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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.

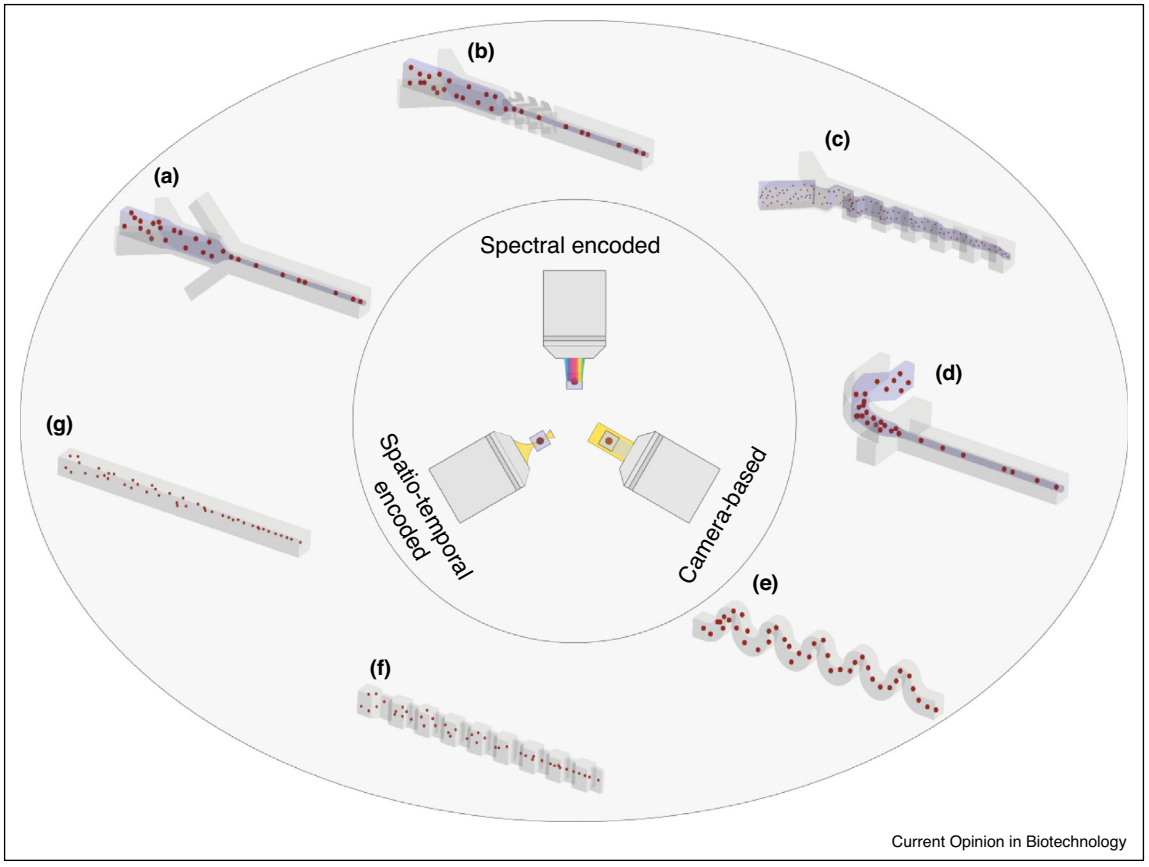
Methods for cell focusing

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

Figure 1



Schematic overview of microfluidic-based cell focusing techniques and the primary imaging detection modules. **(a)** 3D hydrodynamic focusing [16,26]. **(b)** 2D hydrodynamic focusing using chevron shaped grooves to establish 3D sheath flow [28•]. **(c)** A contraction–expansion geometry combined with a sheath flow [29]. (d) Combination of Dean (curved sections) and sheath flows [14•]. **(e)** Serpentine channel [32••]. **(f)** Multi-square contraction–expansion geometry [33] and **(g)** elasto-inertial focusing in a rectangular straight channel [41]. (a–f) Methods for focusing of specimen in Newtonian fluids and (g) non-Newtonian fluids. Central part: the three core techniques currently utilized in imaging flow cytometers are based on spectral encoding, spatial to temporal transformation and multi-spectral based camera imaging.

viscosity PEO solutions [41]. A particularly valuable feature of this method is that cells may be focused without inducing bodily rotation. This is especially useful in morphology-based analysis of disease-infected cells, and bodes well for the realization of next generation imaging cytometry platforms in the short term.

Detection modalities of imaging flow cytometers

The lack of spatial resolution in single point interrogation cytometers has led to the development of new platforms that combine the high-throughput nature of conventional flow cytometry with the spatial resolution of optical microscopy. We now describe the most common detection methodologies used in contemporary imaging flow cytometry (Figure 1, inner circle).

Camera-based imaging flow cytometers

The integration of an imaging modality within a flow cytometer offers significant advantages over a single point interrogation scheme in regard to the collection of morphological and textural information within single cells. A primary challenge faced when integrating imaging-based detection schemes within a flow cytometer is the minimization of optical blur caused by cells moving at high linear velocities. The first commercially available (ImageStream®) imaging flow cytometers made use of epifluorescence excitation schemes and time delay and integration (TDI) cameras to provide for high resolution and multiplexed imaging [27], but at the cost of reduced analytical throughputs (no more than 3000 cells/s) due to relatively long effective residence times in the detection volume.

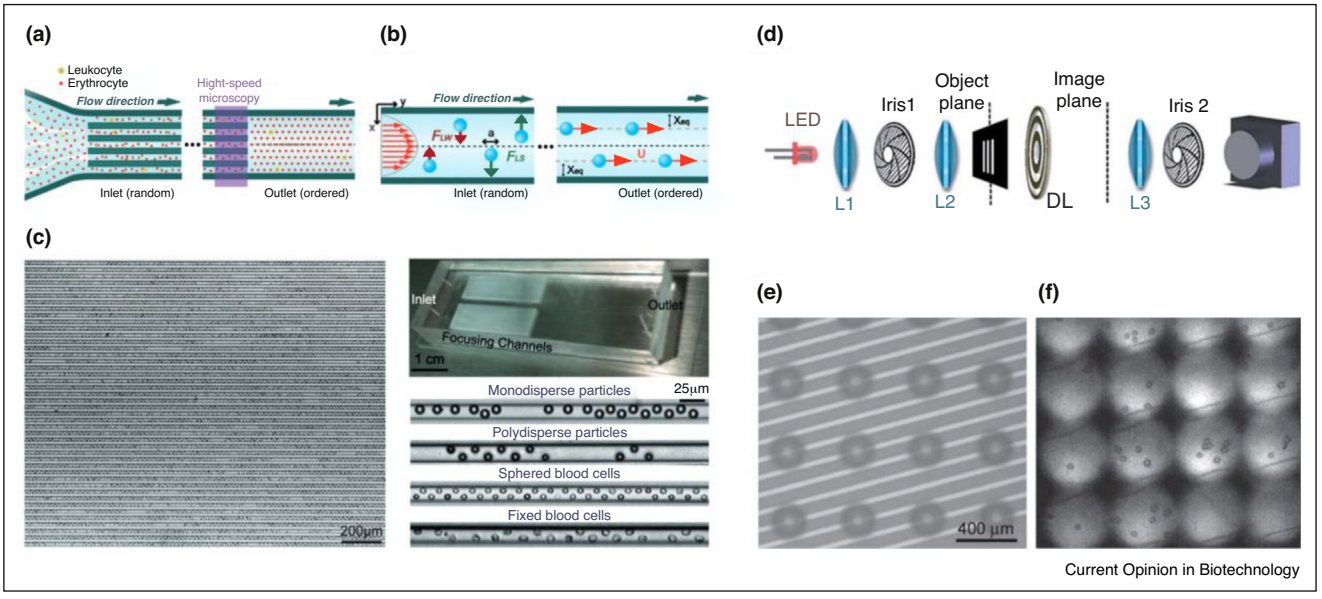
Although conventional imaging flow cytometers provide for efficient analysis of single cells at low-moderate

throughput, they are expensive and require enormous volumes of sheath fluid and trained personnel for operation. Fortunately and as previously described, the basic principles underpinning the method of flow cytometry are directly transferrable to planar, microfluidic formats. For example, Di Carlo and co-workers presented a label-free, high-speed imaging flow cytometry platform integrating sheathless analyte focusing for the analysis of blood cells at extreme throughput [34••] (Figure 2a–c). However, the low resolution associated with bright-field measurements prevented use of the cytometer in morphological or multiparametric studies. Alternatively, Schonbrun *et al.* reported moderate imaging throughput (approximately 2275 cells/s) using a microfabricated platform containing arrays of microfluidic channels and diffractive lenses that generate widefield images with a magnification of 45× and sub-micron resolution (Figure 2d–f) [42•]. Moreover, Heng and co-workers introduced a line-scanning, optofluidic imaging technique incorporating a precisely-defined (line-shaped) focal spot for illumination and a line scan CMOS camera for detection. The combination of both components, enabled the concurrent collection of multiple fluorescence channels, whilst maintaining both high resolution and moderate throughput (1000 cells/s) [43]. An ultra-high throughput imaging cytometer based on inertial focusing (for sheathless manipulation of cells) and stroboscopic illumination (for blur-free imaging at high linear velocities) was recently introduced by Rane *et al.* [38••] (Figure 3a). By leveraging control of the applied volumetric flow rate, a parallel microfluidic architecture and synchronization of the camera frame rate, the authors were able to achieve an analytical throughput of 85 000 cells/s, whilst demonstrating multicolor fluorescence detection and accurate cell sizing at throughputs in excess of 50 000 cells/s. More importantly, the general method enables multi-parametric analysis of cells, incorporating bright-field and dark-field imaging for sizing and morphology, and multi-color fluorescence detection for intra-cellular imaging. To showcase the efficacy of the approach, the authors subsequently applied the technique to the rapid enumeration of apoptotic cells and the high-throughput discrimination of cell-cycle phases for various cell lines. Use of a more powerful excitation source would undoubtedly improve achievable resolution and contrast, and thus stroboscopic imaging flow cytometry represents an appealing platform for high-throughput image-based cellular assays.

Photodetector-based imaging cytometers

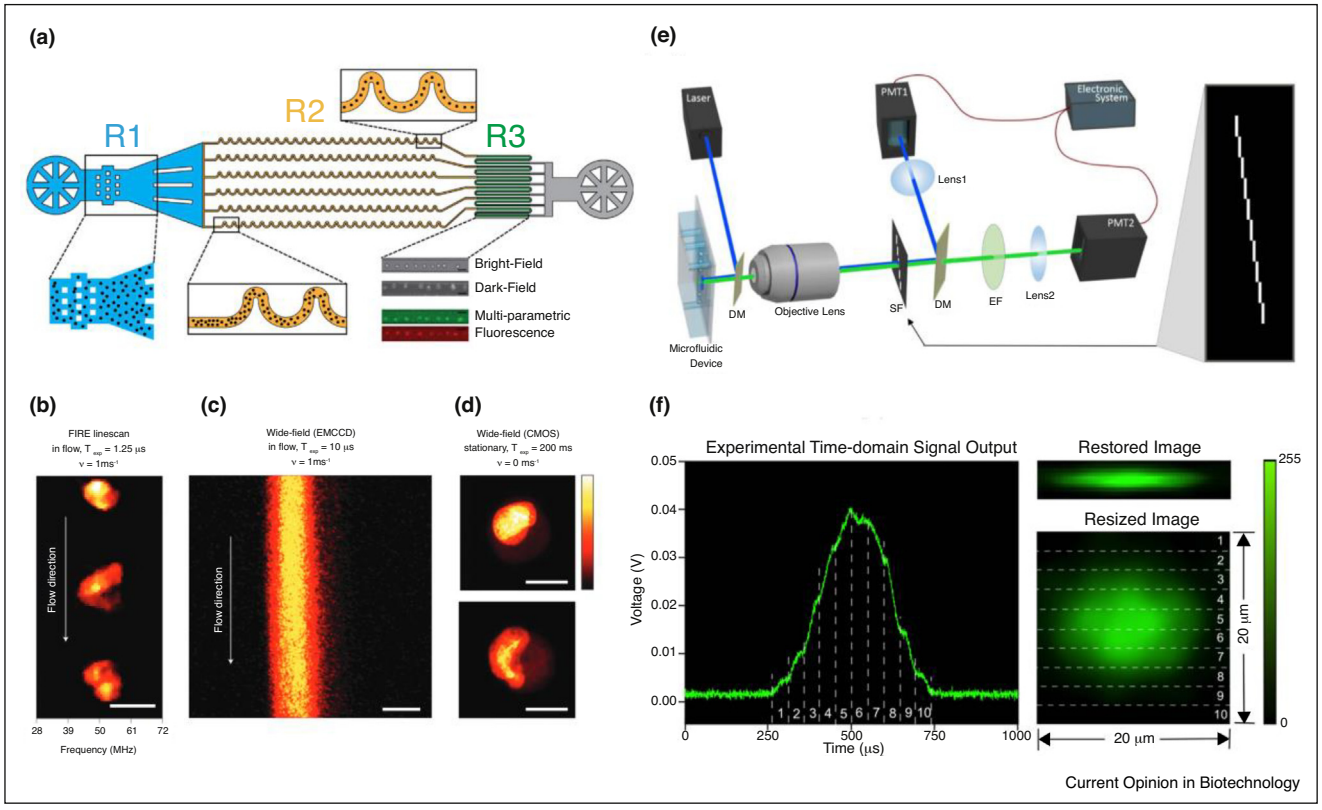
Camera-based approaches offer a direct route to imaging single cells at high analytical throughput. However, such approaches are compromised by the relatively weak fluorescence emission from fluorescent probes and the high speed of cells through the detection volume. In simple

Figure 2



High-speed bright-field microfluidic imaging methods. Left: A high throughput, bright-field imaging flow cytometer. **(a)** Schematic shows high-throughput cell ordering in parallel, straight microchannels. **(b)** The competition of two opposite lift forces results in particle focusing at a defined lateral position. **(c)** The microfluidic device consists of 256 parallel channels (left image) for bright-field imaging of cells and images of particles and blood cells flowing in a single microchannel. (Image adapted from Ref. [34••] with permission from the Royal Society of Chemistry.) Right: Multiple field-of-view imaging flow cytometer. **(d)** Diffractive lens wide-field imaging system. **(e)** Object plane of the optofluidic device consisting of an array of parallel channels and microlenses. **(f)** Sixteen imaging planes of the optofluidic device. (Reproduced from Ref. [42•] with permission from the Royal Society of Chemistry.)

Figure 3



High-speed fluorescence imaging methods. **(a)** Schematic of a microfluidic flow cytometer with inlet port (blue), inertial focusing channels for the sheathless manipulation of the cells (orange), imaging area for the detection (green), and the outlet port for the collection (gray). Representative images from all the possible imaging modalities of the detection: bright-field imaging, dark-field imaging and the two-color fluorescence imaging based on fluorescence labeling. All scale bars represent 30 μm. (Image adapted from Ref. [38••] with permission from Cell Press.) **(b)** Representative FIRE images of cells flowing at a velocity of 1 m/s. **(c)** 10-μs exposure images of individual cells recorded with an EMCCD flowing at a velocity of 1 m/s. Blur is observed in the image due to the long exposure time and the frame transfer nature of the EMCCD. **(d)** Representative widefield fluorescence images of stationary cells. All scale bars are 10 μm. Reproduced from Ref. [48••] with permission from the Nature Publishing Group. **(e)** Implementation of spatial–temporal transformation-based imaging flow cytometry. Schematic diagram of the imaging flow cytometer system with a spatial filter design consisting of slits. **(f)** Time-domain PMT output signal of fluorescence from a cell, corresponding restored fluorescence and corresponding resized fluorescence images showing the true size of the cell. (Reproduced from Ref. [49••].)

terms, this leads to a trade-off between imaging speed and sensitivity. For these reasons, over the past decade, photomultiplier tubes (PMTs) have commonly been used as small field of view detectors in scanning confocal microscopes, and have demonstrated both high spatial resolution and sensitivity when compared to conventional cameras. Additionally, a number of photodetector-based platforms have been proposed for use in imaging flow cytometry. These are largely based on optical time-stretch imaging, which enables the capture of fast events in real time at a line-scan rate of tens of MHz.

A unique microfluidic flow cytometer that combines 384 parallel channels with one-dimensional multicolor confocal fluorescence imaging was introduced by Mckenna and co-workers for use in rare-cell detection applications [44]. Although successful in identifying cells expressing parathyroid hormone receptor, the low analytical throughput (approximately 1000 cells/s) and fluorescence image resolution limited its use to applications not requiring morphology and sizing information. To overcome the challenge of preserving image quality at high throughput, Goda *et al.* developed an ultrafast, continuous imaging technique (based on a point detector and an optoelectronic image-encoding/decoding time-stretching method) for high-throughput image-based cancer cell screening [36••]. The platform could theoretically operate at throughputs approaching 10^5 cells/s, and was able to detect rare mammalian cells (labeled with 1-μm metal beads attached to specific antibodies) in diluted blood samples. Image-based cell identification was sensitive enough to detect one labeled MCF7 cell amongst a million white blood cells, with a false-discovery rate two orders of magnitude smaller than those associated with conventional fluorescence flow cytometry. However, it should be noted that bright-field imaging involves use of

longer wavelengths (1590 nm in the reported studies), and is thus less suited to extracting high-contrast images of cell morphology. Indeed, label-free approaches primarily provide information relating to size and morphology and lack the sensitivity of fluorescence-based methods. As a result, the effective use of time-stretch imaging has to date been limited to particle or cell screening when the cells of interest are labeled with contrast agents. To address this issue, Tsia and colleagues presented a novel imaging technique, called asymmetric-detection time-stretch optical microscopy (or ATOM), as a route to capturing label-free high-contrast cell images, with sub-cellular resolution and at a flow speeds of 10 m/s (which corresponds to a predicted imaging throughput of 100 000 cells/s) [45]. Based on conventional time-stretch imaging, which relies on all-optical image encoding and retrieval through the use of ultrafast broadband laser pulses, ATOM further advances imaging performance by improving image contrast of unlabeled/unstained cells. This is achieved by accessing phase-gradient information, which is spectrally encoded into single-shot broadband pulses. The technique has been applied to high-throughput phytoplantkton screening, with classification based on intracellular texture/morphology [46]. As with the majority of the time-stretch imaging methods, ATOM is not immediately applicable to fluorescence imaging. Nevertheless, it should be noted that a free-space pulse-stretching concept (free-space angular-chirp-enhanced delay, FACED) resembling conventional time-stretch imaging, has recently been used for ultrafast laser-scanning imaging in ATOM, as well as for fluorescence imaging at visible wavelengths [47]. This approach enables ultrafast, high-quality time-stretch imaging at 700 nm, solving the problem of time-stretch imaging in the visible regime.

Interestingly, there have been a small number of recent studies that have addressed the issue of fluorescence imaging detection at high speed. Specifically, Diebold *et al.* reported an imaging technique that realizes real-time pixel readout rates significantly higher than achievable using conventional cameras by mapping images into the radiofrequency spectrum via the beating of digitally synthesized optical fields [48••]. Using such an approach, the authors achieved blur-free fluorescence imaging of cells moving at 1 m/s, and with an extrapolated throughput of 50 000 cells/s (Figure 3b–d). Although the authors were able to achieve blur-free fluorescence imaging at high linear velocities, the complexity of the optical detection system severely limits widespread adoption in non-expert laboratories. Unfortunately, despite its obvious potential, fluorescence imaging using radiofrequency-tagged emission (FIRE) is also limited by signal bandwidth and strong shot noise, which yields poor signal-to-noise ratios. Alternatively, a confocal fluorescence microscope capable of recording at 16 000 frames/s was recently reported by Goda and co-workers [9]. To demonstrate its utility in biomedical applications, the authors presented 3D volumetric confocal fluorescence microscopy of cellular dynamics and confocal fluorescence imaging flow cytometry of hematological and microalgal cells at flow rates up to 2 m/s. Finally, Han *et al.* used a spatial-temporal transformation (via a mathematical algorithm) in conjunction with a specially designed spatial filter to endow conventional flow cytometers with imaging capabilities (Figure 3e,f) [49••]. Using this approach, high quality fluorescence and scattering images of cells moving at 20 cm/s were obtained at a throughput of approximately 1000 cells/s.

Conclusions

Imaging flow cytometry is an emerging experimental tool, with a wide host of applications in cellular diagnostics. In the current article, we have described and discussed some of the most important recent advances in this area, with a focus on the development of both bright-field and fluorescence microfluidic imaging cytometry. In the near future, we expect that imaging flow cytometers will begin to incorporate machine learning algorithms [50,51], so as to allow for high-content analyses in applications such as cell phenotype profiling, personalized medicine and drug development [52].

Conflict of interest

The authors declare that there is no conflict of interest.

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• of special interest

•• of outstanding interest

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