Microfabricated multiple field of view imaging flow cytometry

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The combination of microscopy and flow cytometry enables image based screening of large collections of cells. Despite the proposition more than thirty years ago, adding high resolution wide-field imaging to flow cytometers remains challenging. The velocity of cells in flow cytometry can surpass a meter per second, requiring either sub-microsecond exposure times or other sophisticated photodetection techniques. Instead of faster detectors and brighter sources, we demonstrate that by imaging multiple channels simultaneously, a high throughput can be maintained with a flow velocity reduced in proportion to the degree of parallelization. The multi-field of view imaging flow cytometer (MIFC) is implemented with parallel arrays of microfluidic channels and diffractive lenses that produce sixteen wide field images with a magnification of 45 and submicron resolution. Using this device, we have imaged latex beads, red blood cells, and acute myeloid leukemia cells at rates of 2,000–20,000 per second.

Introduction

Flow cytometry has added a statistical element to the optical measurement of cells that would be exceedingly difficult otherwise. Modern systems can detect fluorescence and scattering signatures from tens of thousands of cells per second. This throughput makes possible the identification of rare sub-populations and a deeper understanding of the diversity of even a single cell population. While the acquisition speed of flow cytometry is extremely high, the amount of information per cell is low. To increase the information content, imaging flow cytometry was first proposed in 1979 as a method to combine microscopy and flow cytometry. Images contain a variety of information that is not easily retrieved from fluorescence or scattering measurements, including morphology, localization of sub-cellular components, and the ability to operate without labels. By operating in a flow cell, an imaging flow cytometer retains several of the advantages of traditional flow cytometry, including high throughput data collection, the ability to monitor single cells, and advantages for studying non-adherent cells, such as blood cells.

Acquiring high resolution images of cells at throughputs common in flow cytometry is challenging. Until recently, one of the bottlenecks was the speed of digital cameras. Recently, however, CMOS cameras have become available that capture images at rates of 2,000–20,000 per second, requiring either sub-microsecond exposure times or other sophisticated photodetection techniques. Instead of faster detectors and brighter sources, we demonstrate that by imaging multiple channels simultaneously, a high throughput can be maintained with a flow velocity reduced in proportion to the degree of parallelization. The multi-field of view imaging flow cytometer (MIFC) is implemented with parallel arrays of microfluidic channels and diffractive lenses that produce sixteen wide field images with a magnification of 45 and submicron resolution. Using this device, we have imaged latex beads, red blood cells, and acute myeloid leukemia cells at rates of 2,000–20,000 per second.

signal to noise ratio images at flow velocities that commonly exceed a meter per second. In order to eliminate motion blur, the effective exposure time needs to be less than the time it takes for the sample to move half the diffraction limited resolution. The fastest digital shutters on CMOS cameras are currently one microsecond, which would correspond to a maximum resolution of 2 μm in a 1 m s⁻¹ flow. One solution is to use flash lamps or ultrafast lasers to strobe the illumination for nano or picoseconds duration. While pulsed illumination can effectively eliminate motion blur, it can still be difficult to obtain enough light in such a short duration for a good signal to noise ratio. Amnis has demonstrated a successful alternative that uses a time delay and integration (TDI) detector to effectively increase the exposure time of fast moving samples. The Amnis system represents the current state of the art in imaging flow cytometry, but has drawbacks that include sensitivity to translation and rotation and speed limitations due to the longer effective time spent in the detection region. Our technique represents another route, which is to exploit the parallelization that is possible by microfabricating both the fluidic and the optical sub-systems.

Microfabricated fluid handling systems have proven to be a powerful and flexible platform for manipulating biological samples. Several reports have shown their utility for implementing flow cytometers. The majority of these use a single sample interrogation region, similar to benchtop flow cytometry. Microfluidics, however, enables much greater flexibility in the design of fluid delivery systems, and recently there have been reports that take advantage of this to implement parallel fluidic systems. Microfabrication of optical components enables the same flexibility. Reports have shown microfabricated optical imaging systems that are capable of collecting images without tabletop microscopes using aperture arrays and in-line...
holography. Integrating lenses into the microfluidic chip itself has primarily been limited to one-dimensional in-plane lenses that can be used for fluorescence excitation and scatter collection, but not for wide field imaging. A few reports have demonstrated refractive and diffractive two-dimensional lens integration, which were used for fluorescence excitation and collection. In this paper, we demonstrate the integration of an array of diffractive lenses that are each capable of relaying wide-field images to a camera. This optical system is capable of exploiting the parallelization that is possible in microfluidics to implement a multiple field of view imaging flow cytometer (MIFC). The ability to image simultaneously in many channels at once proves to be an effective method to circumvent the tradeoff between throughput and exposure time.

**Diffractive lens wide field imaging**

Diffractive lenses offer many advantages over their conventional refractive counterparts in lab-on-a-chip devices. The first is that they can be patterned using a single lithographic step and can be molded into polydimethylsiloxane (PDMS) in much the same way as in the standard microfluidics fabrication process. Diffractive lenses change the direction of incident light by using the geometry of a thin and planar grating pattern, as compared to refractive lenses which use three-dimensional curved surfaces to redirect the light path. Due to their planar geometry, diffractive lenses can therefore be made with tighter tolerances than lenses that require three-dimensional structures. Lab-on-a-chip devices are aligned during their fabrication and frequency the alignment cannot be adjusted during their use. For high resolution microscopes that have a shallow depth of focus, this constraint requires both a large accuracy and precision for the focal length and lens shape of each element in a microfabricated lens array.

In order to obtain accurate control over the diffractive lens geometry, we use electron beam lithography to pattern the diffractive lens master. The minimum required feature size of a diffractive lens is approximately the same as the diffraction limited resolution of an object imaged with the lens. In our case, the lenses have a numerical aperture (NA) of 0.35–0.5, resulting in an outer grating line width of approximately 500 nm. To produce a maximum efficiency for a two layer grating, the lenses are designed so that there is a relative phase shift of $\pi$ for light traveling through PDMS, relative to a neighboring air region. This requires a grating depth of 500 nm for an optical wavelength of 455 nm.

While diffractive lenses have several advantages, chromatic and field aberrations have previously limited their implementation to the deep ultra violet or soft X-ray frequency region where refractive lenses are not effective. The most severe aberration for diffractive lenses is frequently chromatic aberration, which causes different optical wavelengths to have a different focal length. The spectral width that a diffractive lens can tolerate ($\lambda/\Delta\lambda$) is dependent on how many grating periods the diffractive lens has. Macroscopic lenses can have several thousand grating periods, resulting in a required spectral width of a fraction of a nanometre. Microlenses, however, can have a high NA with far fewer grating periods. Fig. 1(a) shows a diffractive lens that has a 200 µm diameter, a focal length ($f$) of 375 µm, and contains 44 rings. For a center wavelength of 455 nm, this lens can accommodate a spectral width of 10 nm, which is comparable to the spectral width of an LED.

Managing field aberrations is also critical in order to obtain high quality imaging. Microscope objective lenses have multiple lens elements and surfaces, and a lot of care goes into the design of flat field lenses that minimize aberrations over a large field of view. Flow cytometry, however, presents an interesting case where aberrations do not need to be minimized over large areas. The field of view needs to be as large but not significantly larger than a single cell, on the order of 20 µm. Hydrodynamic focusing or the channel geometry itself can confine the motion of the cell to this small region.

To characterize the resolution and field of view for the diffractive lens microscope, we first fabricated phase test targets. Fig. 1(c–e) show images collected by the diffractive lens microscope using the optical setup shown in Fig. 1(b). The microscope is a cascaded imaging system, where the diffractive lens first magnifies the object, in this case by a factor of 23, and then the relay lens L3 additionally magnifies the image by another factor of 3. Iris 1 is illuminated by an LED (Thorlabs M455L2) and imaged onto the object plane by L2, thereby confining the illumination to a region smaller than the aperture of the diffractive lens. This illumination scheme is important because the diffractive lens does not have a physical aperture. Fig. 1(c) shows line pairs that have a pitch of 1.0 µm and have a 500 nm line width. Because these are phase objects, only the edges display contrast. As can be seen in the image, the edges of a single line can be resolved, which have a separation of 500 nm. The resolution predicted by the Rayleigh criteria is,

$$w_d = 1.22\lambda / (NA_c + NA_{dl}) = 480\text{ nm},$$

where $NA_c$ is the condenser NA, which is 0.7, and $NA_{dl}$ is the diffractive lens NA, which is 0.45. As shown in Fig. 1(c), 500 nm features can just be resolved, where as 1.0 µm features, shown in Fig. 1(d), are resolved easily. In addition, we can measure off axis field aberrations in this microscope by imaging an extended array of 1.0 µm dots on a 2.0 µm pitch. As shown in Fig. 1(e), at the center of the field of view, the image is diffraction limited, while at distances further from the center, there is distortion and other field aberrations. The image has approximately 10 dots across the field of view that have minimal aberrations, corresponding to a width of 20 µm, which we call the diffraction limited field of view. Consequently, if we restrict the path of cells traveling through the detection region to a width of 20 µm, we can expect to have diffraction limited resolution.

**Parallel imaging flow cytometry**

Using lithography, we then fabricate arrays of microfluidic and microoptical components and integrate them into the MIFC. The microfluidic channel array and the diffractive lens array lie on opposite sides of a 400 µm thick coverslip (Arrayit), as shown in Fig. 2(a). The thickness of the coverslip defines the object to lens distance and cannot be manipulated after fabrication. Consequently, it is critical to accurately define the focal length with a tolerance on the order of the depth of focus of the microscope, which in this case is 4.5 µm, approximately 1% of the focal length. For refractive microlenses this would be a challenging specification to meet, but for diffractive lenses it is not
difficult. The coverslips also are required to have a thickness tolerance on the same order. Fig. 2(b) shows a 4\(\times\)4 lens array aligned to a 16 channel microfluidic device, where each channel is 25 \(\mu\)m wide and 6 \(\mu\)m deep. The microfluidic device has a single inlet that is then split by four cascaded 1\(\times\)2 junctions that result in 16 parallel channels that have a spacing of 97 \(\mu\)m. The lens array is aligned to the channel array at an angle, in this case 14°, so the lens array pitch can be greater than the channel spacing.

Fig. 2(c) shows the image plane of the MIFC for a sample consisting of a suspension of 3.5 \(\mu\)m latex beads. Iris 1 has been replaced by a pinhole array (4\(\times\)4) that is imaged onto the lens array by L1. Each microlens is designed to produce a magnification of 15, which along with an additional magnification of 3 from the relay optics, produces a total magnification of 45. The lens array pitch is 400 \(\mu\)m and is designed to be slightly greater than a magnified image of a 25 \(\mu\)m channel, which is 375 \(\mu\)m. The field size of each image in the array can be controlled by adjusting the aperture size of iris 2 from Fig. 1(b). A similar effect has previously been demonstrated in the context of microlens based scanning confocal microscopes, and is a result of vignetting. Iris 2 can be opened just enough so that neighboring fields of view slightly overlap. Each field of view is disjoint in object space, but the image space is almost continuous. In this way, the sensor array can be used to collect images from specific regions that span a large distance across the device, with few empty pixels.

To investigate the maximum throughput of the MIFC, we flow a suspension of 3.5 \(\mu\)m latex beads with a concentration of 4\(\times\)10\(^8\) beads\(\times\)mL through the device. Video is collected with a CMOS camera (Mikrotron MC1362) having a region of interest of 360\(\times\)360 pixels, running at 3,000 frames per second, and operating with an exposure time of 2 \(\mu\)s, which is the minimum possible for this camera. The camera pixel size is 14 \(\mu\)m, corresponding to an image plane sampling period of 310 nm. The maximum velocity of this device is limited by the field of view of each microlens and by the finite exposure time of the camera. In order to collect images of every bead, the size of the field of view must be greater than the distance that a bead travels in between exposures. For a field size of 25 \(\mu\)m and a time...
between exposures of 333 $\mu$s, the maximum velocity is $75$ mm s$^{-1}$. In addition, the finite exposure time produces motion blur if the object moves a greater distance than the diffraction limited resolution. For a velocity of $75$ mm s$^{-1}$ and an exposure time of 2 $\mu$s, the motion blur is 150 nm and is below the diffraction limit.

Fig. 3 shows data collected at a mean velocity of $50$ mm s$^{-1}$, slightly less than the maximum velocity limited by the field of view size. At this rate, 7038 beads were counted in a duration of 333 ms, which corresponds to approximately 21,000 beads per second. Average throughput in a single channel of our multi-channel device is 1300 beads per second, which is comparable to other imaging flow cytometers that use a single interrogation region.\textsuperscript{5,9} The total throughput for all sixteen channels, however, is more than an order of magnitude faster than other imaging flow cytometers and is comparable to non-imaging flow cytometers. For a single channel imaging flow cytometer to operate at comparable throughput to our parallelized system, at a similar bead concentration, would require an exposure time of 2 $\mu$s/16 = 125 ns. This specification is not possible with any known CMOS camera and would additionally require an extremely bright illumination source, where as we use an off the shelf LED.

Once the data has been collected, it can be analyzed with a variety of image analysis programs. First, objects are isolated from the field of view by looking for pixel values in a fixed window that exceed a threshold. The window is set to a slightly smaller width than the average displacement of a single particle between frames. Because the particles have velocity dispersion, a small percentage of particles are counted twice, while a few others are not counted at all. We have used Morphological functions from the Image Processing toolbox of MATLAB to study the size and shape of the collected images. These functions enable the identification and characterization of each connected region in a binary image. We converted each of the recorded images to binary by applying a low-pass filter and a threshold. Fig. 3(a) shows a scatter plot of the major and minor axis of each identified image region. The primary cluster of the scatter plot has a minor and major axis of 3.4 $\mu$m and 3.6 $\mu$m, respectively.

Examples of the images in this cluster are shown in (b). From the scatter plot, it is also apparent that there is a distribution of images that has the same minor axis length, but a greater major axis length. This can be attributed to two bead aggregates, shown in (c). In addition, there are three bead aggregates in the upper right region of the scatter plot, shown in (d). One surprising feature brought out by the scatter plot was the presence of three larger beads, shown in (e). A flow cytometer or coulter counter would have likely confused these for aggregates, but an image shows that they are 5 $\mu$m single beads, and appear at a concentration of 1/2000 relative to the 3.5 $\mu$m beads.

In addition to latex beads, red blood cells and acute myeloid leukemia cells were imaged separately using the MIFC. Samples of whole blood from healthy donors were obtained from an outside supplier (Research Blood Components). Red blood cells were isolated from whole blood by centrifugation and re-suspended in a standard PBS buffer at a concentration of $4 \times 10^7$ cells mL$^{-1}$. The flow velocity was reduced to a mean velocity of $35$ mm s$^{-1}$ and the camera exposure time was increased to 6 $\mu$s, while all other parameters were the same as the bead experiment. At this concentration, the throughput was approximately an order of magnitude less than that for the beads. Fig. 4(a) shows a scatter plot for 2 s of data, which correspond to 4550 cells, or a throughput of 2275 per second. The throughput can be increased to a rate similar to that for the beads, 21,000 per second, but coincidence detection becomes higher. The approximate coincidence rate at this throughput is 4.7%, which is calculated by measuring the probability that the cell size is greater than 8.5 $\mu$m, which would imply two cells that were spatially overlapping.

The scatter plot in Fig. 4(a) shows a broad distribution of red blood cell morphologies. The major and minor axis of each cell image is calculated independent of the cell’s angular orientation. The peak of the distribution has a minor axis length of 4.8 $\mu$m and a major axis length of 6.2 $\mu$m. As sample images, we choose four gated regions that all have the same minor axis length centered at 4.8 $\mu$m, but have different major axis lengths, centered at 5.2, 6.2, 7.2, 8.2 $\mu$m, and have gate widths of 0.4 $\mu$m. These four regions have increasing aspect ratios, as can be seen in Fig. 4(b–e), where the cells are stretched in the direction of flow. One characteristic shape that has previously been identified in cylindrical and rectangular channels is called a “parachute”\textsuperscript{20} and is apparent in all four of the images in Fig. 4(c). A more thorough discussion of red blood cell shape in the presence of hydrodynamic shear is outside the scope of this paper, but by collecting such large image sets, this device presents a promising method to study red blood cell mechanics.

Malignant white blood cells from a cell culture of acute myeloid leukemia (HL-60, ATCC) were also imaged with the MIFC. Fig. 5 shows two sets of images collected from different culture plates, where both sets were left unstained. The cells in Fig. 5(a) show few sub-cellular features, where as the cells in Fig. 5(b) appear to have developed a microbial contamination, as can be seen from the dark features inside the cell body. Microbial uptake is a common phagocytic function of white blood cells as part of the immune response. These cells range in size from 8–12 $\mu$m, while the microbial fragments that are visible in Fig. 5(b) are sub-micron and separations on the order of 1 $\mu$m can be resolved.
Conclusion

In this paper we have proposed and demonstrated the MIFC based on integrated diffractive lenses. Performing imaging flow cytometry in parallel enables higher throughput or a longer relative exposure time for the same throughput. Imaging with diffractive lenses has been demonstrated to be diffraction limited and capable of resolving sub-micron features. Using the MIFC, we have resolved red blood cell morphology and characterized leukemia cells with and without the presence of a microbial contamination. The MIFC is capable of collecting as high as 20,000 images of discrete particles per second and requires the use of only a simple LED for illumination and a fast CMOS camera for detection. While in this study we have only demonstrated brightfield imaging, fluorescence imaging is also possible, though multiple spectral bands is challenging due to chromatic aberrations. By collecting and analyzing data sets consisting of thousands of cell images, we hope to in the future resolve spatial characteristics of cells that are currently hidden by insufficient statistics.

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References


