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TUTORIAL REVIEW

The Intersection of Flow Cytometry with Microfluidics and Microfabrication

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Menake E. Piyasena[a,b] and Steven W. Graves[a,c]

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A modern flow cytometer can analyze and sort particles on a one by one basis at rates of 50,000 particles per second. Flow cytometers can also measure as many as 17 channels of fluorescence, several angles of scattered light, and other non-optical parameters such as particle impedance. More specialized flow cytometers can provide even greater analysis power, such as single molecule detection, imaging, and full spectral collection, at reduced rates. These capabilities have made flow cytometers an invaluable tool for numerous applications including cellular immunophenotyping, CD4+ T-cell counting, multiplex microsphere analysis, high-throughput screening, and rare cell analysis and sorting. Many bio-analytical techniques have been influenced by the advent of microfluidics as a component in analytical tools and flow cytometry is no exception. Here we detail the functions and uses of a modern flow cytometer, review the recent and historical contributions of microfluidics and microfabricated devices to field of flow cytometry, examine current application areas, and suggest opportunities for the synergistic application of microfabrication approaches to modern flow cytometry.

[a] *Center for Biomedical Engineering, University of New Mexico, Albuquerque, NM USA*
[b] *Department of Chemistry, New Mexico Tech, Socorro, NM USA*
[c] *Department of Chemical and Nuclear Engineering, University of New Mexico, Albuquerque, NM USA*
E-mail: graves@unm.edu; FAX: 15052771979; TEL:15052772043

A. Introduction.

The purpose of this work is to provide a historical perspective of microfluidics within flow cytometry, detail how a modern flow cytometer functions, and to explore how recent efforts in microfluidics and microfabrication might be used to enhance flow cytometry for use in challenging areas. We do not focus

Menake E. Piyasena is an Assistant Professor in the Department of Chemistry at New Mexico Tech University (NMT). He is also an affiliate of the Center for Biomedical Engineering at the University of New Mexico. He received his B.Sc from University of Kelaniya, Sri Lanka and Ph.D. from University of New Mexico. He was a postdoctoral researcher at University of Maryland, and University of New Mexico before joining NMT. His current research interest includes development of microfluidic based diagnostic systems for pathogen detection, malnutrition, and environmental pollutants.

Steven W. Graves is an Associate Professor in the Center for Biomedical Engineering and Department of Chemical & Nuclear Engineering at the University of New Mexico. He has been a member of the National Flow Cytometry Resource (NFCR) at Los Alamos National Laboratory since 1999. He received his Ph.D from Pennsylvania State University in 1998 and conducted post-doctoral research within the NFCR from 1999-2001. He has received several NIH and NSF grants for the development of a variety of new flow cytometers. These efforts have resulted in several awards including an R&D 100 award in 2007.

on the construction of fully microfabricated flow cytometers or the full history of flow cytometry, both of which have been extensively considered. [1-6]

The field of flow cytometry was first conceived in 1934 and subsequently successfully applied in 1947.[7, 8] This early work served to provide the simplest definition of flow cytometry, which is the one by one measurement of cells or particles as they flow through an analysis volume. Such analysis generates a signal vs. time pulse that is proportional to the detection parameter of interest for each particle. The particle-by-particle analysis makes detection of discrete particle populations possible, which is not possible using bulk analysis techniques, and has made flow cytometry the primary method to count specific cell populations.[2, 3]

1. Early microfluidic flow cytometry

The fact that flow cytometry was developed primarily for the analysis of single cells has made it an inherently microfluidic technology. Notably, one of the most prominent examples of an early flow cytometer was also arguably among the first microfluidic devices (Fig. 1).[9] This device was developed by ultrasonically cutting a 100 x 100 μm square cross-section channel into a quartz microscope slide. As cells were drawn through this channel via a syringe pump they passed through the tightly focused spot of light where their absorbance profile was collected via high numerical aperture optics. The collected light was directed through a series of filters that selected for wavelengths of the cellular absorbance spectrum.[9] This instrument was able to analyze at rates of 1000 cells/s. Subsequently, a flow sorter that sorted cells based on their absorbance profile was constructed using ultrasonically machined microfluidic crossed channels that allowed cells of interest to be pulled onto a microfilter for offline microscopic analysis.[10] This enabled up to 300 cells of interest to sorted from large populations of cells and is the first example of a microfluidic cell sorter. This early instrumentation effort was extremely influential and most commercial flow cytometer analyzers use flow cells that have a rectangular channel with a cross section that is hundreds of microns on a side. Subsequent instrument modifications that included the collection of light scatter and fluorescence, use of lasers for excitation, and sheath fluid to focus the sample stream led to what is considered the typical modern flow cytometer.[1-3] However, these early microfluidic flow cytometers ushered in modern flow cytometry and it is clear that microfluidics and microfabrication will continue to play a major role in future advances.

2. Conventional flow cytometry analysis.

In a conventional flow cytometer, sheath fluid and sample are delivered to the flow chamber via pumps or pressure in the sheath tank and sample container (Fig. 2A).[11] Using differential pressures or pump flow rates, the sheath is delivered at a high volumetric flow rate relative to the sample, which

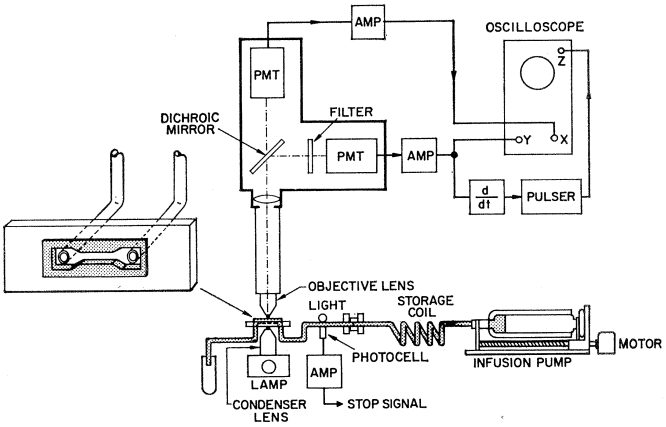

Fig. 1. A schematic of a microfluidic flow cytometer constructed in 1965 by Kamentsky et. al. and used to analyse cells at 1000 cells/s. The bowtie channel shown was ultrasonically cut in a cover slip. The narrow section between the inlet and outlet was 100 μm deep and 100 μm wide. From [L. A. Kamentsky, M. R. Melamed and H. Derman, Science, 1965, 150, 630-631]. Reprinted with permission from AAAS.

hydrodynamically focuses the sample stream in the focusing cone just prior to the flow cell (Fig. 2B). This results in a roughly 5 μm diameter sample stream flowing at linear velocities of up to 10 m/s through the channel of the flow cell, which is typically a few hundred μm on a side (Fig. 2C). This focusing serves to minimize the effects of characteristic parabolic flow profile of pressure driven flow, and ensures consistent linear velocities for particles through the analysis volume to enhance measurement precision (Fig. 2C).[12] Also, as the sample stream diameter approximates the diameter of a typical cell or microsphere, the particles under analysis essentially exclude the sample liquid at the sides of the sample stream and the sample liquid is stretched between the particles during analysis (Fig. 2C). For analysis, a light source, typically a laser, is focused using beam-shaping optics onto the flow cell (Fig. 2A). As the 5 to 10 μm high elliptical laser spot approximates the diameter of a cell or microsphere, the particle under analysis largely excludes sample (top to bottom) from the spot of the laser (Fig. 2D). Thus, the interrogation volume (defined by the intersection of the laser and sample stream) is filled by the cell or microsphere under analysis. This excludes liquid sample from the interrogation volume and enables a flow cytometer to measure many optical parameters, such as fluorescence or scatter, without wash steps [13, 14]. For fluorescence and side scatter, the actual analysis volume is defined by the field of view of the high numerical aperture (NA) optics (Fig. 2D). As such, optical signal from the system as a whole focuses into this analysis volume must be accounted for to maximize signal to background ratios.[15] Once collected via a high NA optical system, such as a gel coupled lens (Fig. 2C), the light emitted orthogonally to the exciting laser is spectrally split along the optical path using dichroic mirrors and filters and finally delivered to sensitive photodetectors such as photomultiplier tubes (PMTs) and avalanche photodiodes (APDs) (Fig. 2A) [3]. The side scatter signal is roughly proportional to cellular granularity while the forward scatter signal is *very* roughly proportional to particle size, though much care must be taken in interpreting these parameters.[3]

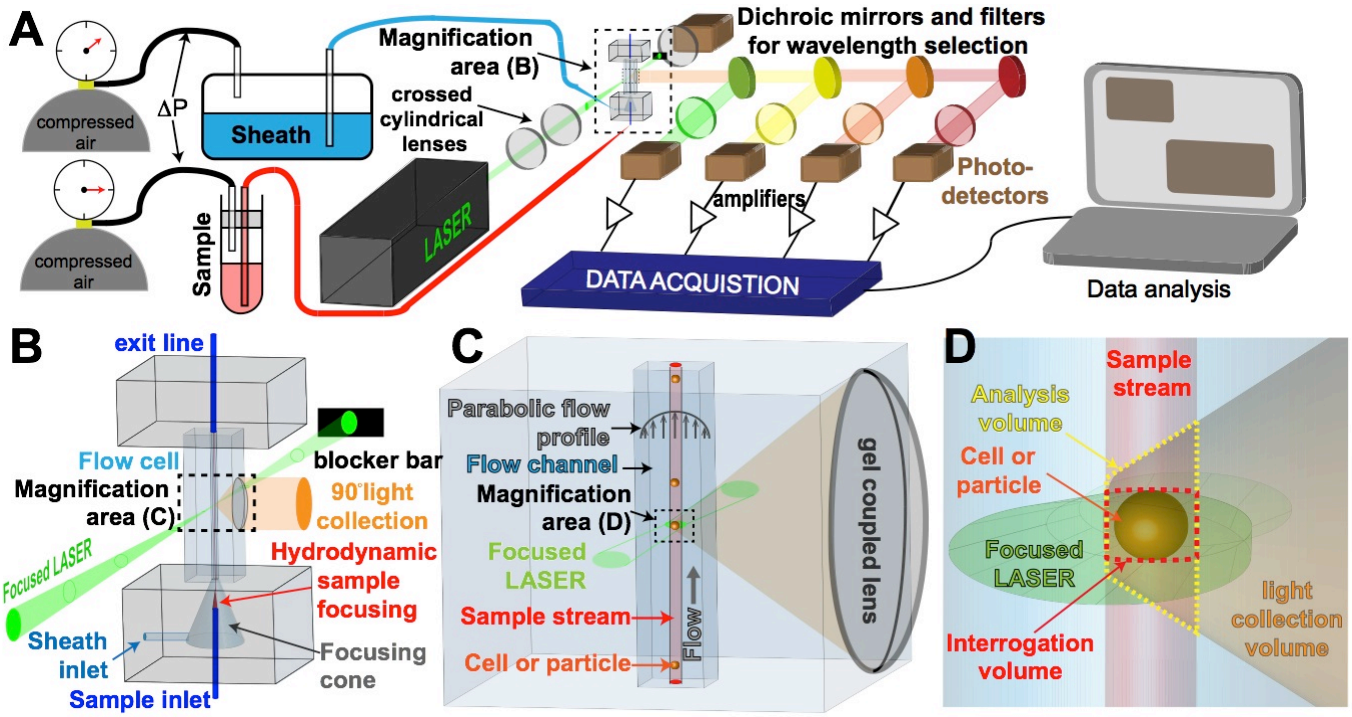

Fig. 2. A common flow cytometer analysis configuration. (A) shows an example using a pressure differential that allows for sample to be delivered slowly into a fast moving sheath stream. The laser is shaped and focused to the flow cell using crossed cylindrical lenses onto the flow cell. The dashed square is magnified in panel B, but the collected light is directed through a set of dichroic mirrors and filters to photodetectors that are connected to the data acquisition system. (B) A magnified view that shows the hydrodynamic focusing cone where the narrow diameter sample tube is surrounded by the fast moving sheath fluid. This results in hydrodynamic focusing of the sample stream as it passes through the flow cell. The focused light strikes a blocker bar in front of the forward scatter detector, which only allows low angle scattered light to reach the detector. (C) 90° light is collected via high numerical collection optics, which is often accomplished via a gel coupled lens. The hydrodynamic focusing ensures that the particle passes through the interrogation volume in the centre of the flow profile. (D) The interrogation volume is made up of the intersection of the laser with the flow stream, while the larger analysis volume is defined by the field of view of the collection optics.

Considering the typical laser beam height of 5 – 10 µm and particle linear velocity of 1 – 10 m/s, particle transit times through the analysis volume occur in a few µs and require high-speed data acquisition systems to accurately record optical signals (Fig 2A).[16, 17] Despite these rigorous technical requirements, commercial flow cytometers can analyze at rates of up to 50,000 particles/s and detect as few as fifty fluorophores per particle.[18, 19] Using reduced linear velocities, detection of a single fluorophore is possible [19, 20]. Furthermore, through the use of multiple interrogation points, lasers, and optical collection paths it is possible to collect as many as 19 optical parameters from a single particle [21].

2. Conventional flow cytometry sorting.

Though early flow cytometry sorters used a microfluidic approach to sort particles, electrostatic droplet sorting offered dramatically increased sorting rates. Though the first sorter was based on Coulter volume,[22] this approach was rapidly adapted to fluorescence based sorting,[23] and is the primary method of sorting in flow cytometry even today.[2] A typical flow sorter has a very similar optical path as in an analyzer (Fig. 2), but the flow cell has been modified to accommodate droplet based sorting (Fig. 3). Sorters can also directly analyze cells in the exiting jet of fluid just prior to droplet breakoff, which offers closer coupling between analysis and the sort decision. Though this offers increased sorting precision, the need for corrective optics and higher power lasers, reduces the sensitivity of this approach. Regardless of the approach for optical analysis, droplet based sorters use a charging collar (not shown in the figure) to charge the droplets, which, when a particle of interest is expected in the droplet, are then deflected using charged plates for collection below. Using variable field strengths, this approach can sort droplets into many collection wells and can sort at rates as high as 50,000 particles per second.[24] These

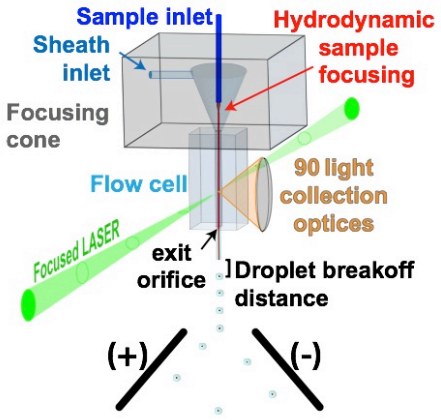

Fig. 3. A common flow sorting flow cell configuration. It is also possible to perform analysis directly in the jet as it exits the focusing region.

features allow for the high speed isolation of multiple particle populations simultaneously from a single sample.[24]

In addition to droplet based sorting there have been several other sorting approaches that include pneumatic, mechanical, and piezo based diversion sorting.[11] Pneumatic sorting has been developed to handle larger particles (> 400 μm) that require exit orifices too large to support traditional droplet based sorting and uses an air jet to divert particles not of interest into a waste chamber.[25] A widely applied method of mechanical sorting moved a sipper tube into the flow stream to collect particles of interest at rates of up to 300 per sec.[26] A fluidic diversion approach activated a piezo drive to induce a pressure wave that diverted the sample stream containing particles of interest into a collection channel as they flowed past.[27] As none of these alternative approaches offers the sorting rates afforded by droplet based sorting, they are not as widely adopted.

B. Microfluidics and microfabrication in flow cytometry.

The analysis and sorting of particles via flow cytometry has several highly interrelated steps, which include sample preparation, delivery, analysis, and sorting. There have been many microfluidic and microfabrication based optimization efforts for each of these steps, but the extreme variety of flow cytometry applications and number of proposed approaches makes describing each step unmanageable if all applications and approaches are considered simultaneously. Therefore, we describe microfluidic and microfabricated solutions for each step using a pertinent example and discuss variations for other notable applications.

1. Sample preparation

Microfluidic sample preparation is a field that is extremely broad and has been well reviewed.[28, 29] While we will not consider general sample preparation steps, we will discuss processes that have unique requirements or opportunities due to their use in flow cytometry analysis. These are optimization of particle concentration and rapid solution exchange around particles to enable washing or rapid mixing for kinetic analysis.

a. Optimization of particle concentration.

Particle concentration optimization in flow cytometry is performed to: a) avoid coincidences in the analysis volume for concentrated sample, b) reduce the event rate within the acceptable limits of the data acquisition system, c) shorten dilute sample processing time.

The most prominent example of an application that is too concentrated is white blood cell (WBC) population analysis in whole blood. This analysis is impossible in a typical flow cytometer due to the presence of about 5 x 10^9 red blood cells (RBCs) per ml of blood.[30] To reduce the concentration of RBCs, the most common approach is osmotic lysis of RBCs, which leaves the 5 x 10^6 WBCs per ml of blood largely intact for analysis.[2, 3] The WBC concentration may be further adjusted via dilution and wash steps may also be used to remove RBC debris (see below).[2, 3] Simple dilution is also possible for many applications, but this requires larger volumes of sample to be analyzed to measure the same number of WBCs. However, dilution is easy and alleviates concerns that osmotic lysis disproportionately affects rare cells.[30, 31] Nonetheless, whole blood must be diluted 100 fold to obtain about 10^7 cells per ml to avoid particle coincidences in the analysis volume and to match the maximum analysis rate of flow cytometers that deliver sample at about 10 μl/min. Emerging rare cell applications, such as counting circulating tumor cells (CTCs), will require detection of less than 100 cells per ml. This will require analysis of several ml of sample to ensure accurate counts of small numbers of cells. Therefore the dilution approach will require greater than 100 ml of diluted sample to be analyzed for very rare cell applications. This is not practical using conventional flow cytometers that deliver at rates of 10 to 100 μl per minute. These concerns are the same for all rare cell applications, which include detection of CTCs, endothelial cells, hematopoetic stem cells (HSCs), and fetal cells in blood samples.[32-34]

Thus, for truly rare cell analysis in blood samples, it is an important goal to develop technologies that can remove the RBC population, yet collect the cells of interest, such as WBCs, CTCs, or HSCs, without loss or modification. For flow cytometry, magnetic immunocapture techniques that enrich cells of interest based on the display of specific cell surface markers followed by analysis are most commonly used.[3, 35] Though effective, this relies on specific cell markers, which may be lost during normal disease progression.[31, 36] As such, label free microfluidic methods that specifically enrich cells of interest prior to flow cytometry analysis remains of interest. These have been well reviewed,[37] but of specific interest are flow through methods that could be included at the front end of a flow cytometry analysis. Such approaches include: a) flow in microfluidic structures such as chevrons or posts. [38] b) inertial migration in narrow microfluidic channels.[39] c) acoustophoresis in microchannels.[40] d) magnetophoretic approaches that separate RBCs using the intrinsic magnetic properties of hemoglobin.[41] Regardless of the method of choice, the overall throughput of the RBC clearing method must allow for processing of many ml of whole blood in a short time frame.

An interesting example that integrates several of the above approaches, is the CTC-iChip, which enables 8 ml/hr and 10^7 cells/second to be processed (Fig. 4A).[42] RBCs are first depleted using size dependent lateral displacement induced via a field of microposts. Larger cells move into a flow path for inertial focusing, and, finally, the magnetically labeled CTC. Also of great interest are acoustophoretic field flow fractionation techniques in microchannels that enrich based on size, density, and compressibility.[43] These have demonstrated

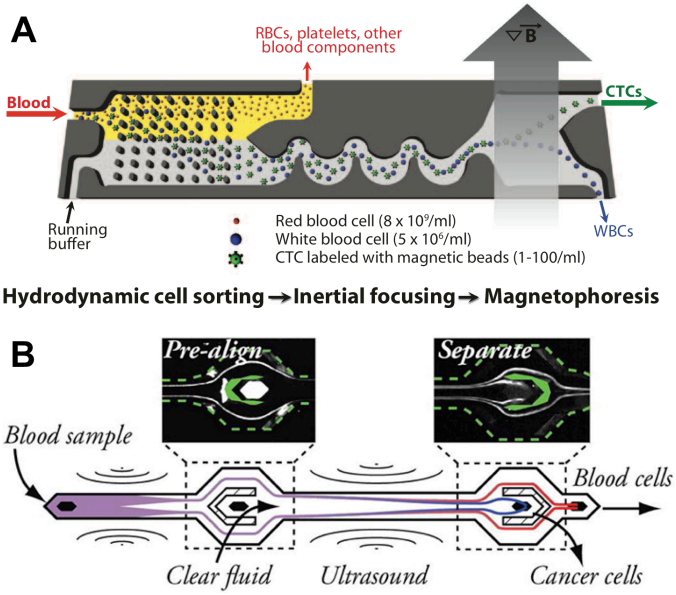

Fig. 4. Online sample preparation of blood cells. (A) The CTC-iChip. From [E. Ozkumur, A. M. Shah, J. C. Ciciliano, *et. al.* Science translational medicine, 2013, 5, 179ra147] Reprinted with permission from AAAS. (B) Acoustophoretic separation of cancer cells from blood using in an etched silicon microchannel. Figure reproduced from [P. Augustsson, C. Magnusson, M. Nordin, H. Lilja and T. Laurell, Analytical chemistry, 2012, 84, 7954-7962] with permission of the American Chemical Society Copyright 2012.

the ability to support ml/minute flow rates and have been used to enrich for prostate tumor cells in blood (Fig. 4B).[44] The flow through nature of both of these approaches give them clear potential for integration with flow cytometry analysis.

Another alternative that may offer flow through cell separation is the use negative acoustic contrast particles that migrate to antinodes in an acoustic standing wave [45, 46]. Such particles are effective for the isolation of both proteins and cells, and are envisioned to be potentially applicable to inline flow cytometry analysis in the future. This concept is also of use for applications that have cells embedded largely in low-density lipid solutions, such as detection of cells in milk [47, 48]. Both of the above approaches drive cells to the node of a standing wave, while driving the negative contrast particles or lipids to the antinode.

Flow cytometry detection of particles of cells in dilute samples, such as pathogen detection, monitoring of algal populations, and detection of blood cells in milk, also would benefit from online concentration approaches. Of the methods mentioned above, acoustic concentration in microchannels is the simplest approach to concentration of particles as most cells and particles are easily concentrated to a node that can be positioned at the center of a microfluidic channel or the center of a cylindrical microcapillary for either direct analysis by flow cytometry or collection for later analysis.[49, 50]

b. Rapid solution exchange.

Though the small analysis volume of a flow cytometer often renders wash steps unnecessary, applications that use fluorescent probes at concentrations greater than about 500 nM, which is necessary for low affinity receptor studies, or have large concentrations of small particulate matter that scatters light, such as detection of rare cells after a lysis step, can cause excessive background that requires a wash step.[3, 14] Wash steps using centrifugation are tedious and slow. Moreover, low affinity ligand receptor measurements require a very fast wash step otherwise the off rate of the bound ligand will prevent accurate cellular measurements.[14] Thus, there is a need for rapid solution exchange around particles.

Stopped flow liquid handling systems have been developed that rapidly mix solutions and deliver them to a flow cytometer with a dead time as short as 120 ms.[51-53] Coaxial mixing capillaries have also been developed that can mix samples inline with flow cytometry analysis with dead times as short as 60 ms.[54, 55] While both of these systems have been used for kinetic assays and rapid washing of samples, the use of microfluidics for direct particle manipulation offers the potential for significant improvements in terms of mixing times and device simplicity. Acoustic standing waves offer the ability to directly move particles from one streamline to another simply by the positioning the acoustic node of the wave in a separate flow stream with the microfluidic devices [43, 56, 57]. These approaches will have clear value for rapid mixing and washing in flow cytometry. It is also possible to use microstructures, as described above, to selectively move particles into new stream lines, but these approaches are not rapid. Therefore, alternative approaches using inertial focusing to rapidly switch particles between streams have been developed.[58] This approach uses wall driven lift to move particles across flow streams in under a ms and will be useful in flow cytometry analysis.[58]

2. Sample delivery

Use of pressure vessels (Fig 2A), pumps (both peristaltic and syringe), and even gravity have been commonly used to deliver sample for flow cytometry analysis.[11] As consistent velocity through the interrogation volume is necessary for precise analysis, it is critical that the sample delivery method provide steady pulse free flow. Pulsatile flow results in varying transit times through the analysis volume, which reduces the measurement precision of the system. This is particularly true when using parameters based pulse area of the signal. The need for steady flow has led to common use of a pressure chamber to deliver sheath fluid for analysis. Pressure is also used to deliver sample, but the volumetric delivery offered by precision syringe pumps enables the direct counting of cells or particles without the use of external microsphere counting standards that are used to precisely meter volumetric delivery rates.[59]

Peristaltic pumps are also effectively used, but care must be taken to dampen fluidic pulses in such systems.[60] Overall, pressure driven flow with external pumps can generate a few µL/min up to several mL/min, as long as the microfluidic system can with stand the high pressure.

a. Microfluidic sample pumping

For microfabricated flow systems, integration of the above off chip pumps is a common solution.[11] However, towards the creation of a fully integrated microflow cytometer, several efforts have used electrophoresis or electro-osmotic flow, on-chip peristaltic pumps, or pneumatic channels to drive sample and sheath for analysis. [61-64] These systems can generate flow rates as high as about 100 µL/min. As there are many microfluidic pump configurations, future efforts for flow cytometry analysis could explore non-pulsatile systems that support µl/min flow rates. Such systems include microfabricated disc pumps, high frequency piezo activated peristaltic pumps, and miniature rotary pumps.[65, 66] It has been demonstrated that these pumps could generate few µL/min up to about 100 µL/min. An interesting alternative on-chip pumping of fluid is the transportation of cells in an electric field, where *E. coli* was transported through the analysis volume of a flow cytometer [67]. As the field lines intersected the interrogation volume the pumping simultaneously moved the cells and provided positioning for analysis.

Each microfluidic pumping mechanism has its own strengths and weaknesses. Absence of moving parts, ease of fabrication and integration, pulse-free flow, low power consumption, low-flow rates, high-flow rates, and ability to use in high back pressure conditions are some of key strengths in various pumping mechanisms mentioned here. On the other hand, limitations due to non-steady flow, need of controlling fluid properties, extra fabrication steps, limited flow rate ranges, and presence of moving parts can hinder the use of a wide array of microfluidic pumps in flow cytometry systems. Thus, the method of sample delivery will need to be carefully matched with the given flow cytometry application.

b. High throughput sampling.

Sample delivery to analysis on the flow cytometer is a time consuming process that is typically performed manually at rates of about one sample analyzed per minute. To automate the processing of multiple samples, current commercial flow cytometry systems routinely employ tube handling systems, plate feeders, and other laboratory robotics. These approaches make handling hundreds of samples a relatively simple and hands free operation.[3] Though such systems speed sampling to an extent, conventional automated sampling remains too slow to sample the hundreds of thousands of samples that are often required for high throughput screening applications. Nonetheless, the high analysis rates of flow cytometry make it an excellent choice for high throughput screening of reactions in or on cells and microspheres.[68] Therefore, high throughput "plug flow" systems capable of at rates of two samples per second have been developed [68-70]. These systems move a sampling capillary between samples arrayed in microtiter plates. The sampling tip is continuously driven or drawn by a peristaltic pump and as it enters each sample and it draws up as little as 2 µl before exiting the sample. As the sampling tip moves to another sample it draws in a small bubble of air that separates each sample. In this way a sampling line delivers a continuous stream of samples to the flow cytometer for analysis (Fig. 5A). Recent iterations of this approach have sampled from up to four 1536 well plates simultaneously. Use of multiplex microspheres for bioassays to screen for activities such as protease cleavage are expected increase screening rates to greater than 1000 experiments per minute and have been routinely used by a single person to screen 60,000 wells per day.[70-72] A typical flow cytometer's maximum sample flow rate of a few hundred µL/min limits the ability of high throughput flow cytometry to process large volume samples. However, the emergence of new sample focusing approaches, such as acoustic focusing, that enable higher sample volume rates (as discussed in section B.3) may allow for screening of larger sample volumes in reasonable time frames. This would make new screening applications, such as screening for response of

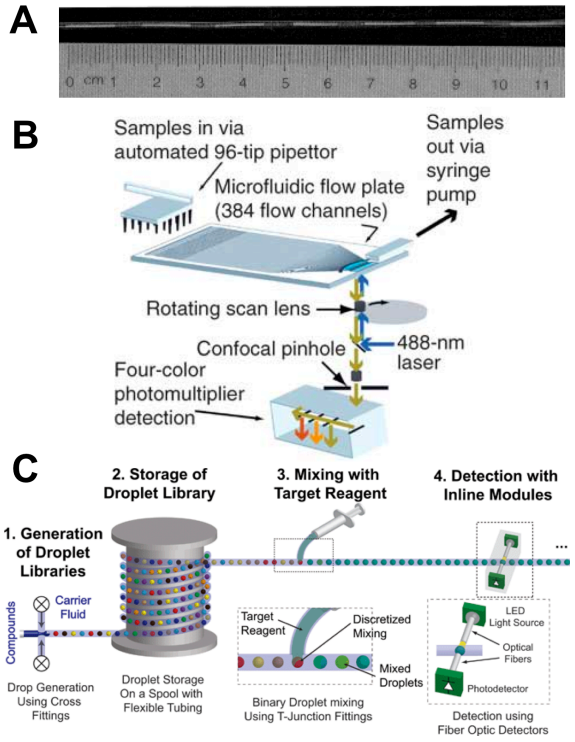

Fig. 5 High throughput and high content screening systems for flow cytometry. (A) An image of samples separated by air bubbles in a delivery line in a sampling system for high throughput screening via flow cytometry. Figure reproduced from reference 68 with permission from John Wiley & Sons, Inc. (B) A system that uses 384 parallel microchannels and a laser scanning detection system to provide images of cells during high content screening. Reprinted by permission from Macmillan Publishers Ltd: [Nature Methods] (B. K. McKenna, J. G. Evans, M. C. Cheung and D. J. Ehrlich, Nature methods, 2011, 8, 401-403), copyright (2011) (C) A system that generates a library of droplets, stores them in a delay line, mixes them with a reagent, and then performs flow based optical analysis. Reproduced from Ref. 81 with permission from The Royal Society of Chemistry.

rare cell populations to lead compounds, possible.

The ability of microfluidics to process small volumes in parallel has long been recognized to provide potential benefit for high throughput screening [73]. This approach has been used to provide image analysis of cells for high content screening in 384 microfluidic flow channels (Fig. 5B) [74, 75]. For this system, laser scanning is used for parallel analysis across each channel. Notably, it demonstrates the concept of increasing flow cytometry sample-to-sample analysis rates through parallelization. Beyond simply increasing sample throughput for screening, the use of microdroplet encapsulation approaches enables the further reduction of reaction volumes for screening and direct inline flow analysis. This can automate many steps of screening, including dose response analysis and sample loading to create multiplex assays. [76-80] The combination of a long capillary line and a microfluidic droplet generation system offers the potential for the generation, storage, mixing and inline analysis of droplets for high throughput screening in a flow format (Fig. 5C) [81].

3. Sample analysis

The analysis region of a flow cytometer requires the precise positioning of a flowing particle and the detection of the particle as it flows past. As discussed above, most flow cytometers use hydrodynamic focusing, but there are also examples of commercial instruments that use microcapillaries without focusing and those that use a combination of acoustic focusing and hydrodynamic focusing [82-87]. For the analysis of positioned particles, optical methods have dominated as scatted enables the discrimination of many cell populations and fluorescence based immunophenotyping makes precise determination of cell subpopulations routine [3]. Nonetheless, the use of non-optical techniques that provide additional particle information, such as Coulter volume sensing, have been and continue to be used in commercially available flow cytometry analyzers.[18, 22, 85, 88]

a. Particle focusing

There are many potential methods to focus particles for flow cytometry analysis in microfabricated systems, which include simple constraint by the walls of a flow channel,[7, 9, 10, 82] use of sheath fluid for 2-D hydrodynamic focusing [89-91], bulk acoustic standing waves (BAW) [92-94], standing surface acoustic waves (SSAW) [95, 96], dielectrophoresis (DEP) [97-99], inertial focusing [100-103], use of microstructures to induce flow path changes [104-106], or a combination of these approaches. These have been well reviewed recently [107-109]. Therefore, we simply discuss the advantages and disadvantages of the various approaches and recent examples of each type.

The most straightforward flow system to fabricate is clearly the non-tapered microfluidic channel, where the sample is simply pumped through the channel for particle positioning. This offers flexibility with regards to channel placement and dimensions to optimize optical or other detection approaches, but it does not precisely restrict the flow path of the particle or the surrounding sample. This results in variations in flow velocity through the analysis volume, as most pressure driven flow systems have parabolic flow profiles [39]. This results in loss of analysis precision, but use of velocity tracking techniques can improve precision [12, 82]. This approach also does not constrain the sample stream, which results in optical interrogation of more of the sample stream and reduces the ability to resolve free vs. bound fluorescent probe.

Hydrodynamic focusing was integrated into flow cytometry in large part to improve the above concerns. However, while it is simply implemented to provide focusing in one-dimension (across the width of the stream), by flowing in a sheath stream in a channel or channels that intersects the sample stream on one or both sides, more complex fabrication approaches must be undertaken to provide focusing in two dimensions (side to side and top to bottom). Multilayer microfluidic systems have made this approach successful in many flow cytometry development efforts [107]. However, recently simple structures have been used to adjust the flow profile, such as chevron structures in PDMS microchannels, have been used to create two dimensional focusing without the need to apply sheath above and below the sample stream [104, 109]. This system uses the chevrons to drive sheath flow from the sides to also focus from the top and bottom. Similarly, a two dimensional focusing system in glass was created by etching micro-weirs above and below the sample stream, which results in tight vertical positioning of the particles in the sample stream [105]. These approaches are promising in that they do not require complex layered fluidics to achieve two dimensional particle focusing.

Despite the advantages of hydrodynamic focusing, it requires liquid sheath and accelerates the particles during analysis. This reduces instrument portability and the increased linear velocity increases the demands on the detection and data acquisition systems [17, 50, 110]. Furthermore, the use of sheath can lead to sample dilution after analysis, though microfluidic demixing approaches show promise in the recovery of sample [111]. For these reasons, sheathless particle focusing approaches have been pursued.

The high flow rates supported by particle focusing using BAW made it a sheathless focusing approach that was shown to be effective for flow cytometry [49, 50]. In this method, particles and cells are driven to the node of a BAW as they are more dense than the surrounding sample fluid [112]. Use of cylindrical capillary makes it possible to place the node of the standing wave axially within the capillary, due the structural excitation of the capillary walls [113]. However it is not simple to microfabricate cylindrical microchannels, but rectangular microchannels are not only easily fabricated, more than one node of the standing wave can be created in transverse direction of the channel. This enables generation of multiple parallel

focused streams of particles in a single channel [92, 93]. A single particle stream can be split into many focused streams in a split channel format (Fig. 6A) or in a single channel format [93] without using multiple sample injectors or flow controls. This makes it simple to generate many flow streams that will enable parallel analysis in the future. Using drive frequencies that match harmonics across the width of the channel and the depth of the channel enables two-dimensional focusing for precise positioning of particles [47, 48]. Acoustic focusing can be implemented in wide range of flow rates, ranging from few µL/min to several mL/min [84, 92, 93]. Conventional silicon microfabrication technologies are well suited to create BAW driven microfluidic channels in silicon and glass with appropriate dimensions required for acoustic manipulation [11, 43]. If polymer materials are the preferred fabrication approach, SSAW based methods can be implemented [114-117]. These approaches create standing acoustic waves analogous to BAW but are not limited to rigid substrates as the standing waves a generated via integrated interdigital transducers (IDTs) (Fig. 6B, 6C) [117]. For example PDMS microchannels with IDTs have been utilized in a SSAW based continuous cell focusing system [96, 117].

Inertial particle focusing in microfluidics has gained interest due to its independence from external forces and its simplicity. In inertial focusing, lift forces manipulate particles in to distinct equilibrium positions along a microchannel [39]. Additionally, channel curvature can be used to induce Dean flow that results in repositioning of inertial focusing equilibrium positions. This enables single streams of particles to be created precisely in microchannels, which has been shown to be effective for flow cytometry analysis [101]. Moreover, this approach induces inter-particle spacing, which eliminates particle coincidences and might allow for higher throughput analysis in the future [101]. As in acoustic focusing, inertial focusing can create multiple focused streams of particles. Recent work demonstrated inertial focusing of cells in 256 parallel microchannels (Fig. 6D, 6E) [102]. Inertial focusing based devices are simple to fabricate in PDMS substrate and are likely to be very valuable in future flow cytometry analysis approaches. However, this technique requires that the particle make up a significant portion of the channel cross-section and can result in size dependent se paration of particles to different equilibrium positions. For these reasons, care needs to be taken when designing inertial focusing flow channels for flow cytometry.

Dielectrophoresis (DEP) is widely used for manipulation of particles. In DEP, particles with different dielectric properties are separated in an inhomogeneous electric field (Fig. 6F and 6G). DEP devices can be fabricated in silicon, glass, and PDMS. However, inherent DEP properties like strong dependence of electrical conductivity of the medium and heat generation in conductive media limits DEP as a universal approach for particle focusing. Nonetheless, several devices have been developed that focus particles for effective flow cytometry analysis using DEP as the focusing force [98, 99, 118, 119].

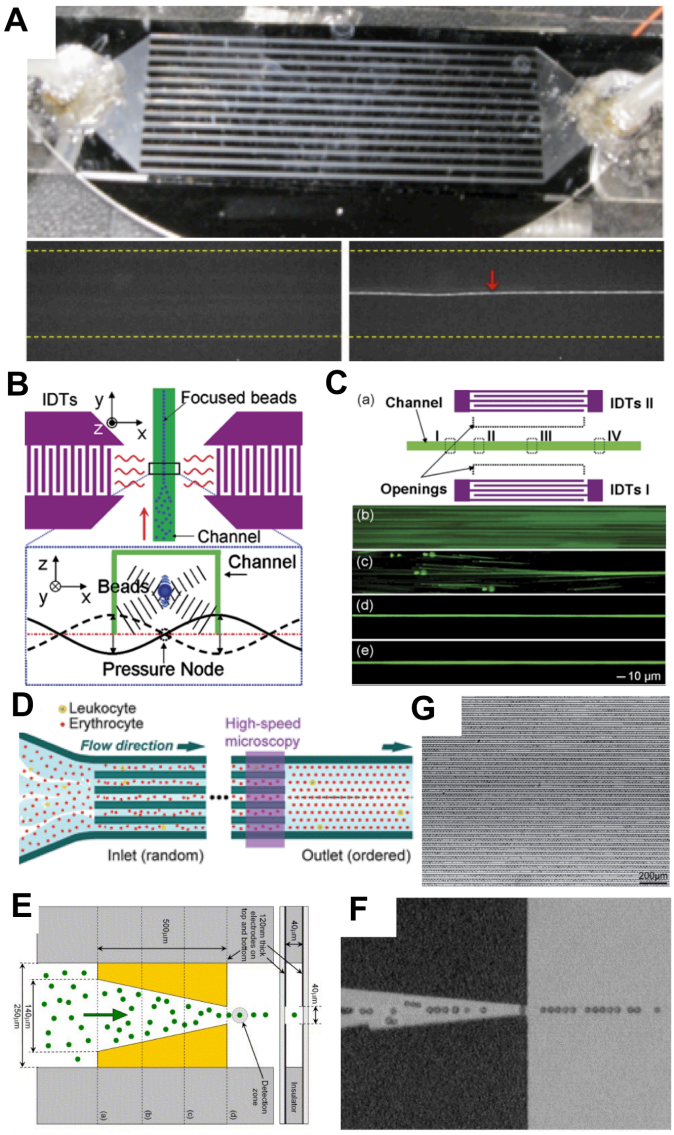

Fig. 6. Sheathless microfluidic focusing techniques. (A). Bulk acoustic focusing in microfabricated silicon devices. A common input is split into many channels and an applied frequency is tuned to generate single focused stream in each channel. Reprinted from *Methods*, v. 57, P. P. Austin Suthanthiraraj, M. E. Piyasena, T. A. Woods, M. A. Naivar, G. P. Lopez and S. W. Graves, One-dimensional acoustic standing waves in rectangular channels for flow cytometry, p. 259-71. Copyright (2012), with permission from Elsevier. (B,C). Surface acoustic focusing in PDMS microchannels. (B) Surface acoustic waves are generated via integrated IDTs fabricated on a piezo substrate that focus 1.9 µm green microspheres into a pressure node. (C) As the microspheres flow from left to right they are acoustically focused into the central stream. The focusing is shown at four points, where the top most image is taken from square I, the next lower from II, the next from III, and the bottom most image from square IV. Figures (B,C) reproduced from reference 117 with permission from The Royal Society of Chemistry. (D,E) Inertial focusing of particles/cells in a highly parallel PDMS microchannel system. (D) Randomly distributed particles/cells introduced from the inlet and inertially focused based on the size, along the length of the channel . (E) A micrograph of inertially focused particles. Figures (D,E) reproduced from reference 102 with permission from The Royal Society of Chemistry. (F, G) Dielectrophoretic particle focusing. (F) A schematic of microelectrodes integrated with the fluidic channels. (G) 6µm particles are focused in the presence of an electric field. Figures (F,G) were reprinted from Biosensors and Bioelectronics, Vol 21, D. Holmes, H. Morgan and N. G. Green, High throughput particle analysis: Combining dielectrophoretic particle focussing with confocal optical detection, Pages 1621-1630, Copyright (2006), with permission from Elsevier.

High throughput flow cytometric analysis in clinical diagnostics is in high demand. Current flow cytometric analysis has a limit of about 50,000 cells/s. This speed is inadequate when it comes to the detection of rare cells like CTCs. Simply increasing the linear velocity in a conventional flow cytometer to increase analytical rates is not possible, as this causes pressures and shear effects that result in cell death. Additionally, this increases demands for highly sensitive and fast response electronics and detectors. Parallel analysis of multiple streams is an attractive alternative. However, it is not easy to generate parallel streams using hydrodynamic focusing, as such systems will require multiple sample inputs and flow control systems. Furthermore, multiple streams in sheath flow focusing will consume increased volume of reagents and generate more waste. Sheathless focusing methods like acoustic focusing and inertial focusing have the potential to generate multiple focused streams in parallel without significant increase in sample flow rate and with minimum device complexity.

Finally, all these focusing techniques have unique advantages as well as limitations. Combining two focusing techniques can compensate some limitations in one technique to some extent. For example, a sheath flow can be introduced into acoustic, DEP, or inertial devices to eliminate orthogonal dispersion of particles [103, 118, 120, 121]

b. Consideration of optical detection in a flow cytometer

Flow cytometers almost invariably collect forward scatter (FSC), side scatter (SSC), and at least a few colours of fluorescence. This basic format is very valuable as bivariate plots of light scatter at multiple angles and accurately differentiates WBCs into three or more distinct cell populations [122, 123]. While FSC is very roughly proportional to a particles relative size and side scatter is proportional the internal granularity of the particle, these parameters are not sufficient for cellular identification in many applications. Addition of fluorescence enables the use of immunophenotyping to further discriminate cell populations. A notable example of this approach is the use of an anti-CD45 fluorescent antibody to discriminate lymphocytes combined with an anti-CD4 fluorescent antibody to discriminate CD4+ T-cells, which is the basis of the Panleucogating method for CD4 cell counts in HIV analysis (Fig 7A) [124, 125]. Using this basic approach, cells can optically split into a variety of populations for diagnostics (Fig. 7A) or for larger scale use of multiple antibodies can determine many cell populations for use in immunological studies (Fig. 7B) [2, 21, 124]. In addition to antibodies for cellular markers, use of other fluorescent dyes such as intercalating dyes and lipophilic dyes enable flow cytometry to measure nucleic acid content, membrane potential, and membrane integrity, among many other cell properties [2]. The collection of multiple parameters of fluorescence is also the basis of suspension microsphere arrays, where up to 500 populations of microspheres are discriminated by a combination of their size, which alters the scatter parameters, and/or their level of

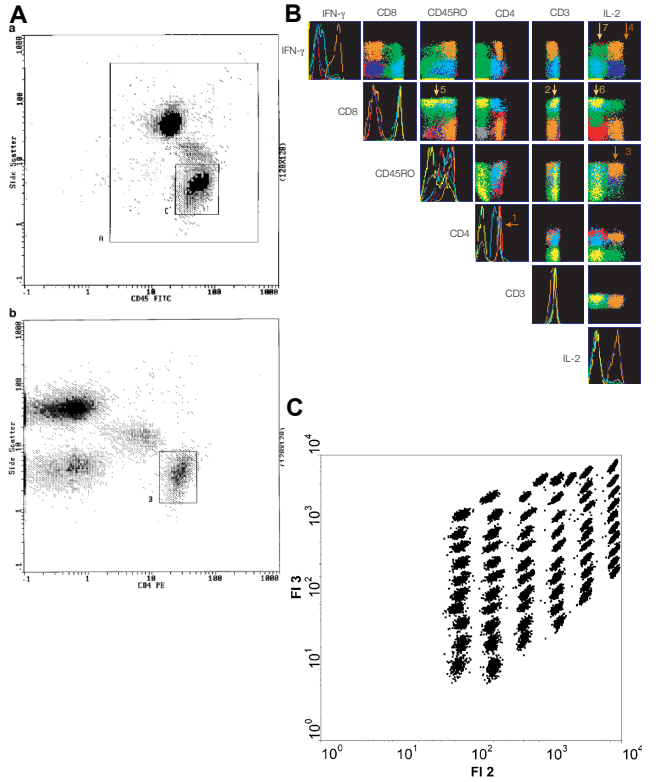

Fig. 7. Examples of optical detection and analysis of typical flow cytometry application areas. (A) The typing of white blood cells by a combination of side scatter and detection of CD45 and CD4 cell surface markers. In the top panel, the leucocyte population is selected for via an electronic gate (the larger rectangle on the bivariate plot of side scatter vs. anti-CD45 generated fluorescence). In the lower panel the resultant CD4+ lymphocyte population is counted via a rectangular electronic gate. The smaller rectangle in the upper panel can be used to count the total lymphocyte population in the sample. This value can be used to provide a percentage of CD4+ lymphocytes, which is useful for paediatric cases of HIV (122). Figure reproduced from reference 125 with permission from John Wiley & sons. Inc. (B) An extensive immunological panel where many markers are being identified simultaneously for cell typing. Up to seventeen colours are used to identify many markers. This approach uses cluster analysis to identify cell populations, which are shown in different colours. Reprinted by permission from Macmillan Publishers Ltd: [Nature Reviews Immunology] (reference 21), copyright (2004) (C) Each population of a 64-plex microsphere set resolved by their response in two colours of fluorescence. The populations can be gated on as shown for cells in panel A and the reporting fluorescence for the assay on the microsphere can be discriminated from the overall population. Figure reproduced from reference 129 with permission from John Wiley & Sons, Inc.

fluorescence staining with one or more colours of fluorescence (Fig. 7C) [126-129]. Using the surface of a such microsphere, it is possible to perform multiplexed ELISA like immunoassays, single nucleotide polymorphism detection, and a variety of enzymatic activity assays [126-128, 130, 131]. In these assays, the type of assay is identified by the microsphere position in the array, and the assay result is read out in a separate fluorescence channel that has been gated to only measure the values from the microsphere of interest.

Sensitive and accurate analysis of fluorescence on a flow cytometer requires both calibration of the intensity and spectral response of the instrument. Detection of multiple fluorophores on a single instrument requires consideration of fluorescent

crosstalk. In flow cytometry this is generally accomplished using a process known as compensation, which is a relatively complex mathematical process to deconvolve the individual contribution of each fluorophore in each detector that has been extensively discussed elsewhere, but must be considered for accurate flow cytometry measurements [132]. As sensitivity and resolution is also critical to instrument performance, there have been several attempts to standardize evaluation of flow cytometers for these parameters. A well accepted approach is to use a set of calibrated microsphere standards that allows for precise estimation both the sensitivity and resolution of a flow cytometer's fluorescence measurements [19, 133]. Using this approach to estimate sensitivity, many commercial flow cytometers now detect as few as 50 fluorophores per particle [18]. This extremely sensitive performance has required the use of high numerical aperture collection optics and stable laser sources, but systems with performance sufficient for some flow cytometry applications have been built with very simple components such as laser pointers or LEDs for excitation and fiber optics for collection [110, 134, 135].

Beyond fluorescence intensity, there have been several efforts to extract additional optical information such as fluorescence lifetime, Raman spectra, and complete images [136-138]. These approaches require the addition of high-speed modulation, CCD spectrometers, or digital cameras, but they provide significant additional information content that makes them valuable for applications such as apoptosis studies or highly multiplexed analysis. High speed flow based imaging has been widely adopted and current commercial systems can image bright field organisms flowing in a capillary at high volumetric flow rates as well as fluorescently image hydrodynamically focused cells at rates of 2000 cells/s [136, 139]. Even faster imaging systems that use time encoded signals to achieve imaging rates and inertial focusing as high as 100,000 s^{-1} in a flow cytometer [140]. Another promising approach is the addition of Raman spectral collection to flow cytometers. The extremely narrow Raman spectral features in combination with surface enhanced Raman scattering nanoparticles and advanced data analysis approaches is anticipated to dramatically increase multiplexing and decrease optical probe cross-talk [141].

c. Optical detection using microfabricated systems.

While there have been many microfluidic flow cytometers built, most have used stand off optical detection akin to a typical flow cytometer. Due to the similarity of these systems in principle to a conventional flow cytometer, they will not be reviewed here. There also has been extensive work on the integration of other micro optical components, such as filters from dye doped PDMS substrates, liquid micro-lenses with tunable focal length and transmission properties, and optical waveguides into microfluidic systems. [142-144] Though there are many promising efforts in this area, here we will specifically discuss microfabrication approaches that may offer improved simplicity and/or affordability without use of complex fabrication approaches.

One of the simplest approaches to optical detection proposes a patterned mask integrated into a flow channel wall [145]. As the particle flows through the channel it is directly excited and its emission pattern is recorded in time as it passes the mask, which provides a known temporal modulation to the emission signal [145]. The known modulation is used to selectively deconvolve the particle-based fluorescence from background. Use of coloured optical masks enables the detection of multiple fluorophores. This approach will dramatically reduce the need for alignment and use of complex spectral selection elements.

Though direct fiber optic collection and delivery have been long proposed for microfluidic flow cytometry [146], the performance of such systems still significantly pales to that of a conventional flow cytometer. This performance lag is shown in the > 5 fold increase in the coefficient of variation seen between fiber optic systems and a modern flow cytometer [147]. Though fiber systems will offer more robust performance due to their simplicity and rigid construction, the low numerical aperture of most such systems is currently highly limiting to fluorescence detection. Nonetheless, the robust alignment afforded by this approach has made it possible to create simple flow cytometers that effectively perform bead based detection of bacteria and toxins [148, 149]. However, the inherent optical limitations of the numerical aperture of most fibers has been recognized by the microfabrication community, which has fuelled the development of on chip lenses for light collection or use of lenses incorporated directly into fiber systems. These systems show improved light collection for flow cytometry [144], but they add complexity to the microfabrication process.

One of the areas of opportunity for microfluidic flow cytometry is expected to be parallel analysis of samples. To that end technologies that faithfully reproduce the flow cytometry analytical volume are of interest. One microfabricated approach that can be applied over many parallel channels is the use of a microball lens array, which has been shown to enable sensitive collection over many flow channels and provides a tight analytical volume for each analysed stream [150]. This or similar approaches may be highly effective for high throughput flow cytometers.

d. Impedance detection

Some of the earliest flow cytometers incorporated Coulter volume sensing as a measurement parameter [151]. Coulter volume sensing works by measuring the impedance change across a small orifice as a cell or particle passes through the orifice [152]. The measurement of particle impedance as it displaces a volume of solution is extremely sensitive and it is commonly used to measure particles ranging from submicron to millimetres in diameter [153]. In addition to simply measuring the impedance change, which is proportional to the volume of the

particle as it passes through, many microfluidic cytometers are pursuing the use of impedance spectroscopy as a method of particle analysis [154]. This approach has been demonstrated to successfully differentiate leucocyte populations in flow and to successfully count CD4+ cells [155-157]. As these approaches do not require optics they may be both effective in low cost and high throughput application areas.

4. Sorting

As can be seen from the introduction, microfabricated flow sorters have been a component of flow cytometry from its inception [10]. There are many reasons that droplet based sorting became the dominant sorting technology, which include: simplicity of implementation, robust performance, sorting rate, and sorting purity [158]. These advantages are largely retained today, but this approach generates aerosols that can negatively impact biosafety [159], results in shear that reduces cell viability, and is not simple to construct in parallel. While solutions such as aerosol containment hoods [159] and moderately parallel droplet sorters can be constructed [160], there have been numerous efforts to create microfluidic cell sorters using valves, acoustics, DEP, and optical traps. Here we consider the most recent microfluidic approaches to flow sorting of particles.

a. Microvalve sorting

A common approach to creation of a microfluidic flow sorter is the use of on-chip microvalves, which was used in early work to selectively sort particles at rates of 20 per second [61]. Though many valve approaches have been developed, they all share the principle that a valve is opened or closed to divert a particle into collection channel. These systems are inherently limited by the activation rate of their valves and the displacement volume of the valve, but recent microfabricated magnetic valve based sorting systems can achieve kHz activation frequencies with extremely small displacement volumes, which makes it a promising approach for both parallel and microfluidic sorting approaches [161].

b. Field based sorting

The use of acoustics has a long standing role in flow sorting, including the use of piezo drive to launch acoustic pressure waves into a y-shaped microchannel, where pressure wave diverts particles of interest at rates approaching 1000/s [27]. In addition, to this approach SSAW has been used to sort particles into multiple collection streams in microfabricated chips [162]. This approach may enable direct incorporation of sorting in a variety of microfabricated flow cytometers and will be parallelizable, but will require increased linear velocities beyond the current 2 mm/s to achieve high sorting rates. Alternatively, surface acoustic waves can be used to induce acoustic streaming that directly imparts energy into a liquid. This approach has been used in a microfabricated sorting system that combines hydrodynamic focusing to position the flow stream as it enters the sorting region [115]. The flow stream is then programmatically oscillated at frequencies of several kHz by the imposition of SAW waves, which results in the movement of the stream across the intersection of two collection channels. This has enabled accurate and gentle sorting of cells at rates as high as 2000 s^{-1}.

c. Droplet sorting

It is also possible to encapsulate cells in aqueous droplets within an oil stream, which enables flow cytometry measurements on the droplets as they flow past. In this case SAW can directly move the aqueous droplets in oil and has been used to sort cell containing droplets at rates of 2000 s^{-1} [115, 163]. Dielectrophoresis has also been used to effectively sort encapsulated cells at similar rates [119]. Though these approaches add the complexity of encapsulating cells in droplets, they enable examination of enzymatic activities external to the cell and obtain kHz sorting rates.

C. Integration of microfluidics and microfabrication into flow cytometers

While it can be argued that every flow cytometer that makes use of flow channels that are hundreds of microns in dimension is an example of an integrated microfluidic system, there are many efforts to integrate more extensive examples of microfluidic sample handling and microfabricated optics [164]. These efforts can be categorized as commercially available or those that are still research prototypes.

1. Research prototypes

There have been many examples of research prototypes that include fluorescence flow sorters, simple analysers, and highly parallel scanning flow cytometers [61, 74, 101, 164]. For the purposes of this review we are defining an integrated system, all aspects of the flow cytometer are fully accounted for including sample delivery, focusing, detection, data acquisition, and sorting (if needed). As in any system design, trade offs are made depending on the intended use of the instrument. For example, in flow cytometers designed for routine use in the field where service is difficult and usage may be rough, it may be desirable to include a robustly aligned optical pathway or alignment free detection at the expense of sensitivity and resolution. This has been done both with patterned optical masks and fixed fiber optics [145, 148, 149]. Another common approach is to simplify an instrument through the use of field based or inertial particle focusing. This both removes sheath, which eliminates an entire fluidic pathway, and reduces the linear velocity of the particles. The reduced linear velocity makes use of low power and low cost excitation sources, detectors, and data acquisition systems possible, which makes these approaches of particular utility where cost and portability are critical [156]. Their also have been

several excellent systems created that use either microstructures or inertial forces to focus particles in one dimension, and hydrodynamic focusing to focus them in the other direction. This simplifies chip construction and has been coupled with embedded fiber optics to make simple to use robust flow cytometry prototypes [121, 148, 149, 164]. The use of microfluidics also makes it possible to consider parallel flow streams as a pathway to increased throughput analysis [74, 92, 102]. However, the only truly integrated parallel flow system has used a complex laser scanning approach [74].

2. Commercial systems with integrated microfluidics

The Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA USA) has been commercially available for over a decade (Fig. 8A) [165-167]. This instrument can perform several routine diagnostics using a variety of microfluidic chips, one of which is a flow cytometry chip that enables direct cell or particle analysis based on fluorescence and scatter properties. This chip uses on chip buffer to perform hydrodynamic focusing to position particles to one side of the flow channel where they are interrogated with a red diode laser or a blue LED. Though this system has low linear velocities that lead to low analysis rates, it has been successfully used for many applications.

A newer instrument that has been available for several years is the Fishman-R (Fig. 8B) (On-Chip Biotechnologies Co. Ltd., Tokyo, Japan) [168]. This instrument uses hydrodynamic focusing on a microchip but has several novel capabilities. First, it collects the outgoing sample in tightly positioned channel that allows for sample recovery with minimal dilution. Second, it uses a chip-based wave-guide to collect both scatter and fluorescence efficiently, which simplifies alignment of the system and provides excellent sensitivity (200 mean equivalents of FITC per particle) and analysis rates (4000 s^{-1}). Finally, there is a sorting version available that uses a simple crossed channel and pressure driven flow to divert cells of interest at rates reported at 10 sort events s^{-1}.

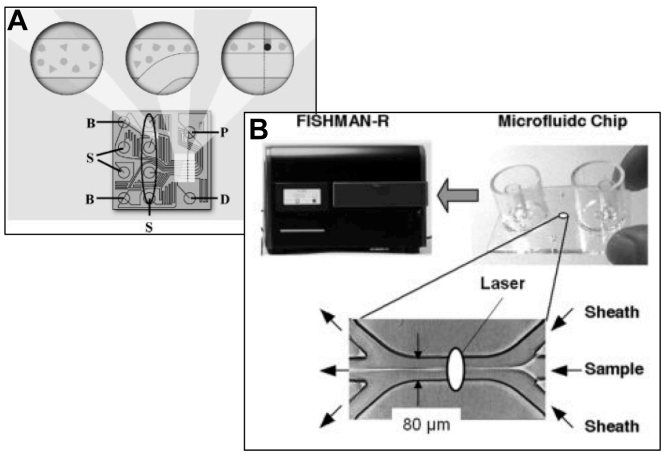

Fig. 8. Commercial microfluidic systems. (A) The Agilent 2100 Bioanalyzer cell analysis chip The letter B denotes where the buffer is input. The S denotes the sample input. Whereas the P and D denote the priming well and reference dye well respectively. The particles from the sample are delivered in a microchannel (left hand circle), focused against the channel wall by sheath fluid (center channel), and analysed using a red diode laser or blue LED for excitation (right hand circle). Figure reproduced from reference 167 with permission from John Wiley and Sons, Inc. (B) The Fishman-R microfluidic flow cytometer. Figure reproduced from reference 168 with permission from John Wiley and Sons, Inc.

The final commercial microfluidic flow cytometer discussed here is the SH800 (Sony, Champaign, IL USA) [169]. This system uses a multilevel microfluidic chip that achieves two-dimensional hydrodynamic focusing. The chip is available with a variety of exit orifice sizes to facilitate optimal sorting of a variety of particle types, has an on chip optical detection window, and uses droplet based sorting principles after the sample exits the chip to achieve conventional sorting rates. The use of this chip also enables the system to automatically align the chip for optimal optical analysis and use barcodes to optimize sorting rates. The disposable nature of the chips makes them simple to replace in the case of a clog, which is a common problem in sorting that requires significant user intervention [3].

Conclusions

As flow cytometry has matured over the last century, it has continued to adopt innovations and has led to the wide spread use of microfluidic technology both in the research lab and in commercial instrumentation. However, in the immediate future, three areas of need stand out where microfluidic and microfabrication approaches may make the greatest contribution to flow cytometry.

1. Though there are now several commercial flow cytometers, that are robust, portable, and relatively affordable, there remains a need for further reduction of cost, size, and complexity of flow cytometers [156]. Thus, it is expected that microfabrication efforts will continue to be of value to provide effective flow cytometers for clinical applications, such as CD4+ T-cell counting, in resource poor areas of the world.

2. The increasing need for high throughput analysis in rare cell and high throughput screening applications will make microfabrication of parallel flow cytometry systems to increase analysis and sorting rates an attractive goal. Though many parallel focusing approaches have been developed, creation of simple methods to analyse the many flow streams will be critical. For rare cell applications specifically, this need may also be addressed by the development of inline cell separation methods that simplify the isolation and delivery of rare cells to more conventional flow cytometry analysis.

3. Though immunofluorescence and other fluorescent probes are highly informative, flow cytometry will benefit from higher information content assays. While flow based imaging provides a solution in part, there are many cellular properties that cannot be seen via probes alone. An interesting example where microfluidics has provided increased information content is the recent work that explored the deformability of cells by imaging cell shape during compression between two flow channels [170, 171]. In combination, with high speed imaging this approach may provide a method to directly measure mechanical properties of cells. Such approaches, that provide measurements of intrinsic

properties of cells are likely to require the cellular micromanipulation capabilities that are a strength of microfabricated systems.

Finally, flow cytometry is one of the most powerful clinical diagnostics that has been made possible through the application of microfluidics. It is clear that an immense amount of effort continues to further optimize flow cytometry diagnostics through careful application of microfabricated systems, which will result in important improvements to medical diagnostics and biomedical research worldwide.

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