seal a microfluidic device,” in Proceedings of 3rd International Conference on Multi-Material Micro Manufacture (Borovets, Bulgaria, 2007), pp. 3–5.
29. Popescu G et al., “Optical imaging of cell mass and growth dynamics,” Am. J. Physiol.-Cell Physiol. 295(2), C538–C544 (2008). [PubMed: 18562484]
30. Shaked NT, Finan JD, Guilak F, and Wax A, “Quantitative phase microscopy of articular chondrocyte dynamics by wide-field digital interferometry,” J. Biomed. Opt. 15(1), 010505 (1-2 2010).
31. Guo Q, Park S, and Ma H, “Microfluidic micropipette aspiration for measuring the deformability of single cells,” Lab Chip 12(15), 2687–2695 (2012). [PubMed: 22622288]
FIG. 1. QPI system diagram.
Quantitative phase imaging system: Path-matched sample (S) and reference (R) beams create off-axis interferograms imaged by using a CMOS camera, beam splitter (BS), retroreflectors (RRs). Inset: Amplitude and phase images of red blood cells flowing through the chamber.
FIG. 2. Influence of defocus.
(a) Images of tumbling RBCs at different orientations. Scale bar = 5 μm. (b) Optical volume of RBCs in (a) calculated at different propagation distances.
FIG. 3. OV of the tumbling cell refocused using different parameters. 
(a) Focusing distance for a tumbling cell calculated using different parameters: MV, MG, GI, 
and TC. (b) Optical volume of the tumbling RBC propagated by focusing distances 
calculated using different metrics shown in (a). C1–C5 correspond to the five time points 
shown in Fig. 2(a) throughout the flow.
FIG. 4. Optical volume change using different metrics.
Optical volume changes between horizontal and vertical RBCs during flow using focusing distances determined by MV, MG, GI, TC, and minimum range of OV (N = 50). Inset: Example images of horizontal and vertical RBCs, respectively. Scale bar = 5 μm.
FIG. 5. Different RBC orientations.
Different RBC positions and orientations as it tumbles through the chamber.
FIG. 6. Phase and amplitude images of horizontal and vertical RBCs.
(a) Phase map of a horizontal RBC, (b) phase map of a vertical RBC, (c) amplitude map of a horizontal RBC, (d) amplitude map of a vertical RBC. Scale bar = 5 μm.
FIG. 7. Phase and amplitude images of RBC.
Phase maps of a RBC at the refocus distance calculated using minimum range of OV, MV, MG, GI, and TC at the top and corresponding amplitude maps at the bottom. Scale bar = 5 μm.