Label-free imaging flow cytometer for analyzing large cell populations by line-field quantitative phase microscopy with digital refocusing

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Abstract: We propose a line-field quantitative phase-imaging flow cytometer for analyzing large populations of label-free cells. Hydrodynamical focusing brings cells into the focus plane of an optical system while diluting the cell suspension, resulting in decreased throughput rate. To overcome the trade-off between throughput rate and in-focus imaging, our cytometer involves digitally extending the depth-of-focus on loosely hydrodynamically focusing cell suspensions. The cells outside the depth-of-focus range in the 70-µm diameter of the core flow were automatically digitally refocused after image acquisition. We verified that refocusing was successful with our cytometer through statistical analysis of image quality before and after digital refocusing.

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1. Introduction

Imaging flow cytometry [1] has recently emerged as a technique for accessing large populations of cells for research and clinical use from the point of view of the localization of fluorescence-tagged molecules in a cell or the cell’s morphological information provided by fluorescence microscopy, bright-field microscopy including phase-contrast, differential interference contrast (DIC), and quantitative phase microscopy (QPM) [2,3]. However, these techniques result in slow imaging speed when compared to conventional non-imaging flow cytometry, which performs at speeds of 1,000 to 100,000 cells per second. State-of-the-art ultra-fast label-free [4–8] or labeled [9,10] imaging flow cytometers use a single-pixel photo diode for sensing serially encoded images because of taking advantage of its temporally high bandwidth. A two-dimensional (2D) array of photo-diodes also resolves the throughput-rate limitation of labeled [11,12] or label-free [13–15] images by means of multiplexed imaging because of taking the advantage of its spatially high bandwidth. The drawback of a 2D-array sensor is discrete overlapping of images for stitching subsequent images or non-seamless images due to dead time for image transfer. A linear-array sensor, however, can seamlessly acquire an image without overlapping images for stitching them together. Bianco et al. [16] used a linear-array sensor in digital holographic microscopy (DHM) in the spatial domain [17] for biological samples in a flow. DHM or QPM provides quantitative information about optical path length (OPL). Over the past two decades, many research groups
provided novel and accurate strategies to investigate cells by using label-free quantitative phase imaging (reviewed in [15]). One advantage of DHM or QPM among bright-field microscopy is focus tracking [18,19] with digital refocusing. Various automatic digital refocusing methods were proposed and compared [20–24] because they suffer from the lack of generality for specimens of different optical transmission functions [25]. Label-free classification of cells based on physical parameters and subcellular structure [7,26–28] requires focused images of cells [29].

We propose a line-field quantitative phase-imaging flow cytometer in the time domain on transmission-mode of a sample. To the best of our knowledge, quantitative phase images retrieved from temporal varying interferograms (i.e. in the time domain [17]) have not been acquired using a linear-array sensor. Line-scan imaging requires the sample to be translational in the flow; otherwise, the images of the translational sample with rotation will have motion (rotation) blur. To avoid the rotation of cells near the wall of a microfluidic channel, we used a sheath-flow technique to hydrodynamically focus on the interrogation position at the center of the microfluidic channel. The drawback of this technique is the decrease in the throughput rate due to dilution of the cell suspension by sheath flow. Therefore, we also carried out loosely hydrodynamical focusing and digital refocusing of images to overcome the trade-off between spatial throughput rate and image quality.

2. Materials and methods

2.1. Sheath flow

Hydrodynamic focusing of cells is a very common technique in flow cytometry. Cell suspension is focused by using a buffer solution flowing around a stream of the cell suspension. This co-axial flow is called sheath flow. The temporal throughput rate \( n_t \) depends on the average flow velocity \( V \) and spatial throughput rate \( n_s \) and expressed in Eq. (1).

\[
n_t = V n_s = V(CA_c),
\]

where \( C \) is concentration of cell suspension, and \( A_c \) is an area across the core flow. Generally, hydrodynamic focusing increases coefficient variances (accuracy) in conventional non-imaging flow cytometry because it aligns cells just in a line where an excitation laser spot is focused. To increase the \( n_t \), the sheath-flow rate \( q_{sh} \) will increase, while the ratio of the core-flow rate \( q_c \) to \( q_{sh} \) [30] is constant to maintain the diameter \( d_c \) across the core flow, resulting in aligning cells on a line.

\[
d_c = d_t \left[ \frac{q_c}{q_c + q_{sh}} \right]^{1/2},
\]

where \( d_t \) is the diameter of the microfluidic channel.

The acquisition rate (line rate) in imaging flow cytometry is 1,000 times lower than non-imaging flow cytometry. In this situation, to increase the \( n_t, q_c \) can be increased because of multiplexed imaging lateral to the flow direction. We controlled the \( n_t \) by adjusting the \( q_c \) (or \( A_c \)) under the assumption that \( V \) and \( C \) are constant in Eq. (1). A flow-cell module (J12800-000-103, Hamamatsu) conducts laminar sheath flow by feeding \( q_{sh} = 30 \mu L/min. \) and \( q_c = 2 \mu L/min. \) to the flow-cell from two micro syringe pumps (ICX07100, Chemyx Inc.). The inner and outer squares of the flow-cell are 250 \( \mu m \times 250 \mu m \) (equivalent diameter \( d_t = 282 \mu m \)) and 4 mm \( \times 5 \) mm, respectively. According to Eqs. (1) and (2), the theoretical \( d_c \) is 71 \( \mu m \), and the theoretical spatial throughput rate is 2.7 cells/mm when \( C \) is \( 0.7 \times 10^6 \) cells/mL. The theoretical temporal throughput rate is 23 cells/sec when \( V = 8.5 \) mm/sec. We conducted an observation of flowing cells in eight hours (total sample volume and cells are 960 \( \mu L \) and 672,000 cells, respectively).

2.2. Set-up

Imaging flow cytometry with a linear-array sensor exploits the translational movement of the target to obtain a 2D image. High-speed imaging generally requires a high-powered light source.
to obtain signals in a short exposure time ($\Delta t$). Suppose that a 2D (rows and columns is $M$ and $N$) array sensor images an object with $\Delta t$, which is shortened to $\Delta t/M$ to acquire an image with the same signal level when the same powered light source is brought into focus in a line ($1 \times N$). In other words, line-field imaging has an advantage in terms of high-speed imaging. We used a heterodyne Mach-Zehnder interferometer [17,31] with field illuminations of reference and sample lights. Our set-up is illustrated in Fig. 1. The light (optical-fiber coupled He-Ne laser, center wavelength 633 nm, 6 mW) was split into two arms as reference and sample lights. The reference light is frequency-shifted using two acousto-optic modulators (AOMs) before the light is brought into focus on a line in the conjugate plane of the image plane ($P3$) by using a cylindrical lens ($CL1$). The sample light is brought into focus on a line in the sample plane ($P1$) by using another cylindrical lens ($CL2$). The scattered light from a flowing cell is corrected by the objective lens ($O1$) equipped with a correction collar (Plan Flour ELWD, Nikon, 20×, NA0.45). The diffraction-limited resolution of $O1$ is theoretically about 0.9 $\mu$m. The magnification (M) of our set-up is 25× because the focal length of the tube lens ($T$) is 250 mm. These lights interfering with each other form temporally varying interferograms on the line. The heterodyne frequency ($\Delta \omega$) was determined by the driving frequencies ($\omega_1$, $\omega_2$) of the two AOMs. One is driven at $\omega_1 = 200$ MHz, and the other is driven at $\omega_2 = 199.980$ MHz. The interferogram, temporally blinking at the $\Delta \omega$, as shown in Fig. 2(a), was acquired with a linear-array sensor (raL2048-80km, Basler, 1 pixel × 2,048 pixels, 7 $\mu$m × 7 $\mu$m) at 80,000 lines per second (lps) at 12-bit depth resolution and $\Delta t$ of 6 $\mu$s. Note that the $\Delta \omega$ and frame (line) rate of the camera ($f_c$) have the relationship $f_c = 4\Delta \omega$. The line-shaped interferograms ($N = 1,100$ pixels) are grabbed using a grabber card (PCIe-1433, National Instruments) installed on a desktop computer. The images are stored in two solid-state disks (SSDs) in RAID0 configuration. The successive scan lines build up the 2D interferogram shown in Fig. 2(c), where an area in the yellow rectangle was cropped in Fig. 2(d). The interferograms contain information of the OPL, and optical density (OD) of cells. We also use OD (called amplitude in this paper) to search for the best focused images in digital refocusing.

Fig. 1. Schematic of interferometer set-up. $A1, A2$: acousto-optic modulators (AOMs), $CL1, CL2$: cylindrical lenses, $O1$: objective lens 20×, NA0.45 having correction collar, $O2$: objective lens 20×, NA0.50, $T$: tube lens $f = 250$ mm, FC: flow cell, inner square 250 $\mu$m × 250 $\mu$m, $P1$: object plane in focus, $P2$: Fourier plane, $P3$: image plane, $P$: linear-array sensor having 1 pixel × 2,048 pixels whose square size $\Delta p$ is 7 $\mu$m × 7 $\mu$m.
2.3. Image processing

Each line-shape interferogram \( (I_i) \) has a \( \pi/2 \) difference between consecutive line images. The amplitude and phase images are retrieved from interferograms by using Eq. (3).

\[
\begin{align*}
\nu_n &= (I_{4n+1} - I_{4n+3}) + i(I_{4n+2} - I_{4n+4}) \\
\end{align*}
\]  

(3)

The background of the acquired phase image typically has curvature because of temporal fluctuation, and the mismatch in the curvatures of the wave-fronts of the sample and reference lights. After the curvature is removed, the backgrounds of the image are uniform, so it is easy to segment each cell on the basis of the triangle threshold method \([32]\). The size of the cropped image of the segmented cell was 150 pixels \( \times \) 150 pixels in the phase-image domain. In the interferogram-domain (Figs. 2(c) and (d)), the size corresponded to 150 pixels \( \times \) 600 pixels for the purpose of phase retrieval based on the temporally four-step phase-shifting algorithm. A retrieved amplitude and phase image are shown in Fig. 4(c). Image processing was carried out using the open-source image-processing software ImageJ and its custom-made plugins written in Java.

2.4. Digital refocusing

In imaging flow cytometry, it is important to acquire an high-fidelity image of an object (image quality). In other words, the image should be just in focus. We overcome the trade-off between spatial throughput rate and image quality by digital refocusing after acquiring images when hydrodynamic focusing does not perform perfectly. QPM records the complex amplitude so that digital refocusing can be carried out using the propagation method. A recorded hologram \( o(x, y, d) \) is propagated for the distance from the in-focused plane \( d \) by using a convolution-based method.

\[
\mathcal{F}[o(x, y, d)] = O(u, v, d) = O(u, v, 0) \exp(i\varphi),
\]

(4)

\[
\varphi = \pi \frac{\lambda}{n} d(u^2 + v^2),
\]

(5)

where \( \lambda \) is the center wavelength, \( n \) is the refractive index of the surrounding media (\( n = 1.33 \)), \( d \) is the distance from in-focus plane (the refocus distance), \( (u, v) \) are the coordinate variables in the Fourier plane, and \( \varphi \) is a quadratic phase term.

Another side-effect of non-ideal hydrodynamic focusing is various image distortions. Suppose that a cell is a true sphere and that the focus plane of the imaging system is in the center of hydrodynamic focusing where the velocity of the stream is maximum (2\( V \) when the flow is supposed to be Hagen–Poiseuille flow); thus, a given cell outside the center is slightly slower...
than in the center and its image becomes elongated when the $2V$ is equal to product of the pixel size of the linear-array sensor ($\Delta p$) and sampling frequency of the sensor ($f_s$). No metrology for velocity of cells in our set-up exhibits distortion on the y-axis. We compensated for this image distortion by using the aspect ratio of the major and minor axes of the elongated image. The resolution in the x-direction ($\Delta x$) is $\Delta p/M$, where $M$ is the magnification of the optical system. The resolution in the y-direction ($\Delta y$) is $a/b \Delta x$, where $a$ and $b$ are the major and minor axes of the ellipsoid shape of the cell image in Fig. 3(a).

$$F_u = \frac{M}{\Delta p}, \quad F_v = \frac{b}{a} F_u$$

This unbalanced resolution between the x- and y-axes results in the ellipsoidal quadratic phase shown in Fig. 3(b). The defocused hologram (amplitude and phase) of a 4.5-µm microsphere in flow (Polybead 17135, Polysciences, Inc.) in Fig. 3(d) are acquired by our set-up and digitally refocused by minimizing the total absolute first derivative of median-filtered (two-pixels radius) amplitude images by using an absolute gradient operator (GRA [21]). This operator detects the cumulative edge of an image. For a 3D phase object like a non-stained cell, GRA of the amplitude of a hologram is minimum when the center of the cell in depth is brought into focus. We searched the minimum GRA in the defocus-distance range of –66.5 to +66.5 µm. The minimum GRA in Fig. 3(c) was at $d = +27$ µm. Figure 3(e) shows its digitally refocused hologram. Assuming that the refractive indices of the bead and the surrounding medium are 1.59 and 1.33 respectively, the maximum phase value is theoretically 11.6 radians. However, the actual phase value does not reach the value due to spatial undersampling, which causes sharp phase difference exceeding the principal interval [33].

![Fig. 3.](image)

**Fig. 3.** (a) Schematic of acquired image of a sample. (b) Ellipsoidal quadratic phase in Eq. (5). (c) The minimum search of GRA in the defocus distance range of –66.5 to +66.5 µm. (d) Original (defocus) hologram of a bead. (e) Automatically digitally refocused hologram of the bead. In each panel, the left sides are amplitude images, and the right sides are phase images. The yellow profiles are the cross-sections on the white lines.

### 2.5. Sample preparation

Human-colorectal-tumor cell lines HCT116 were purchased from the European Collection of Authenticated Cell Cultures (ECACC, England, UK) and maintained in Dulbecco’s modified...
Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37°C in a humidified atmosphere containing 5% CO₂.

3. Results

![Diagram](image)

**Fig. 4.** (a) Ellipsoidal quadratic phase in Eq. (5). (b) The minimum search of GRA in the refocus distance range of –66.5 to +66.5 µm. (c) Original (defocus) hologram of a single cell. (d) Automatically digitally refocused hologram of the single cell. In each panel, the left side is an amplitude image, and the right side is a phase image. The yellow profiles are the cross-sections on the white lines.

We conducted an observation of flowing cells in eight hours. The total number of cells to be observed was 401,849 among 672,000 cells. The rest of the cells may be stacked somewhere including tubes and joints. 194,177 cells out of observed cells were digitally refocused, analyzed, and are plotted in Figs. 5 and 6. The rest of the cells mainly failed to unwrap due to sharp phase exceeding the principal interval [33].

Figure 4 shows digital refocusing of the representative hologram (amplitude and phase) retrieved from the interferogram of a single cell shown in Fig. 2(d). The same procedure for the bead experiment in Fig. 2 was done for cell experiment. The minimum GRA in Fig. 4(b) was at \( d = -21 \) µm. The amplitude in Fig. 4(d) shows that diffraction pattern around the cell is less clear than the original hologram while the edge of the cell is clear. The phase in Fig. 4(d) shows that the expected hemisphere shape can be observed.

Figure 5 shows lateral and longitudinal distributions of positions of the flowing cell. Figure 5(a) plots a 2D histogram of the positions of flowing cells across the microfluidic channel (x-z plane). This graph shows how the loosely focused effect widens the cross-section of the focused stream. The diameters (6σ) of the core flow was about 77 µm in lateral (x-axis) and 63 µm in refocus distance (z-axis). The average of the two diameters agrees with the theoretical value (71 µm) calculated from Eq. (2). The asymmetric distribution shows the focused stream walks around in time (Visualization 1). This may cause very low velocity of flow.

For the longitudinal (y-axis) distributions, the \( d_y \) is defined as the distance between any two successive cells in the y direction. The inverse of \( d_y \) means the spatial frequency of the cells in a unit length (counts of events per unit). The resolution of the y-axis in the center of the channel...
**Fig. 5.** Lateral and longitudinal distributions of cells in the microfluidic channel. (a) Lateral (x-z plane) distribution of positions across the microfluidic channel, and horizontal and vertical projections of the distribution in bar-graphs. (b) Probability (bars) and its cumulative probability (red curve) of the inverse of $d_y$. Upper horizontal axis represents the distances (1,000 $d_y$) between any two successive cells in the y-direction. Lower horizontal axis represents the spatial frequency of cells in the y-direction.

**Fig. 6.** Changes in the ratio of projection areas and standard deviations of cell images before and after digital refocusing.
is calculated by \(8V/f_c\). Figure 5(b) shows the probability (left vertical axis) and its cumulative probability (right vertical axis) of the \(1/d_y\) (lower horizontal axis) and \(d_y\) (upper horizontal axis). The cell size is estimated by measuring the pixel counts in the x-direction. The distribution of cell size is shown in the inset in Fig. 5(b). The average size of cells is \(14 \pm 1 \mu m\). The distance of more than 98.5% out of any two successive cells is more than \(20 \mu m\). In other words, the coincident arrival of cells is less than 1.5%. The average distance \((d_y)\) between any two successive cells is \(1.47 \text{ mm}\), which corresponds to 0.68 cells/mm.

Digital refocusing is expected to obtain in-focus images for better image quality. To validate the digital refocusing, two representative parameters were extracted from cell images before and after the digital refocusing. One is a projection area \((A)\) of a cell and the other is a standard deviation \((\sigma)\) of a refractive index map of a cell relative to that of the surrounding media. As optical path-length and physical path-length (thickness) of a cell are defined as OPL and PL, respectively, the relative refractive index map is calculated by OPL/PL. In our previous paper [27], a standard deviation of the OPL/PL of a single cell was dominant for label-free classification of cells.

Figure 6 shows a histogram of the ratio of projection areas \((A_{DR}/A_0)\) versus the ratio of standard deviations \((\sigma_{DR}/\sigma_0)\), where the subscripts 0 and DR mean before and after digital refocusing, respectively. The distortion in the y-direction due to the lack of the line rate of the camera was canceled by taking the ratio of projection areas before and after digital refocusing. It turns out that qualitatively most of the projection areas shrank after refocusing while standard deviations increased.

4. Discussion

Typical non-imaging sheath-flow cytometry involves a core diameter of 10 \(\mu m\) and speed of 10 m/s. Under these conditions, its temporal and spatial throughput rates are 785 cells/s and 0.0785 cells/mm, respectively [34]. Imaging flow cytometry, however, suffers from decreased temporal throughput rate due to the bandwidth limitation of linear-arrayed sensors. Intuitively, a bandwidth of a 1,000-arrayed linear sensor is one thousand times narrower than a single-pixel sensor, such as a photomultiplier or photodiode. To overcome the bandwidth limitation in imaging flow cytometry, many researchers carry out multiplexing of flowing channels or imaging and shorten the distance between flowing cells in sheathless flow. Our loosely focused sheath-flow cytometer involves the average core diameter of 70 \(\mu m\) \(6\sigma)\, and the flow rate ratio of core and sheath is 1:15 to increase the spatial throughput rate. However, a loosely focused streamline of cell suspension adversely affects image quality from the points of view of defocus and coincident arrival of cells [35], which may cause image overlapping issue. Our results in Fig. 5(b) show 98.5% of the cell population spaced more than 20 \(\mu m\) apart from each other on the focused stream. The rest of the cells overlapped or aligned on the horizontal axis (x-axis in Fig. 2). This image overlapping issue will be solved by using 3D label-free quantitative phase imaging flow cytometry [36–39]. We compensated for the defocus issue by using a digitally refocusing technique and characterized the flow condition across the microfluidic channel in Fig. 5. Digital refocusing reveals the dynamics (Visualization 1) of the 2D distribution of flowing cells in the cross-section of the microfluidic channel in Fig. 5(a). Figure 5(b) shows that the spatial throughput rate increased, and its average was 0.68 cells/mm, which is about 9 times 0.0785 cells/mm of typical non-imaging flow cytometry. However, the experimental spatial throughput was four times less than the theoretical value (2.7 cells/mm) because the dynamic diameter of the core flow was about half the static average diameter (70 \(\mu m\)). Figure 5(b) also shows that the coincident arrival of cells was less than 1.5%. We statistically proved that the process improved the image quality of a large population of cells (194,177 cells), as shown in Fig. 6.

In principle, line-scan imaging requires the cell to be translational (not rotational) in the flow. It is well-known that a rotation occurs for non-focused flowing cells [14]. The two opposite sides
of the cell, the one close to the wall and the other closer to the channel center, will experience two different flow velocity values. As a consequence, a torque will act on the cell and a rotation will be induced [40]. When the flow in the channel is supposed to be Hagen–Poiseuille flow, the profile of velocity is parabolic. The difference \( \Delta V \) in the velocity at the two opposite sides of the cell is 1 mm/sec. The worst case occurs at the edge of focused stream (35 \( \mu \)m in radius), where the rotational angle during the line-scan of a whole cell is \( \Delta V/V \sim 0.12 \) radians (7\(^\circ\)) corresponding to \( \sim 1 \) \( \mu \)m in the edge of the cell. Consequently, the rotation issue was negligible in our experiment.

While for a modern imaging flow cytometer [41], the speed can reach up to 1,000 cells/sec, our set-up reaches only 23 cells/sec at \( C = 0.7 \times 10^6 \) cells/mL and \( q_c = 2 \mu \)L/min. The ways to increase the temporal throughput rate in our current set-up are (a) replace the camera with a high line-rate camera (200,000 lps camera is now commercially available), (b) condense the cell suspension up to \( 25 \times 10^6 \) cells/mL [12], and (c) increase the flow rate of the sample \( (q_c) \). We believe that these modifications will increase the temporal throughput rate up to 1,000 cells/sec in our set-up.

5. Conclusion

We proposed a line-field quantitative phase-imaging flow cytometer and used it to carry out digital refocusing of quantitative phase images and statistical analysis of a large population of cells flowing in a microfluidic channel. To overcome the spatial throughput-rate limitation in sheath-flow cytometry, we set the streamline of cell suspension in a manner of loosely hydrodynamic focusing to increase the spatial throughput rate. We successfully compensated for the degradation in image quality by using a digitally refocusing technique. Digital refocusing supports morphological analysis of large populations of cells from the point of view of high spatial throughput rate and image quality. The temporal throughput rate will increase by sheathless focusing such as inertial or acoustic focusing. We believe that label-free imaging flow cytometry will pave the way for intact single-cell analysis in regenerative medicine and detection of circulating tumors cells in cancer patients’ blood.

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Disclosures

The authors declare that there are no conflicts of interest related to this article.

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