On-chip light-sheet fluorescence imaging flow cytometry at a high flow speed of 1 m/s

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Abstract: We present on-chip fluorescence imaging flow cytometry by light-sheet excitation on a mirror-embedded microfluidic chip. The method allows us to obtain microscopy-grade fluorescence images of cells flowing at a high speed of 1 m/s, which is comparable to the flow speed of conventional non-imaging flow cytometers. To implement the light-sheet excitation of flowing cells in a microchannel, we designed and fabricated a mirror-embedded PDMS-based microfluidic chip. To show its broad utility, we used the method to classify large populations of microalgal cells (Euglena gracilis) and human cancer cells (human adenocarcinoma cells). Our method holds promise for large-scale single-cell analysis.

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1. Introduction

Imaging flow cytometry is an emerging technique of importance in microbiology, immunology, and cell biology, capable of acquiring high-resolution images of cells with high throughput [1–4]. By virtue of high information content in images, imaging flow cytometry enables us to perform more multiparametric analysis of heterogeneous cell populations than conventional non-imaging flow cytometry [5,6]. Among various techniques of imaging flow cytometry, fluorescence imaging flow cytometry, which acquires fluorescence images of cells, is particularly powerful as it provides information about cell surface expression and intracellular molecules due to the high specificity of fluorescent labelling and, thus, has a wide range of applications in cell studies including cell morphology, cell-cell interactions, cell population classifications, cell signaling, co-localization, cell death, and autophagy [5].

Unfortunately, the throughput of fluorescence imaging flow cytometers is much lower than that of non-imaging flow cytometers, which places a limit on its practical applications. For example, a commercially available product, ImageStream® Mark II, has a throughput of ~1,000 cells/s at a pixel size of 0.3 μm × 0.3 μm [6,7], which is one order of magnitude lower than that of non-imaging flow cytometers. As it uses time-delay integration to acquire...
sufficient imaging sensitivity, which extends exposure time using the charge transfer sequence of a CCD image sensor, the throughput is limited by the data transfer rate of the CCD image sensor (typically <100 MHz). Replacing the CCD image sensor with a CMOS image sensor can, in principle, overcome the speed limit by virtue of its higher data transfer rate (>100 MHz), but unfortunately, current CMOS image sensors are not compatible with time-delay integration, such that its low signal-to-noise ratio (SNR) accompanied by short exposure time practically limits the cell throughput.

Additionally, despite a high demand for it, implementation of high-throughput fluorescence imaging flow cytometry on a microfluidic chip remains technically challenging, making it difficult to conveniently add various functions (e.g., sorting, manipulation, cultivation) to conventional flow-cell-based flow cytometers. Realization of on-chip microfluidic operation for high-throughput fluorescence imaging flow cytometry would, therefore, further extend its capabilities [8]. Several recent studies have reported on-chip fluorescence imaging flow cytometry, but have not achieved both cell throughput comparable to conventional non-imaging flow cytometry (i.e., ~10,000 cells/s) and microscopy-grade image quality (i.e., high spatial resolution and high SNR) simultaneously [9–16].

In this paper, we present on-chip fluorescence imaging flow cytometry at a high flow speed of 1 m/s by light-sheet excitation on a mirror-embedded microfluidic chip that overcomes the above limitations. To implement the light-sheet excitation of flowing cells in a microchannel, we designed and fabricated a mirror-embedded microfluidic chip made of polydimethylsiloxane (PDMS) and a coverslip. To precisely embed and position the mirror (which looks seemingly simple, but difficult in practice and is the key to the implementation of the light-sheet excitation and fluorescence imaging), we developed a highly effective fabrication process using a prism mirror and mold structure. Compared with standard wide-field excitation, the light-sheet excitation on the chip boosted the fluorescence intensity at each image pixel by a factor of ~10. This allows us to obtain fluorescence images of cells flowing at a high speed of ~1 m/s at a pixel size of 0.325 μm × 0.325 μm (corresponding to a high throughput of up to 10,000 cells/s, depending on the cell size and sample concentration assuming the 100-μm cell spacing), which is comparable to the flow speed of conventional non-imaging flow cytometers (1-5 m/s). To show its broad utility, we used the method to classify large populations of microalgal cells (Euglena gracilis) and human cancer cells (human colorectal adenocarcinoma cells and human breast adenocarcinoma cells) cultivated under different conditions. Our method holds promise for large-scale single-cell analysis in metabolic engineering and cancer biology.

Fig. 1. Principles of light-sheet excitation in comparison with wide-field excitation. (a) Schematic of the light-sheet excitation of flowing cells in the microchannel on the mirror-embedded microfluidic chip. (b) Schematic of the wide-field excitation of cells flowing in a microchannel on a standard microfluidic chip. (c) Schematic of the cross section of the light-sheet excitation beam. (d) Schematic of the cross section of the wide-field excitation beam.
Fig. 2. Schematic of the high-throughput on-chip light-sheet fluorescence imaging flow cytometer. FG: function generator; TL: tube lens; DM: dichroic mirror; M: mirror; SL: spherical lens; CL: cylindrical lens; OL: objective lens; Inlet 1: sample flow inlet; Inlet 2: sheath flow inlet.

2. Materials and methods

The principles of the light-sheet excitation are shown in Fig. 1(a). An aluminum-coated glass is embedded in the PDMS. The light-sheet beam reflects on the aluminum-coated surface via the objective lens and selectively illuminates the focal plane of the objective lens in a similar manner to light-sheet microscopy. The microchannel is designed such that cells flow in the
focal plane and, thus, are excited by the light-sheet beam. Fluorescence emitted from the cells is collected by the objective lens. On the other hand, in the case of standard wide-field excitation, the excitation beam illuminates the whole field of view (FOV) from the back of a microfluidic chip as shown in Fig. 1(b). The width of the light-sheet excitation beam in the depth direction of the imaging optics is comparable to the depth of field (DOF, typically a few micrometers, Fig. 1(c)) while the width of the wide-field excitation beam in the lateral direction is comparable to the width of the microchannel (typically tens of micrometers, Fig. 1(d)). Then, assuming that the power of the excitation laser is identical in both cases, the light-sheet excitation configuration has much higher excitation efficiency due to the smaller cross section of the excitation beam, leading to a significant improvement in the SNR of fluorescence imaging and hence allowing high-speed fluorescence imaging of fast-flowing cells.

A schematic of the on-chip imaging flow cytometer is shown in Fig. 2. The source of the light-sheet beam is a semiconductor laser (Cobolt 06-MLD, $\lambda = 488$ nm). The laser light passes through a pair of cylindrical lenses ($f = 26$ mm, $f = 100$ mm) and a spherical lens ($f = 125$ mm) so that a desired beam profile is obtained in the focal plane of the objective lens as depicted in Fig. 1(a). The designed width and thickness of the excitation beam were 832 $\mu$m and 4 $\mu$m ($1/e^2$ beam diameter), respectively, which correspond to the FOV in the flow direction and the light-sheet range (double Rayleigh length) of 52 $\mu$m, respectively. This indicates that, considering that the thickness of the beam is larger than the focal depth of the imaging setup (~2 $\mu$m) and the wide-field excitation requires the excitation beam width of ~52 $\mu$m to cover the same FOV, the light-sheet excitation has a higher excitation efficiency approximately by a factor of 52/4 = 13 than the wide-field excitation. The objective lens (Olympus UPLSAPO 20x) has a numerical aperture (NA) of 0.75. The fluorescence collected by the objective lens passes through a dichroic mirror (Semrock ff509-fdi01, cut-off wavelength: 509 nm) and two notch filters (Semrock NF03-488E, center wavelength: 488 nm, OD6) and forms an image in the sensor plane of the scientific CMOS (sCMOS) camera (pco. edge 5.5) through a tube lens ($f = 180$ mm). Since the pixel size of the camera is 6.5 $\mu$m $\times$ 6.5 $\mu$m, the corresponding pixel size in the object space is 0.325 $\mu$m $\times$ 0.325 $\mu$m. A combination of a dichroic mirror (Semrock ff580-fdi01, cut-off wavelength: 580 nm) and a mirror having a slight relative angle difference is placed in the fluorescence light path so that images of different colors are produced at different positions in the direction perpendicular or parallel to the flow direction. The sCMOS camera is operated in the global reset mode. The timings of the camera’s exposure and laser beam illumination are synchronized by controlling the laser emission using external signals from the function generator triggered by the camera’s external output signal so that the fluorescence image is incident on the camera during its exposure time. The pulse duration of the excitation beam is set as ~300 ns, which corresponds to the pixel dwell time of the fluorescence image at a flow speed of 1 m/s. The region of interest (ROI) of the camera is 2,560 $\times$ 160 in the flow and transverse directions, respectively, which corresponds to the FOV of 832 $\mu$m $\times$ 52 $\mu$m. The maximum ROI is chosen in the flow direction to take the full advantage of the data transfer rate of the sCMOS camera. The ROI in the transverse direction is chosen so that the maximum frame rate is large enough to capture all the cells in the 1-m/s flow. In fact, the maximum frame rate with the ROI of 2,560 $\times$ 160 is ~1,250 frames/s, enabling image capture with a spatial interval of 800 $\mu$m, which is smaller than the FOV in the flow direction.

Figure 3(a) shows a schematic of the microfluidic chip that consists of a hydrodynamic focuser and an optical interrogation region. The hydrodynamic focuser employs Inlet 1 for the sample flow and Inlet 2 for the sheath flow [phosphate-buffered saline (PBS) for cancer cells and water for microalgal cells] to focus cells in the in-focal-plane (y) direction. The width of the sample flow in the y direction is determined by the ratio of the sheath flow to the sample flow (typically, 4.5 $\mu$m in our experimental conditions), while the width in the depth (z) direction of the sample flow is the same as the height of the microchannel. The optical
interrogation region consists of a microchannel, an aluminum-coated glass, and an alignment guide for accurate fabrication accompanied by its dimensions.

The fabrication process of the mirror-embedded microfluidic chip is shown in Fig. 3(b). First, a mold structure is created on a silicon wafer by a soft lithography process using a photoresist and a mask pattern. The mold structure is used for creating a microchannel and placing an embedded mirror at an accurate position. Second, a trapezoidal prism having an aluminum-coated slant surface is placed on the silicon wafer. Since the 90-degree edge of the prism is dabbed against one edge of the mold structure when it is placed on the wafer, the mirror is positioned with a high precision of ~10 μm, which is determined by the precision of fabricating the mold structure and prism. Third, uncured PDMS is poured onto the silicon wafer, degassed in a vacuum chamber, and cured by the following steps: (i) leaving it at room temperature for one day and (ii) baking it at 80°C for 4 hours. Finally, the PDMS slab is cut and peeled from the silicon wafer. The peeled PDMS and a glass substrate are exposed to oxygen plasma and bonded. Figure 3(c) shows a picture of the fabricated microfluidic chip while Fig. 3(d) shows a zoomed picture of the microchannel and aluminum-coated glass, indicating the precise alignment of the aluminum-coated glass to the microchannel.

For a proof-of-principle demonstration of the high-throughput on-chip imaging flow cytometer, we used *E. gracilis* as a test sample. *E. gracilis* is a species of flagellated eukaryotes in Excavata found in freshwater and has been used in biological research as a model organism for decades [17–20]. Specifically, *E. gracilis* NIES-48 was provided by the Microbial Culture Collection at the National Institute for Environmental Studies [21]. The cells were cultured in a nitrogen-deficient medium for 5 days and are regarded as *E. gracilis* in the “nitrogen-deficient condition” [17]. For the detection of intracellular lipid bodies, we prepared a stock solution of 1 mM BODIPY 505/515 (Thermo Fisher Scientific, USA) in dimethyl sulfoxide with 1% ethanol. Both the nitrogen-sufficient and nitrogen-deficient *E. gracilis* cells with a concentration of ~10^6 cells/mL were prepared by concentrating cultured cells with a centrifuge and were stained with 10 µM BODIPY 505/515 in de-ionized water followed by incubation in the dark for 30 min. The concentration before the centrifugation was confirmed using a cell counting plate. The cells were pumped by a syringe pump (Harvard Apparatus, 75-4500) from a syringe (Helmet, 60373-860) to the mirror-embedded microfluidic chip through a PEEK tube (Upchurch Scientific, 53500-746).

We also used human cancer cells [human breast adenocarcinoma (MCF-7) cells and human colorectal adenocarcinoma (HT-29) cells] as test samples. Both cells were routinely cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) (Wako Pure Chemical Industries, Ltd.) supplemented with 10% fetal bovine serum (MP Biomedicals), 1% penicillin streptomycin (Wako Pure Chemical Industries, Ltd.), 1% Sodium Pyruvate (Thermo Fisher Scientific, USA), and 1% GlutaMAX (Thermo Fisher Scientific, USA) at 37°C in a 5% CO₂ atmosphere. 3 days after seeding, the adherent cells were washed with PBS (Wako Pure Chemical Industries, Ltd.) and peeled off via trypsinization. The suspended cells (~10^6 cells/mL) were stained with 20 µM of CellTracker Red (Thermo Fisher Scientific, USA) and 0.2 µM of SYTO16 (Thermo Fisher Scientific, USA) in PBS and followed by incubation in darkness for 1 hour at 37°C in a 5% CO₂ atmosphere. The excess fluorescent reagent was removed by centrifugation. The cells were introduced into the microfluidic chip in the same manner as above.
3. Experimental results

In order to validate the sensitivity improvement of our method, we obtained fluorescence images of the microchannel filled with a fluorescein isothiocyanate (FITC) solution under the light-sheet illumination and wide-field illumination as shown in Fig. 4(a). It is clear that fluorescence signals were obtained in the entire FOV of 832 μm × 52 μm with the light-sheet illumination. The intensity of the fluorescence at the corners of the FOV with the light-sheet illumination was ~10% of that of the center of the FOV, which is consistent with the theoretical estimation.

Next, we obtained autofluorescence images of unstained *E. gracilis* cells [Fig. 4(b)] to validate the imaging capability with the sensitivity improvement shown in Fig. 4(c). The intracellular distribution of chlorophyll was obtained by both wide-field excitation and our method, but an average of the maximum intensity of 10 cell images in the vicinity of the center of the FOV was improved by a factor of ~10 with the light-sheet illumination with an identical power of the excitation laser. This improvement is due to the higher energy density of the light-sheet excitation beam than that of the wide-field excitation beam and is consistent with the designed enhancement factor of the excitation efficiency \((13)\) as described above. Accounting for the sensor noise level of the sCMOS camera and the photon noise (shot noise), the SNR improvement was estimated to be ~3.6.

To demonstrate high-throughput imaging flow cytometry, we performed classification of *E. gracilis* cells cultivated under nitrogen-sufficient and nitrogen-deficient conditions. We stained intracellular lipids of the cells with BODIPY505/515. We loaded the cells suspended in water to the sample inlet at a flow rate of 30 μL/min and water to the sheath flow inlet at a flow rate of 300 μL/min. We extracted rectangular image areas of the two fluorescence colors (green channel for lipids and red channel for chlorophyll) for each cell from an acquired raw image \((2,560 \times 160 \text{ pixels})\), leading to two-color images of the cells flowing at a speed of 1 m/s. This value corresponds to a cell throughput of 10,000 cells/s assuming 100-μm cell spacing. As shown in Fig. 5(a), we obtained a large number of two-color fluorescence images \((N = 3,000)\) for both cultures. The areas of fluorescence were digitally segmented by the following steps: (i) low-pass filtering, (ii) standard automatic segmentation implemented in LabVIEW’s Vision Development Module, (iii) removal of small subareas caused by noise components of the images. Their morphological features (area, perimeter, average intensity, etc.) were calculated and recorded as a vector data set for each cell. Figure 5(b) shows a scatter plot of the cells with two morphological features of the cells (lipid area and chlorophyll area) where a support vector machine (SVM) was applied to classify the cells.
The two cell types were successfully classified with a high accuracy of 83.1%. Additionally, we used the SVM with ~100 morphological features obtained from each cell to find a meta-feature that optimally classifies the two cell types, providing a further improved classification accuracy of 98.6%. A histogram of the cells with the meta-feature is shown in Fig. 5(c), which represents the improved classification accuracy by virtue of the increased number of morphological features. These results indicate the ability of our method to differentiate between different cell types via the identification and SVM of the morphological features of the lipids and chlorophyll, which is not possible with conventional non-imaging flow cytometry.

Moreover, we performed classification of cancer cells using human breast adenocarcinoma (MCF-7) cells and human colorectal adenocarcinoma (HT-29) cells. We stained their cytoplasm and nuclei using CellTracker Red and SYTO16, respectively. We loaded the cells suspended in water to the sample inlet at a flow rate of 30 μL/min and water to the sheath flow inlet at a flow rate of 300 μL/min. We extracted rectangular image areas of the two fluorescence colors (green channel for the nuclei and red channel for the cytoplasm) for each cell from an acquired raw image (2,560 × 160 pixels), which correspond to two-color images of the cells in a flow at a speed of 1 m/s. As shown in Fig. 6(a), we obtained a large number of two-color fluorescence images (N = 2,800) for both types. Again, the areas of fluorescence were digitally segmented by the same method shown above. Their morphological features (area, perimeter, average intensity, etc.) were calculated and recorded as a vector data set for each cell. Figure 6(b) shows a scatter plot of the cells with two morphological features (cell area and nucleus-to-cytoplasm ratio), manifesting the statistical difference between the two types. Again, we used the SVM with ~100 morphological features obtained from each cell to find a meta-feature that optimally classifies the two cell types, providing a high classification accuracy of 91.5%. A histogram of the cells with the meta-feature is shown in Fig. 6(c), which represents the improved classification accuracy by virtue of the increased number of morphological features.

![Image](image-url)
4. Discussion

The imaging sensitivity of the present setup is ultimately limited by the saturation and photobleaching of fluorescence emission. If photobleaching is negligible, which is usually the case, the sensitivity can further be improved by using an excitation laser with higher output power. In fact, the saturation typically occurs at the excitation light intensity of \( \sim 10^5 \text{ W/cm}^2 \), which corresponds to the excitation light power of \( \sim 12 \text{ W} \) at the sample [24]. It should be noted that the saturation power decreases as the excitation light is more confined, indicating that the sensitivity limit tends to be lower in an imaging setup with a higher magnification. On the other hand, if photobleaching occurs during the excitation, the sensitivity improvement is suppressed even with an excitation laser with higher output power.

Although we demonstrated the highly accurate classification of cell populations using our imaging flow cytometer, the accuracy can further be improved. First, compensating for the non-uniformity of the excitation beam intensity in the FOV will suppress errors in the morphological features related to the fluorescence intensity. This can be done by correcting the pixel values of cell images using the excitation beam profile obtained as an image of a fluorescence dye solution such as Fig. 4(a). Second, cell focusing in the depth direction will decrease the number of out-of-focus images in the image data set, suppressing errors in morphological features such as cell area and perimeter. This can be performed by cell focusing techniques such as 3D hydrodynamic focusing or acoustic focusing [25,26]. In addition, it will also improve the effective cell throughput because the cells are efficiently aligned in the focal plane, which will also effectively improve the classification accuracy due to an increased number of collected images. Third, optimization of the digital segmentation process may improve the classification accuracy.

While we employed the light-sheet excitation configuration in our imaging flow cytometer for sensitive imaging with a DOF comparable to a standard wide-field imaging setup, the imaging flow cytometer can also be used for obtaining sectioned images, dark-field images, or 3D images in a similar manner as typical light-sheet microscopy, which further extends potential applications of the method [11,27–29]. The sectioned images or 3D images can be obtained by an excitation light-sheet beam having thickness smaller than the DOF of the imaging setup at the cost of the FOV in the light propagation direction. In fact, implementation of 3D light-sheet microscopy using a mirror-embedded sample holder has been reported previously [11,28,29]. Additionally, a higher sectioning capability can be obtained while retaining the FOV by employing non-diffractive beams such as Bessel beams or Airy beams [30,31] although a careful optical design is needed for high sensitivity.

Similar to other imaging flow cytometers, the limited DOF of our imaging flow cytometer can potentially cause errors in image data analysis due to the lack of information about cell structure in the out-of-focus regions. A typical example where the errors will be apparent is fluorescence in situ hybridization (FISH), which requires accurate localization of fluorescent spots in a nucleus [6]. In such a case, extended DOF methods such as wavefront coding is highly effective, which can readily be incorporated to our setup [6].

By virtue of flexibility in designing a PDMS chip, our mirror-embedded microfluidic device has a wide variety of applications in life science and bioengineering [32]. For example, the device can be integrated in a large-scale PDMS-based lab on a chip as a cell analysis unit together with other functions such as cell culturing or cell sorting [33]. The device itself can also be used as a sample holder for light-sheet microscopy by appropriate modification of the channel dimensions and the spatial profile of the excitation beam [28].
Fig. 6. Image-based classification of MCF-7 (human breast adenocarcinoma) and HT-29 (human colorectal adenocarcinoma) cells. (a) Obtained images of the cells. Top: MCF-7 cells, Bottom: HT-29 cells. Green: nucleus stained by SYTO16. Red: cytoplasm stained by CellTracker Red. (b) Scatter plot of the cells (N = 2,800 for each culture). (c) Histogram of the cells with a meta-feature obtained by a support vector machine.

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**Disclosures**

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