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High-throughput microfluidic imaging flow cytometry

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Recently, microfluidic-based flow cytometry platforms have been shown to be powerful tools for the manipulation and analysis of single cells and micron-sized particles in flow. That said, current microfluidic flow cytometers are limited in both their analytical throughput and spatial resolution, due to their reliance on single point interrogation schemes. Conversely, high-speed imaging techniques can be applied to a wide variety of problems in which analyte molecules are manipulated at high linear velocities. Such an approach allows a detailed visualization of dynamic events through acquisition of a series of image frames captured with high temporal and spatial resolution. Herein, we describe some of the most significant recent advances in the development of multi-parametric, optofluidic imaging flow cytometry for the enumeration of complex cellular populations.

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Introduction

Flow cytometry is a widely used analytical technique for counting, examining and sorting cells suspended within a stream of fluid. Because of the quantitative and multi-parametric nature of the basic method and analytical throughputs of up to 50 000 cells/s, flow cytometry is rightly considered to be the gold standard method for counting and identifying cells within heterogeneous samples. In its most basic embodiment, flow cytometry involves the 'one-by-one' measurement of cells or micron-sized objects as they move through an optical probe volume at high velocity. Such an analysis yields a signal during the time-of-passage that is proportional to a particular parameter of interest. This basic approach allows measurement of a number of properties of individual cells, in a manner not possible using conventional techniques [1]. Unsurprisingly, flow cytometry has been used to measure a diversity of physical and chemical characteristics of individual cells [2] in a range of applications, including the diagnosis of blood cancers [3], DNA sequencing [4], T cell phenotyping [5] and the detection of rare cells [6].

All flow cytometric methods incorporate two basic steps, namely cell focusing and cell detection. Put simply, cells must first be focused into a single file arrangement within the center of fluid flow, and then made to pass through the focal plane of a detection system such that no more than one cell occupies the detection volume at any instant. This approach enables the serial examination of cells of interest through their optical (fluorescence emission or scatter) signature. Significantly, the approach enables examination of size, morphology (e.g. shape, internal complexity) and biochemistry (e.g. cell cycle distribution and DNA content) in a non-destructive, high-throughput and quantitative manner [7]. Unfortunately, although conventional fluorescence cytometers allow for the high throughput quantitation of cellular populations, they are costly, mechanically complex, consume large sample and reagent volumes (due to the use of sheath flows) and require trained personnel for both operation and maintenance [8]. Indeed, current designs incorporating sheath fluids and conventional optics are bulky, expensive to operate and difficult to parallelize. These features drastically limit the use of conventional flow cytometers in key biological applications, such as the identification of circulating tumor cells in blood, which are typically present at abundances of less than one per one billion cells [9].

To address the aforementioned issues, much recent activity has focused on the development of microfabricated flow cytometers that integrate inexpensive optical components, but are able to rapidly count cells and probe cellular populations at the single cell level. With regard to cell-based analyses, such microfluidic systems offer a range of advantages, including reduced instrumental footprints, minimal sample/reagent consumption, low unit cost and facile implementation in remote environments [[10,11,12,13,14•]. Additionally, the adoption of planar, chip-based microfluidic formats enables the direct integration of a variety of optical and detector components (such as microlenses) for probing cellular

characteristics such as nuclear morphology [15]. Despite the fact that many microfluidic flow cytometers are characterized by exceptional sensitivities, analytical throughputs rarely exceed a few thousand cells per second [16]. Indeed, optical detection schemes based on single point illumination are inherently serial in nature, and thus will always be limited in terms of analytical throughput due to pressure and shear effects [17]. In addition, conventional flow cytometers are unable to extract spatially resolved information that is often critical in quantifying complex cellular phenotypes [10,18,19]. To this end, the development of image-based microfluidic flow cytometers provides an opportunity to develop novel and high-efficiency platforms that combine the high-throughput nature of conventional flow cytometry techniques with the spatial resolution of optical microscopy. As noted, integration of an imaging modality within a flow cytometer offers significant advantage over single point interrogation or illumination schemes, since cellular morphology analysis plays an important role in the identification and assessment of a range of disease states [20,21••,22,23]. Additionally, signals arising from coincident events or debris within the analytical sample, that are often highly problematic in conventional flow cytometry, can be eliminated through real-time acquisition and analysis of cellular images.

Methods for cell focusing

A microfluidic flow cytometer should incorporate robust (and simple) fluidics for the efficient manipulation, ordering and positioning of cells. Figure 1 provides an overview of some of the most important microfluidic-enabled tools for focusing and imaging large numbers of single cells in flow. As previously noted, robust control over the position of each cell within the flow path ensures that each cell transits the optical detection volume with a consistent trajectory and at a constant velocity. This is a particularly critical aspect for imaging flow cytometry since the limited depth of field of a microscope means that all the cells to be imaged should be tightly confined to the imaging plane (whose depth is defined by optical characteristics of the objective).

Under normal circumstances, particles or cells entering a microfluidic device are distributed in a random fashion across the channel cross-section, and owing to the parabolic nature of hydrodynamic laminar flows, cells will travel downstream at widely varying velocities [24]. Unsurprisingly, hydrodynamic focusing using simple sheath flows has often been used to control cell position in a direct manner [25]. For example, 3D sheathing strategies (Figure 1a) have been realized via the fabrication of multi-layered microfluidic devices [16,26]. Commercial imaging flow cytometers such as the *Amnis Imaging Flow Cytometer* range provided by Merck Millipore use such an approach to focus cells and subsequently employ wide-field illumination and CCD camera detection to collect blur-free images of cells moving at moderate to high velocities [27]. Nevertheless, it should be noted that the use of multiple sheath flows is problematic, since fluctuations in input flow-rates will lead to disturbances in the focused stream, which may in turn direct it away from the detection plane.

It is well recognized that microfluidic architectures are highly efficient in inducing vorticity and local disturbances in the flow in a passive manner. Such a strategy has been leveraged to good effect by Golden *et al.*, who incorporated chevron shaped grooves and alcoves within microfluidic channels to sculpt and position sheath flows around a sample flow (Figure 1b) [28•]. Importantly, advection generated by the grooves can be used to precisely define the size, shape and position of the focused flow with only two inlets. In addition, Lee *et al.*, used a series of channel expansions and contractions to induce a secondary Dean flow that is able to control the position of the sample stream (Figure 1c) [29]. Similarly, Huang and co-workers developed a microfluidic flow cytometer that uses a combination of hydrodynamic sheath and Dean flows to control the axial position of the cells within a microfluidic channel (Figure 1d) [14•,30]. Inertial focusing within low Reynolds number environments provides an efficient and sheathless approach to controlling the position and velocity of cells moving through microfluidic channels, and is therefore of significant utility in developing robust microfluidic flow cytometers [31]. Moreover, extended channel curvature can be used to concurrently induce both Dean forces and shear-stress induced lift forces to control the position of cells within continuous flows (Figure 1e,f) [32••,33]. It is important to note that the use of inertial forces has already shown significant utility in passive cellular manipulation in ultra-high-throughput imaging-based flow cytometry [34••]. Here, lateral migration can be used to robustly position cells in the center of a microfluidic channel, while passive self-assembly ensures that the frequency of passage (or packing) of single cells through the optical detection volume is maximized to ensure high throughput measurements [35]. The passive manipulation of cells, without the use of external fields or sheath flows, engenders uniform flow velocities, facile cell positioning and exquisite control over cell spacing, and in many ways represents an ideal solution to achieving, high-throughput single cell analysis [36••,37•,38••]. However, it should be noted that pure inertial focusing is normally only accessible when operating at high volumetric flow rates. In this respect, the adoption of elasto-initial focusing techniques [39,40] allows for efficient focusing at significantly lower volumetric flow rates, which is advantageous for high resolution imaging applications (Figure 1g). Interestingly, Holzner and co-workers elegantly demonstrated the utility of viscoelastic fluids for enhanced elasto-inertial focusing of cells within straight, rectangular cross section microfluidic channels, using low molecular and low