Diffraction Phase Cytometry:
blood on a CD-ROM

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Abstract: We demonstrate Diffraction Phase Cytometry (DPC) as a single shot, full-field, high throughput quantitative phase imaging modality, dedicated to analyzing whole blood smears. Utilizing a commercial CD as a sample substrate, along with dynamic spatial filtering via a liquid crystal spatial light modulator, we have developed a compact instrument capable of making quantitative, physiologically relevant measurements. To illustrate the ability of the system to function as a highly sensitive cytometer we imaged a large number (N=1,537) of live human erythrocytes in whole blood without preparation. We retrieved a comprehensive set of geometrical parameters including cell volume and surface area, which are not directly available using existing cytometers. Furthermore, we retrieved the minimum cylindrical diameter, through which red blood cells can pass, and deliver oxygen. These initial results prove the concept for an inexpensive lab-on-a-chip blood screening device.

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1. Introduction

The human red blood cell (RBC) membrane, a fluid lipid bilayer tethered to an elastic 2D spectrin network, provides the principal control of the cell's morphology and mechanics [1, 2]. In turn, these properties influence the ability of RBCs to transport oxygen in circulation. Four decades ago, Canham and Burton [3] painstakingly measured the geometry of erythrocytes by photographing the cells hanging on their edges from a slide and then tracing the membrane profile to calculate volume, surface area and diameters. Even though other methods such as micropipette aspiration [4] and ektacytometry [5] have been developed since then to calculate erythrocyte geometry, none provide a direct optical measurement of geometrical parameters such as surface area. Other microscopy methods that offer optical measurements, such as confocal microscopy, rely on exogenous contrast agents or fixation processes [6].

The use of the optical phase shift through a sample as an endogenous contrast agent has served as a powerful technique in microscopy since the advent of techniques such as phase contrast (PC) and differential interference contrast (DIC) microscopy [6]. Even though these modalities greatly enhance the ability to observe details within transparent objects such as living cells, it was not until the development of Quantitative Phase Imaging (QPI) techniques that the phase shift through a sample was able to provide quantitative information [7]. Furthermore, with the development of common path interferometry and methods such as diffraction phase microscopy [8], we can obtain quantitative phase images that are sensitive to sub-nanometer changes in optical path lengths over broad temporal scales.
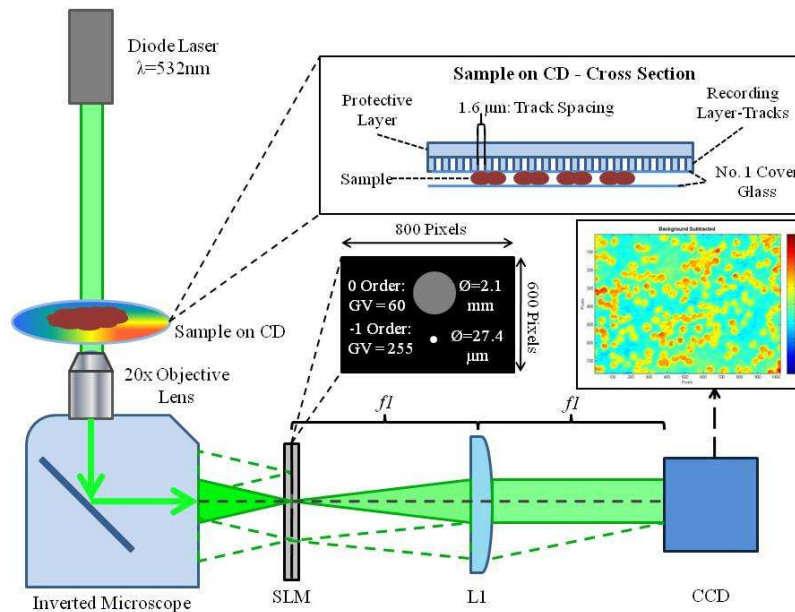

Fig. 1. DPC setup. SLM, Spatial Light Modulator; L1, lens; focal length, *f1*=300 mm. GV, Grey Value of pixels; Ø, Diameter of pinholes.

In this paper, we present diffraction phase cytometry (DPC) as a single shot, full-field, high throughput QPI technique that is capable of taking direct measurements of cell morphology by measuring the optical path length difference between the cell and its

surrounding medium. We demonstrate the principle of implementing blood cytometry in a "lab-on-a-chip" instrument which will be inexpensive and highly portable. With further refinements, such an instrument will make QPI technology available to any clinic with a modern computer and allow for a detailed analysis that is currently not possible without access to expensive instruments such as flow cytometers.

2. DPC Experimental Setup

The DPC setup is illustrated in Fig. 1. A frequency-doubled Nd:YAG laser (λ=532 nm) was used as the illumination source for an inverted microscope (MEIJI Techno TC5200) equipped with a 20X, 0.4 NA objective. With the tube lens of the microscope removed, the Fourier transform of the microscope image is projected on the SLM (Spatial Light Modulator) plane which is used as a spatial filter as shown in Fig. 1. The SLM used is a liquid crystal display (LCD) sandwiched between a polarizer and an analyzer and was extracted from a commercial digital LCD projector (EPSON). The SLM is controlled via the green channel of a standard RGB monitor signal [9]. Each pixel on the SLM is 13.7 μm^2 with a total size of 800 x 600 pixels, and has a dynamic range of 256 grey values. The lens L1 acts as the inverse transformer and projects the magnified microscope image plane onto its front focal plane, where it is recorded by the CCD. The CCD is operated in 2x2 binning mode and the image resolution is 1024 x 768 pixels where each pixel is ~ 5x5 μm^2; the field of view of the image is 180 x 135 μm^2, and the total magnification of the system is ~ 30X.

A commercial blank compact disk with its label peeled off is placed at the object plane of the microscope. The tracks on the CD serve as a 1.6 μm pitch transmission diffraction grating (see Fig. 1 inset), which generates several diffraction orders at the SLM plane; each containing full spatial information about the sample placed on the CD [8]. The SLM is then used to spatially low pass filter the 1st order beam by passing it through a pinhole mask with a diameter of $\emptyset$=27.4 μm so that it is proportional to the spatial average of the image field and is thus appropriate to use as the reference field in a common-path Mach-Zender interferometer [8]. The entire frequency content of the 0th order beam is allowed to pass and thus serves as the object field; all the other orders are blocked entirely. Since the 0th order beam has a significantly higher intensity than the 1st order, we adjusted the SLM voltage such that it selectively attenuates and levels the intensities of the two beams, for maximum fringe contrast. The ability to adjust the contrast between the two interfering fields represents a significant improvement with respect to using a conventional pinhole. This setup provides spatial sampling of the image at 16 CCD Pixels/Period. Quantitative phase information is then retrieved from the interferograms as described in Ref. [8].

In order to correct for aberrations and background noise, we estimated and subtracted the background [10], a constant characteristic of the instrument, using a least squares regression fit to a polynomial of the form: $B(x,y)=b_0 x^2 + b_1 y^2 + b_2 xy + b_3 x + b_4 y + b_5$ (via a MATLAB routine developed in-house). The subtraction is used to remove the low-frequency non-uniformities in the image (due to residual aberrations, etc). This background does not vary significantly over the size of the cell and, thus, does not affect the volume measurement. However, having a flat background provides a common reference for estimating the cell volumes automatically for a large number of cells.

3. DPC of live erythrocytes

To demonstrate the ability of the DPC system to perform detailed cytometry type measurements we imaged mature erythrocytes in peripheral blood smear samples from a healthy adult male and retrieved medically relevant geometric parameters such as volume (V), surface area (SA), sphericity index, and minimum cylindrical diameter (MCD) as described by Canham and Burton [3]. Erythrocytes or red blood cells (RBC's) are the dominant cell type in whole blood and are thus a good candidate for testing our DPC system. RBCs are relatively

simple in structure, with a homogenous interior and are also an excellent indicator of the physiological condition of a patient [10]. The blood smears were prepared by simply sandwiching a droplet of whole blood, obtained by finger prick, between two No. 1 cover slips. The sample is then placed between the CD and the objective as shown in Fig. 1. Imaging is done immediately after preparation of the sample to minimize effects from drying. From the reconstructed quantitative phase maps we can simply relate the measured phase shift $\Delta\varphi(x,y)$ to sample height by:

$$h(x, y) = 2\pi\lambda\Delta\phi(x, y) / \Delta n , \qquad (1)$$

where $\Delta n = n_{plasma} - n_{RBC}$ is the difference in the indices of refraction of the erythrocytes and the plasma, $n_{plasma} = 1.3515$ and $n_{RBC} = 1.3871$ [11, 12]. For comparison purposes we also measured 35, 4.5μm diameter polystyrene microspheres (Polybead) suspended in immersion oil (Zeiss). The standard deviation for the microspheres is reported by the manufacturer as $\sigma = 0.236$ μm and the reported index of refractions are $n_{microspheres} = 1.59$ and $n_{oil} = 1.518$ for the microspheres and oil respectively. The measured height distributions for both samples are shown in Fig. 2 below. From the microsphere measurements (4.44 ±0.243 μm) shown below it is clear that the DPC system correctly retrieves the height of a sample if the refractive indices are known.
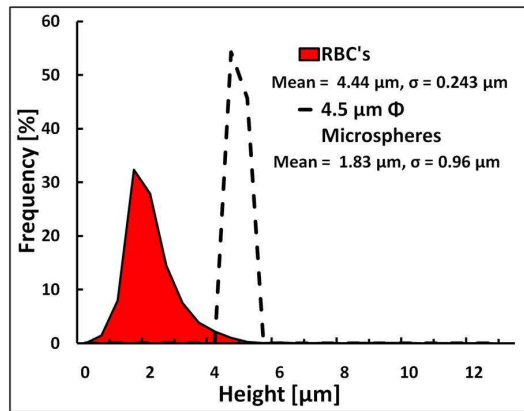

Fig. 2. Unbiased height frequency distribution for RBCs and a control sample of 4.5 μm polystyrene microspheres.

To aid the analysis, image processing software was developed in MATLAB to automatically detect and analyze the erythrocytes. In order to retrieve relevant information from the phase maps the program implements a typical particle detection scheme utilizing a watershed algorithm, a distance transform and a H-minima transform [13] to obtain a list of coordinates occupied by individual erythrocytes. Considering the broad nature of the height distribution, to differentiate between individual cells, cell aggregates, debris and other cell types present in the samples only regions with mean heights between 1.25 and 1.8 μm were considered. These values were chosen based on the observed mean values of erythrocytes with the refractive index values reported in the literature. For future studies with whole blood samples, this restriction can be refined to also include populations of leukocytes and platelets. The methods used to calculate the three dimensional parameters such as volumes, surface area, sphericity and the minimum cylindrical diameter are described below, whereas parameters such as diameter, projected area, perimeter and eccentricity were retrieved directly using region property descriptors available in MATLAB (Fig. 3).

Volume: The volumes of individual erythrocytes were calculated by integrating the height map over the area of the cell. The histogram of the measured volumes is shown in Fig. 3(a). The mean cell volume reported here (69 +/-23 fL) is smaller than the average values in

literature (80-90 fL) [15], which might be due to small refractive index deviation from the assumed value [11]. The shape of the volume distribution and its relative modifications with respect to normal conditions have been widely used for clinical diagnosis and shown to be a reliable indicator of various physiological conditions ranging from anemia and vitamin B12 deficiency to nicotine and alcohol addiction [10].

Surface Area: The surface areas of individual erythrocytes were measured by calculating a three dimensional triangular mesh that conforms to the surface of the erythrocytes and then calculating the area of this mesh. An example of this mesh is shown in the inset to Fig. 3(b). Since our measurement is only sensitive to changes in optical path length, the area of the cell membrane was approximated as $SA = 2 * SAC + P * h_{min}$, with *SAC* the mesh surface area, *P* is the perimeter of the cell and h_{min} is the minimum optical path difference in the region occupied by the cell. The resulting distribution is shown in Fig. 3(b). To our knowledge, this is the first measurement of erythrocyte surface areas using a quantitative phase technique.

Sphericity: The sphericity of an erythrocyte is a dimensionless parameter first described by Canham and Burton [3] and is a measure of how spherical a cell is, with values between 0 and 1, for a sphere and laminar disk, respectively. It indicates the ratio of the surface area of a sphere with the same volume as the cell to the actual surface area of the cell,

$$Sphericity = 4.84 \frac{V^{2/3}}{SA} \qquad (2)$$

Since erythrocytes are required to be flexible enough to squeeze through small capillaries and it is known that a small increase in the area of a membrane results in hemolysis [14], the "non-sphericity" of red blood cells is essential to their ability to deform. The mean sphericity index calculated for all 1,537 cells is 0.72, and the histogram of sphericities is shown in Fig.2c.

Minimum Cylindrical Diameter (MCD): The MCD is a theoretical geometric parameter that indicates the thinnest cylindrical channel that a given erythrocyte could squeeze through. The MCD for an erythrocyte population is based on the idea that the most efficient shape for a cell to pass through a narrow capillary is similar to that of a hotdog [3]. The MCD is related to the volume and surface area as:

$$V = SA * MCD - \frac{\pi MCD^3}{12} \qquad (3)$$

Calculating the smallest cylinder an erythrocyte can pass through is relevant since aged or diseased cells are removed from the blood stream though a filtering system which depends on geometric constriction [3]. Fig. 3(d) shows the histogram of the measured MCDs.

The distributions shown in Fig. 3 for Sphericity and MCD differ significantly from those reported by Canham and Burton [3], (superimposed on Fig. 3 for comparison). These differences are expected and unavoidable due to differences in the measurement techniques, especially sample preparation. A comparison of the data reported by Canham and Burton for subject R.S. and our measurements can be found in Table 1 below. It is clear that the cells measured by DPC are on average, both smaller in diameter and thinner. This difference could be due to either difference in sample preparation or a bias towards thicker, rounder cells in the measurements of Canham and Burton [3].

Table 1: Comparison of DPC data with subject R.S. from Canham and Burton [3].

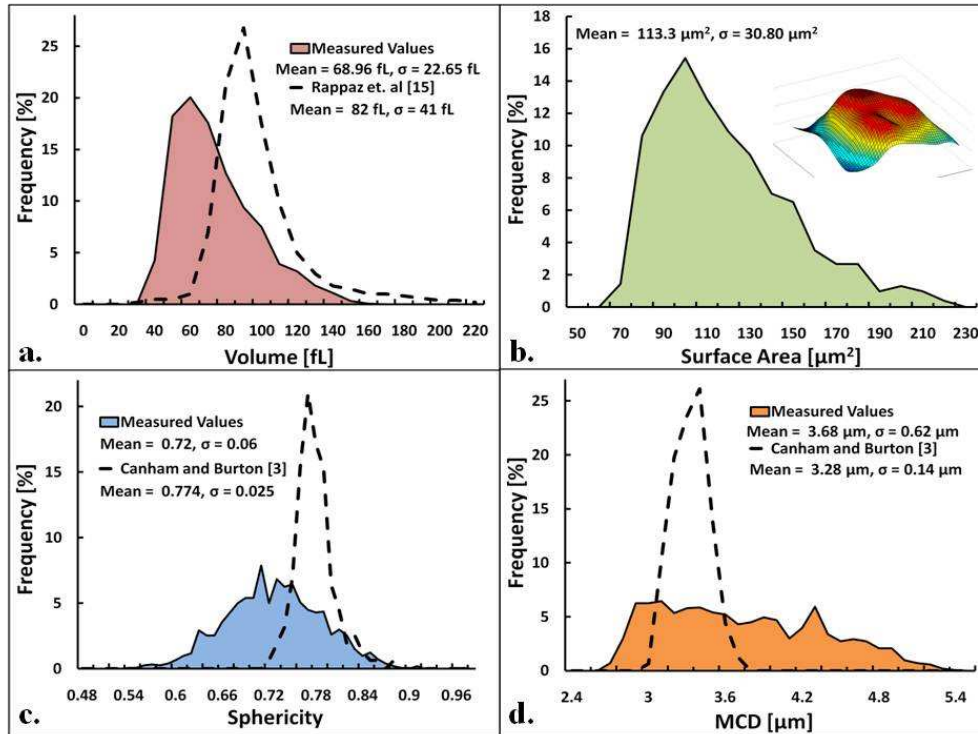|	DPC	Canham and Burton
Volume [fL]	68.96 ±22.65	105.3 ± 17.2
Surface Area [μm²]	113.30 ±30.80	139.4 ± 18.0
Diameter [μm]	7.53 ± 1.11	8.083 ± 0.56
Sphericity	0.72 ± 0.06	0.77 ± 0.025
MCD [μm]	3.68 ± 0.62	3.28 ± 0.14

Fig. 3. Frequency distributions a) Volume distribution, the dashed line shows volume data from Rappaz et al. [15] , b) Surface Area; inset shows the calculated surface mesh for a slightly deformed cell, c) Sphericity, d) Minimum Cylindrical Diameter; dashed lines show data from Canham and Burton [3].

The relationship between cell volume, surface area and shape determines their ability to deform and thus successfully deliver oxygen by squeezing through capillaries much smaller than their diameter. From the distributions shown in Fig. 3 it is difficult to determine the nature of these geometric constrictions and it is therefore necessary to study the correlations between the parameters measured [3]. The plots of Volume vs. Diameter, Surface Area vs. Diameter and Surface Area vs. Volume are shown in Fig. 4(a) and (b). The plots indicate a linear relationship rather than higher power law dependence as previously shown by Canham and Burton [3]. It is also evident from these geometric constrictions and from the description of cellular shape by the sphericity index that cells with larger volumes should tend to be thinner than those with smaller volumes. The fact that there is an upper limit on the sphericity as the cell volume increases is illustrated in Fig. 4(d). Furthermore, from the constant MCD lines superimposed on the Surface Area vs. Volume plot in Fig. 4(c) it can be seen that Eq.3 predicts the linear boundary that limits the distribution.

It is remarkable that despite the differences in the histograms of the individual parameters between our measurements and those of Canham and Burton, the relationships between the measured parameters are very close. The deviations that exist are reasonable based on the differences between test subjects, this is supported by the differences in the data between the subjects tested by Canham and Burton. Since the accuracy of our technique is supported based on the 4.5µm microspheres measured and since the previously defined relationships between RBC geometrical parameters hold true, the dissimilarities between our measurements and those reported in literature could be attributed to all the reasons discussed above, including: measurement techniques, sample preparation and physiological conditions of test subjects.
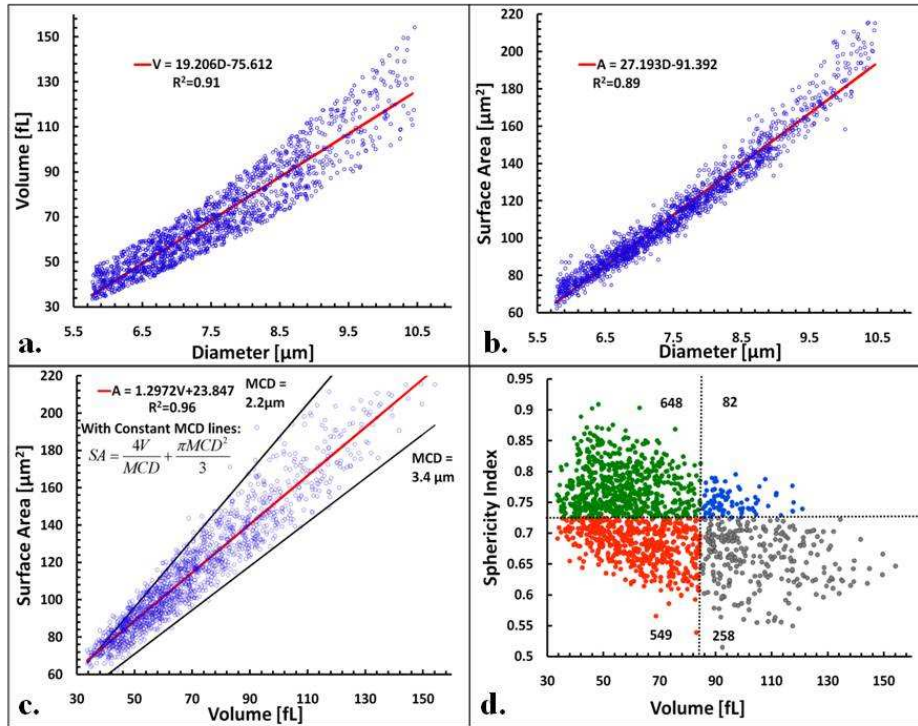
Fig. 4. Red lines are linear fits with R^2 values shown in the legend a) Volume vs. Diameter, b) Surface Area vs. Diameter, c) Surface Area vs. Volume with constant MCD lines shown in black, d) Sphericity Index vs. Volume, quartered to show that cells with larger volume are thinner.

4. Summary

In summary, we presented a novel adaptation of diffraction phase microscopy by utilizing a commercial compact disk as a diffraction grating in the sample plane. A dynamic spatial filtering system was implemented using spatial light modulation via a commercial liquid crystal projector, which allowed us to attenuate the object field in order to achieve maximum fringe contrast. We demonstrated the ability of the DPC system to take cytometry measurements of human erythrocytes and calculated several parameters. Out of these parameters, i.e. volume, surface area, sphericity and minimum cylindrical diameter, only the first is commonly available from most hematological measurement methods such as flow cytometry and centrifugation. We show that the correlations between the measured volume, surface area and diameter display the same linear relationships that have been reported previously, including the geometric constriction on the data predicted by the minimum cylindrical diameter model.

In the future we plan to make this technique more feasible as an inexpensive "lab-on-a-chip" type medical instrument by integrating DPC with existing CD-ROM drive technology. A CD-ROM based method of medical diagnosis would make this technology immediately available to the over 1 billion people that have access to modern computers and would allow for quick and easy diagnosis for a large variety of physiological conditions.