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The good, the bad, and the tiny: a review of microflow cytometry

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Abstract

Recent developments in microflow cytometry have concentrated on advancing technology in four main areas: (1) focusing the particles to be analyzed in the microfluidic channel, (2) miniaturization of the fluid-handling components, (3) miniaturization of the optics, and (4) integration and applications development. Strategies for focusing particles in a narrow path as they pass through the detection region include the use of focusing fluids, nozzles, and dielectrophoresis. Strategies for optics range from the use of microscope objectives to polymer waveguides or optical fibers embedded on-chip. While most investigators use off-chip fluidic control, there are a few examples of integrated valves and pumps. To date, demonstrations of applications are primarily used to establish that the microflow systems provide data of the same quality as laboratory systems, but new capabilities—such as automated sample staining—are beginning to emerge. Each of these four areas is discussed in detail in terms of the progress of development, the continuing limitations, and potential future directions for microflow cytometers.

Keywords

Flow cytometry; Microfluidics; Fluid focusing; Integrated optics; Cell sorter

Introduction

In the last 55 years [1], flow cytometry has become a standard analytical method in cell biology and medicine. As flow cytometers became commercially available [2], they began to increase in complexity, employing multiple lasers and an expanding number of detectors to meet requirements for multi-color fluorescence analyses. In the last decade, however, advocates of point-of-care and on-site analysis have created a market for flow cytometers that are simple to use, inexpensive, and portable [3]. An intermediate class of flow cytometers is currently available that are relatively simple to use [4,5], but these systems are still expensive and at least the size of a typical laser printer. A number of investigators in the microfluidics community have focused on using microfluidic technologies and incorporating small optical components to create microflow cytometers with very small footprints; commercial availability is on the horizon.

The microfluidics approach to creating a flow cytometer has concentrated on four main areas:

1. focusing the particles to be analyzed in the microfluidic channel,
2. miniaturization of the fluid-handling components,

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3. miniaturization of the optics, and
4. integration and applications development.

Strategies for focusing the particles, as they pass through the interrogation region in front of the laser beams, include the use of focusing fluids, nozzles, and dielectrophoresis. Strategies for optics range from the use of microscope objectives to polymer waveguides or optical fibers embedded on-chip. While most investigators use off-chip fluidic control, there are a few examples of integrated valves and pumps. To date demonstrations of applications are primarily used to establish that the microflow systems provide data of the same quality as laboratory systems, but new capabilities—such as automated sample staining—are beginning to emerge. Each of these four areas will be discussed in detail to give the reader an understanding of the progress of development, the continuing limitations, and potential future directions for microflow cytometers.

General theory of the microflow cytometer

There are several requirements for a microflow cytometer to be able to analyze particles effectively (either biological or synthetic). The first requirement is that each particle must be isolated in some way so that it can be interrogated individually within a detection region. This is most effectively accomplished by focusing a stream of suspended particles down to a diameter where particles travel in single file through an interrogation region. By utilizing the characteristics of laminar fluid flow in microfluidic devices, fluids can be used to ensheath and focus other fluids without mixing, and thus to create confined streams within a channel. Ideally, the sample stream is sufficiently confined by a sheath fluid such that it is extremely rare for two independent particles to pass through the detection region at the same time. Figure 1 shows a schematic diagram of the detection region in a common flow-cytometer configuration. The interrogation volume is the region of space where the particle is detected. It is the union of the core stream, the volume illuminated by the excitation light and the volume from which the detection optics can detect light. Examples of specific techniques that researchers have utilized to control the interrogation region will be discussed in terms of both fluidics and optics.

The convention used in this paper to describe focusing schemes is somewhat different from that in much of the literature. Devices that can focus the sample stream from the sides are often referred to as two-dimensional focusers, while those that can also focus the sample stream from the top and bottom are said to be focusing in three dimensions. Since focusing from two opposing directions in the same axis is localizing the sample stream in only one dimension, the convention used in this paper will be to refer to systems that sheath in the horizontal *or* vertical dimension (but not both) as 1-D systems. Those that focus in both the horizontal *and* vertical dimensions are referred to here as 2-D systems. If the sample stream fills the channel completely, it is referred to here as unfocused.

Sample interrogation is most commonly achieved using optical detection (via light scatter, fluorescence, or absorption), but electrical impedance also is sometimes used. The measurements taken from the particles can be a simple yes/no count, but are frequently quantitative. The signal from a particle is detected as a pulse. The width and geometry of the pulse is a function of the particle velocity and the length of the interrogation region. It is possible to simply quantify information from the peak maximum directly, but much higher signal-to-noise ratios are possible if the peak area is used. This option, however, is not available if the particles are not traveling at a uniform speed when detected.

The channel architecture and method of pumping liquid govern the fluid velocity profile through the channel. As such, particles traveling at different locations in the channel will have different velocities. The great advantage of flow focusing is that it forces all of the particles to

travel in the same location in the channel and therefore to have the same velocity. In addition, focusing typically moves the particles to the center of the channel, where the flow profile is relatively flat, so small changes in position do not have a large effect on the velocity.

However, forcing the sample into a tightly focused stream can create another challenge. Halving the diameter of the sample stream requires that the linear velocity be quadrupled to maintain the same volume throughput. While microfluidics are frequently endorsed for low volume requirements, there are times when the lower limit in sample size is set by the concentration of the target. If a cytometer is to detect a cell that may only be present at a few cells mL^{-1} , sampling only 500 nL will most likely fail to see that cell. One could simply increase the flow velocity, but as particles pass through the detection region faster, the sampling electronics must become faster, and the total number of photons available for detection decreases. A complete solution for these issues related to throughput has not yet been presented.

Fluid handling

The development of a fully integrated microflow cytometer relies, in large part, on precise handling of fluids for effective sample manipulation and analysis. In order to analyze a sample particle by particle, a flow cytometer must introduce a single-file stream of particles into an interrogation region that usually contains precisely aligned optics for detection. Techniques such as flow focusing and dielectrophoretic focusing are commonly used to orient particles in this fashion before entry into the interrogation region. Such particle-focusing techniques play an integral role in almost all cytometers, and this critical feature is evident in microfluidic versions also.

Methods for pumping

Stable and well aligned fluid flow requires pumping methods that avoid the appreciable pulsation that often accompanies standard pumps such as peristaltic pumps and membrane driven pumps [6]. Additionally, the flow velocity profile, resulting from the method of pumping, channel architecture, and flow scheme, also governs such factors as the alignment, particle speed, stability of flow, and particle detection rates. The position and speed of a particle as it passes through the interrogation region are especially critical as they determine the pulse shape, width, and height of the detected signal. If these parameters are not well defined and reproducible for each particle, the breadth of information obtained from data analysis is greatly reduced.

Most microflow cytometry studies rely on external components to control fluid flows in a microchip. This is due to the inherent obstacle faced by the field of microfluidics—effective on-chip miniaturization of all the components necessary for manipulation of fluids. External pressure-driven pumps often used in microflow cytometry applications include syringe pumps [7–13] and positively or negatively pressurized reservoirs using a compressed gas source or vacuum pump [14]. Passive pumping methods such as gravity-driven flow have also been used [15–17]. Groisman et al. [17] demonstrated the use of gravity-driven flow to create a high-throughput microfluidic cytometer with detection rates up to an astounding 17,000 particles s^{-1} —a rate that approaches the limits of conventional commercial cytometers (1,000–50,000 cells s^{-1} , depending on analysis and accuracy requirements). Such solutions meet the essential cytometer requirements for precise non-pulsatile control of flow rate. In some cases, these relatively cumbersome solutions to integration are acceptable, demonstrating the ever-present trade-off between size and function that is often encountered when developing portable analytical tools.

There are few examples of robust fully integrated on-chip pumping methods employed in microfluidic devices, and fewer used in microflow cytometers due to the additional constraints

of these systems. Several microflow cytometers have utilized multi-layer soft lithography of polydimethylsiloxane (PDMS) to create pneumatically actuated valves and peristaltic pumps that produced flow velocities of up to 14 mm s^{-1} [18–20].

A significant number of microflow cytometers have turned to fluid-handling techniques that can be characterized as non-mechanical pumping. Electrokinetic techniques [21–23] utilize strong electric fields to move particles through the device via electroosmosis and/or electrophoresis. From the standpoint of focusing, electrokinetic techniques behave in a way very similar to pressure-driven flow. Models for both pressure-driven [24] and electrokinetic focusing [21,22,25] are well developed. In some cases, electroosmotic flow is suppressed by modifying the channel surface charge [26], which means that the particles simply follow the lines of the electric field. This offers some key advantages over many micropumping methods. In particular, fabrication is simple as pumps can be avoided. Electrodes may be embedded in the system by standard microfabrication techniques, but care must be taken to avoid or control bubble formation by electrolysis. While pressure-driven flow produces a nominally parabolic profile, electroosmosis produces flow which is much more plug-like. In some cases, this partly relieves the increase in variance associated with relatively large sample streams, because all the particles are traveling at the same rate, regardless of their position. However, since the particle velocity is now a function of the surface zeta potential, variations due to sample complexities must be accounted for. Also, many microfluidic chips are made by bonding together two separate layers, which may not be the same material. If this is the case, differences in zeta potential between the two materials will cause a non-uniform velocity field within the channel [27], giving rise to the same problem.

Munyan and coworkers [28] offered an interesting alternative solution to the problem of integrating a microfluidic pump for portable applications. The group demonstrated an electrolytically actuated micropump that offers a low-power alternative for continuous flow applications. This method utilized controlled electrolytic production of gas in a fluidic reservoir as a pressure source to control fluid flow rate in a microchannel. This type of pumping mechanism could be a good candidate for microflow cytometry applications, as it provides a pulseless, pressure-driven flow, and reservoirs and electrodes can be integrated directly into a microfluidic chip.

Particle focusing

From the theoretical standpoint of individually interrogating particles, there is actually little need for focusing. It is possible to manufacture a sufficiently narrow channel to force the particles into a single file. A handful of examples have been reported where focusing was not used [27,29–37]. While successful, these were primarily proof-of-concept papers aimed at demonstrating an innovation in optical or electrical [37] detection and not intended to represent fully functional cytometers. The use of a narrow channel raises serious dangers of clogging and surface fouling and is not really effective if there is a broad range of particle sizes. The channel must be big enough to accommodate the largest particle or aggregate found in the sample to avoid clogging, which may mean that the smallest particles (which may be targets of the study) can pass through the detection region in groups. There are also significant back pressure and shear stress associated with driving fluid through such small channels, which increases instrument costs and can damage cells. When the sample stream is not completely sheathed, particles can come into contact with the channel wall; fouling can alter the detectable target concentration or interfere with optical or electrochemical measurements. Channel surfaces have been treated with compounds such as bovine serum albumin [38,39], hydroxypropylmethylcellulose [16], or covalent coatings such as polydimethylacrylamide [25], or trichlorohexadecylsilane [40] to reduce fouling.

One-dimensional flow focusing

Traditional cytometers create sheathed flow by placing a drawn glass capillary or other small tube inside a larger one. While highly effective at introducing a sample stream into sheath flow, this kind of structure is extremely difficult to microfabricate.

The earliest attempts to approximate sheathed flow used a simple “cross” intersection. Sheath fluid is introduced on either side of the sample stream, focusing it laterally. The idea of using this style of focusing for cytometry was introduced by Jacobson and Ramsey [23] using electroosmotic flow, although cytometry was not demonstrated at that time. Cytometry capability was later demonstrated by Blankenstein and Larsen [3], who used pressure-driven flow, and by Schrum et al. [41], who used electroosmotic flow for the interrogation of polystyrene beads. Later McClain et al. demonstrated the same system with bacteria [25], and this cross has become the most widely used sheathing system for microcytometry [9,12,17, 21,22,24,25,38,41–52]. The sample stream is introduced into the top of the intersection while sheath streams are introduced from the sides. The ultimate width of the sample stream depends on the relative flow rates of the sample and sheath streams and is largely independent of the geometry of the intersection. Lancaster et al. [12] reported a 1-D focused flow in the vertical direction. Using a three-layer input, they created a thin ribbon of sample to be imaged using a microscope.

In most cases that do not use microscopy and video imaging, a major difficulty with 1-D focusing is that the illumination and collection efficiency are not uniform over a broad detection volume. The passage of the particle is registered as a pulse of light by the detector. Whether detection is by fluorescence or scattered light, the intensity of the detected pulse is proportional to the intensity of the illuminating light, which typically varies spatially across the beam. If particles are allowed to travel through any part of the beam, any attempts to obtain quantitative information from them will be confounded by variations in illumination. In the extreme case, where the sample stream is greater than the beam diameter, some particles may miss the beam entirely and not be detected. A similar rule applies to the collection optics, where the efficiency of collection from different regions of the sample stream may vary.

This problem is not just theoretical. Pamme et al. [45] were able to collect light scatter of 6- μm monodisperse latex particles at 15 and 45 degrees, but reported relative standard deviations as high as 30%, which they attributed to the variation in excitation intensity across the interrogation region. The broad scatter in their data inhibited their ability to differentiate between 2 and 9 μm particles. Chung et al. [46] demonstrated both 1-D and quasi-2-D focusing. The quasi-2-D focusing was achieved by making the sheath channel deeper than the sample channel, so that some of the sheath fluid went over the sample stream as well as on each side. This sheath flow then pushed the sample downward against the bottom of the channel. By comparing the two sheathing systems with all detection parameters held constant, they were able to demonstrate reduced variance in the fluorescence intensity when the sample stream of 10 μm fluorescent beads was more effectively confined.

Fu et al. [47] experienced 11% variances in signal when particles were passed through a channel focused in 1-D. Red blood cells showed even greater variance, 38%, probably due to their nonsymmetrical shape. The authors suggested that higher dimension focusing would help solve the problem.

Two-dimensional flow focusing

By tightly focusing the sample stream in two dimensions, the particles can be made to travel single file along a single trajectory, further reducing variance and eliminating the risk of contaminating the channel surface. Relatively few groups have achieved this in a microfluidic

device, and the fabrication and plumbing of the chips can be complex. Sundararajan et al. [42] used soft lithography to create a vertical chamber made in five layers with as many as six sheath inlets. The final design was able to focus the sample into the middle of the outlet channel. However, this approach is quite complex and only works if the layers are well aligned.

Simmonet and Groisman [17,49] created two closely related designs that were able to fully sheath the sample. Their designs used a deep main channel intersected with a series of shallow side channels with inlets near the bottom of the main channel. In the first design, the sheath stream was introduced into the main channel, and sample stream was introduced from either side by a pair of shallow channels so that it filled the lower portion of the deep channel. Additional pairs of channels introduced more sheath fluid to lift the sample stream off the bottom and to confine it laterally for complete sheathing. In the second design, they added another set of outlets to remove fluid from the region where the greatest diffusion could take place. This improved the focusing in the vertical direction. In addition, the detection channel was very broad, so that the sample stream could be focused into a wide ribbon, up to 700 μm wide, but less than 1 μm deep. A microscope was used to image particles passing through the channel.

In a minor modification to the approach of Simmonet and Groisman [49], Chang et al. created 2-D sheathing using just two layers in PDMS [50]. As shown in Fig. 2, two separate channel levels were created with contiguous, perpendicular flow channels. Sample and sheath were introduced into the lower portion of the main channel from perpendicular channels in the lower layer to focus the sample stream in the vertical direction. Then a more standard cross in the upper layer was used to introduce sheath fluid into both sides of the channel and focus the sample stream in the horizontal direction. The result was a roughly rectangular sample stream located in the center of the channel.

The above 2-D designs require at least four and as many as six inlets to fully ensheath the sample stream. The numerous sheath inlets can be viewed as useful where it is desirable to steer the sample stream within the channel. In most situations, however, it is more important that the position of the sample stream remains constant, and the requirement to evenly control flow into so many inlets is a significant problem.

Wolff et al. [9,51,53] presented a novel design in which the sample stream comes up through a “chimney” to be injected into the middle of the channel. While better than the previous cross designs, some sample still comes into contact with the top of the channel. It is likely that a similar structure with a higher aspect ratio could fully sheath the flow.

Yang et al. [52] created a quite complicated structure that currently comes closest to reproducing the classic annular design. The fabrication required three masks, and four exposures. Three exposures were tilted, requiring use of prisms and index-matching liquids. The result was a structure with a vertical wall containing a small via at mid-depth. It is through this via that sample stream entered the main channel, and sheath fluid was introduced on either side.

For a well focused stream, the greatest limitation on sensitivity may be the burden placed on data acquisition; a sufficient number of signals must be collected from particles as they pass through the interrogation region at high speed. One possible solution to this problem was presented by Bang et al. [54], who demonstrated that after a sample has been focused into a stream with a width of the order of the particle diameters, all information about the initial distribution of the particles in the channel is lost. Once this happens, it becomes possible to widen the channel again without spreading out the particles. This slows the particles down and essentially concentrates them in a narrow central region of the wider channel. A more common solution to alleviate the burden of throughput on data acquisition, is integration of analog and

digital electronic components; the significance of which will be discussed further in the cytometer electronics section.

Dielectrophoretic particle focusing

In some circumstances, it is possible to perform focusing without a sheath fluid. Dielectrophoresis (DEP) provides another mechanism for focusing particles into a tight stream. When in a nonuniform alternating electric field, a particle experiences a force either toward (positive DEP) or away from (negative DEP) the points with the greatest field gradient. The magnitude and direction of the force depends on a variety of factors, including the slope of the field gradient, the frequency of the electric field, the particle diameter, and the difference between the polarizabilities of the particle and the surrounding solution.

All the DEP-based focusing experiments to date have used negative DEP. Because the strongest field gradients occur at the surface of the electrode, particles experiencing positive DEP tend to be simply pulled toward the electrodes, which can damage cells. Ensuring that negative DEP takes place frequently requires that the sample be diluted with solution of the appropriate ionic composition.

As shown in Fig. 3, Lin et al. [55] used a cross-style fluid intersection to focus particles horizontally and then DEP to focus them vertically. This provided an excellent demonstration of the importance of 2-D focusing. They were able to turn the vertical focusing on and off and observe the effect it had on the amplitude and spread of the signal as the particles passed a pair of embedded optical fibers. The signal obtained using 2-D focusing was both more intense and more precise for human red blood cells and for two sizes of latex beads.

Eschewing hydrodynamic focusing altogether, Holmes et al. [56] created a funnel using two pairs of electrodes on the top and bottom of the channel. In this kind of configuration, the particles were repelled from entering the space between the electrodes. They were able to fully focus a stream of latex beads at their highest potential of 20 V. The slanted surfaces of their funnel did not go all the way to the edge of the channel, and at lower potentials, particles that were outside the sloped region were able to escape focusing. The drag force of the flowing solution was sufficient to overcome the repulsion of the electrodes when the particles met it head-on.

Optics

Once core-sheath flow has been achieved in a cytometer, optical elements provide a method for interrogating, detecting, classifying, and identifying the particles based on both fluorescence and light-scattering criteria. Following the trends of commercial instruments, many microflow cytometers still use traditional bulk optics and typically require a confocal microscope stage for mounting commercial high-powered objectives. Lately, there has been a trend toward the introduction of integrated optical elements within these devices which is essential for the realization of a portable or hand-held device [57,58]. This section will focus on both commercial and unique solutions to the problems presented by this transition to integrated optical components.

Waveguides

In many microcytometer designs, waveguides are used to precisely deliver excitation light and collect both scattering and fluorescent signals in a controlled manner. One common method of incorporating integrated waveguides into microcytometers is to simply add lengths of commercially available optical fibers into pre-fabricated passively aligned elements [34,45, 47,59]. One end of the fiber is easily connected to the light source or detector, and the other end can be inserted directly into the device. Inspired by silicon optical bench technology in

which grooves are etched into wafers for the passive alignment of parts, PDMS, plastics, glass, and other substrates can be machined with channels and windows for the passive alignment and support of these fibers. While efficient at guiding light with little attenuation (losses of the order of 0.2 dB km^{-1} have been reported), optical fibers can prove cumbersome to integrate and are not well suited to systems incorporating multiple excitation sources, filters, or detectors.

As an alternative to using commercial fibers, waveguides can be fabricated directly into microdevices using a variety of optically transparent or reflective materials, or the waveguides can be cast or molded into the device after other fabrication steps are complete. In the latter case, common materials for molding include PDMS and UV-curing optical adhesives, both of which are available with a variety of different refractive indices. For example, Lien and coworkers demonstrate that it is possible to take advantage of these commercially available materials to create an all-PDMS device with integrated PDMS waveguides [60]. Although the refractive index differences in commercial materials can be large, very small changes can be produced for custom waveguide fabrication. Godin and co-workers report that it is possible to blend commercially available grades of PDMS to create small refractive index changes [35]. Alternatively, Chang-Yen and co-workers have created small refractive index changes in PDMS by curing at elevated temperatures [61].

As opposed to molding, photolithography has been used to make well defined waveguides out of optically transparent photoresists such as SU-8; these waveguides are integrated directly during the fabrication steps of the device [8,44]. Alternatively, buried waveguides have been fabricated directly onto silicon wafers in a variety of materials including silicon dioxide [62], silicon oxynitride [57], and doped glasses [63]. Although buried waveguides are precisely defined by the micromachining process, they tend to be thin in the vertical direction due to the limitations of fabrication techniques and may not match up with the height of the fluidic channel to be interrogated. Waveguides fabricated in this manner tend to leave a ridge on the surface of the device where the waveguide is buried, making final device bonding difficult. In order to alleviate this problem, further processing such as depositing a thick top layer of glass followed by planarization by chemical-mechanical polishing is required. Finally, coupling these waveguides to external fibers can be problematic, although they are well suited to cases where the detectors or sources are mounted or fabricated directly on the wafer.

Molded and specially microfabricated waveguides have many advantages over commercially available optical fibers. However, these integrated waveguides typically have an attenuation at least five orders of magnitude greater than that of optical fibers (of the order of dB cm^{-1}), necessitating their use in short segments. Most of this loss is probably due to roughness in the sides of the waveguide [64]. Since an average roughness of just 1% of the width of the waveguide results in 0.01 dB cm^{-1} attenuation, it may be necessary to shield optically sensitive areas of a microdevice from radiation lost from the sides of rough waveguides. Inhomogenities in refractive index of such materials or scattering centers in the bulk of the waveguide can make losses even worse. Careful fabrication techniques will be necessary to reduce integrated waveguide attenuation to acceptable levels.

Fluidic channels have been used as waveguides in some instances. Waveguiding through air or aqueous solution is difficult because the core of the waveguide must have a refractive index higher than the cladding in order to achieve total internal reflection. Unfortunately, most cladding materials used in device fabrication have a refractive index higher than 1.33 (pure water); only a few materials such as fluoropolymers have refractive indices this low. Fluoropolymers are difficult to machine, although they can be used as coatings for liquid-filled waveguide channels [65]. Alternatively, “leaky mode” waveguides are sometimes used in biosensors, in which light is allowed to leak from the core due to the absence of total internal reflection. Despite these losses, guided modes still exist, due to partial reflection between the

core and the cladding. In one example, light was launched into a liquid-core leaky waveguide using a prism coupling [29]. In a second case, anti-resonant Fabry–Perot mirror coatings were used as a replacement for the total internal reflection condition. Bernini and co-workers deposited controlled thin layers of silicon nitride and silicon dioxide layers in the underlying substrate [13], creating a waveguide that essentially combined the fluidic and optical paths.

Finally, non-ablative laser machining has been used to directly write waveguides and pre-pattern microfluidic channels in the bulk of monolithic glass and fused silica substrates. Using femtosecond laser pulses, the refractive index of glass can be changed by small amounts, of the order of 1%. As these structural changes only occur at the focused part of the beam, buried waveguides can be directly written into these substrates without the need to bond pieces together. One company, Translume, has commercialized this process [66]. Alternatives to use of femtosecond lasers are specially doped glasses that can be written in the absence of femtosecond pulses. In both cases, the laser-machining process also changes the etch rates of the glass in HF by a factor of 20 or more, so that microfluidic channels can be pre-patterned in the interior of a substrate with a laser, and then later fabricated by wet etching.

Optical detectors

Depending on the source and intensity, a variety of optical detectors are commonly used in microflow cytometers: photomultiplier tubes (PMTs), avalanche photodiodes (APDs), CCD cameras and CMOS imaging arrays, and PIN photodiodes. Traditionally, PMTs and APDs have been used in commercial cytometers that measure fluorescence intensity due to their very high sensitivity, including the ability to count photons. The internal gain of a detector is the most important signal amplification step, as it increases the signal with the smallest effect on noise. Therefore, PMTs are typically chosen for very low light applications because they have internal gains of many orders of magnitude with low noise. However, PMTs are relatively large. APDs have even more intrinsic sensitivity than PMTs, but only modest internal gain compared with PMTs (typically 3–4 orders of magnitude). Photon counting is possible in Geiger mode, and modest voltage levels are required for implementation as compared with most PMTs. A significant advantage of APDs is their size. PMTs are bulky, because of their vacuum tube structure, whereas APDs are solid-state electronic devices. A significant disadvantage of APDs is their sensitivity to temperature changes. Compensating circuitry must be added, although complete compensation and control electronics packages are commercially available. The disadvantage of both types of detector is their cost. When more light is available, PIN photodiodes can be used for detection and such elements are well suited for integration into microdevices. However, no internal gain is available, limiting their sensitivity. While not ideally suited for collecting fluorescence signals, which can be smaller than 1 nW, they are well suited to collecting light-scattering data. Additionally, PIN photodiodes can be fabricated with a hole in the center, positioned in such a way as to let the excitation beam pass through [67]. This particular geometry makes a beamstop unnecessary in forward-scattering applications and can allow the excitation beam to enter the system directly through the detector for backscatter measurements.

Any signal from a photodetector can be externally amplified. However, the inherent noise in the signal also increases when amplified in this way. Several classical methods have been implemented to increase the signal-to-noise (S/N) ratio from optical detectors in microdevices, potentially making fluorescence measurements possible without expensive photodetectors. One approach is lock-in amplification, where the excitation source is modulated at a known frequency. Because the signals of interest (fluorescence and light-scattering) also cycle with this frequency, they can be isolated from the background by use of standard electronics. Significant improvements in S/N have been realized with lock-in amplification. As an example, Tung and co-workers [38] developed a microflow cytometer, with integrated commercially

available fibers, that was capable of performing two-color fluorescence measurements utilizing standard PIN photodiodes. The disadvantage of such a method is the additional burden placed on data-acquisition operations. Custom-designed data-acquisition circuitry and signal-processing electronics will certainly help alleviate the demand on microprocessors for a portable device using this *S/N* enhancement technique. As an alternative, successive measurements on the same particle with time-of-flight analysis have been demonstrated in a PDMS cytometer with integrated PDMS waveguides of a higher refractive index [68]. Such a device takes advantage of the fact that the velocity of particles flowing through the core of the cytometer should be constant. An 80-dB increase in signal-to-noise ratio, as measured by a CCD camera, was reported for 5- μm fluorescent beads. In separate work, this research group integrated an 8 $\times$ 1 optical demultiplexing device directly into their cytometer, also with significant improvement in signal-to-noise ratio [32].

CCD cameras and CMOS imaging arrays have been used for studies of a number of microflow cytometers. They boast reasonable sensitivities and have the advantage of being able to image objects in the flow. However, the largest disadvantage of this technology is the speed at which it can run. Most cytometers using CCD cameras as detectors can operate at flow rates of less than 100 cells s^{-1} ; commercial instruments run significantly faster. Despite their current availability in bulky packages, it is possible to integrate these types of imagers directly into the microdevice. Nieuwenhuis and co-workers used both a two-photodiode strip sensor and an integrated linear array of photodiodes for imaging the projection of PVC test particles as they moved through their cytometer [69]. Hartley and co-workers [70] describe the integration of a custom-built CMOS chip directly into a flow cytometer device by both PDMS encapsulation and direct insertion, and flip-chip bonding to the rear of their transparent glass device, shown in Fig. 4. Interestingly, the direct incorporation of this CMOS bare die not only eliminated the use of a microscope or waveguide coupling to obtain data, but also included a microprocessor that performed spatial filtering and eliminated the need for external data acquisition and analog signal-conditioning electronics.

Lenses

A number of microflow cytometers described in the literature use bare optical fibers or waveguides without additional lensing. Light emerging from such a fiber will be in the shape of a cone dictated by the numerical aperture (NA) of the fiber, causing an increase in beam size on the excitation side and fixed collection efficiency on the reading side. For devices choosing to operate in such a way, optical fibers with a variety of both core sizes and NAs are available, to help control the beam size and collection area. However, lensing is highly desirable, especially in cases where sensitivity is decreased due to the small size of samples (such as for increasing intensity of fluorescence emission). Focusing light down to a small spot can increase fluorescence emission, and the use of lenses can increase the NA of the collection optics. While fibers with lensed ends are commercially available, little variety currently exists in the types of fiber that can be obtained in this way. Fiber tip etching can also be used to create lensing effects, although fibers so modified may be fragile and difficult to insert into microdevices. Other commercially available solutions exist. Ball lenses (standard or GRIN) and cylindrical GRIN lenses do exist that can be added to microdevices to collimate or focus the beam. However, it is difficult to obtain such lenses on a size scale smaller than approximately 200 μm .

Literature exists for the top-down fabrication, from a variety of materials, of lensing arrays and individual elements at much smaller sizes than are commercially available. PDMS [71], SU-8 [8,72], fluid-filled [35], and polymer lenses [73,74] have been fabricated for use in microfluidic devices. Microlenses can be fabricated for use with integrated in-plane optics, especially for coupling to waveguides. As an example, Camou and co-workers [71] describe the fabrication

of a PDMS waveguide with a curved end, acting as a lens to focus light into a fluidic channel. Although this technique is excellent for producing lenses with pre-defined focal lengths and fixed optical alignment, there are two significant disadvantages. The first is that lenses made in this way are inherently cylindrical, since the top-down machining techniques allow for no continuous variations in the z-direction. Cylindrical lenses can only focus light in a single dimension. Second, depending on the fabrication method, it can be difficult to control roughness down to subwavelength levels, so that scattering from these optical elements can be problematic. Finally, note that any lens designed to be fabricated by casting an elastomer such as PDMS can shrink on curing, altering its optical properties from the original design.

In many cases, lensed systems provide a creative method of obtaining signals that are difficult to obtain directly with fibers. For example, angle-specific light scattering data can be difficult to collect with optical fibers, because only one small part of the angular section can be observed with a fixed fiber. However, Singh and co-workers demonstrate a microfluidic device capable of obtaining light-scattering data over a wide angular range by use of a hemispherical lens [29]. The authors fabricate a 30- μm channel using photoresist, and launch light directly into the waveguide using a prism and the phase-matching condition. The liquid in the device itself serves as a leaky waveguide through which the light excites the beads. Scattered light is collected through a commercially obtained hemispherical lens and is projected directly on to a CCD array. Using this configuration, it is theoretically possible to obtain all light-scattering data from 0–180°, although the authors note that in practice such collection would be difficult due to the inherent brightness of the forward scattering angles compared with other angles. The authors demonstrate that by de-centering the beads from the axis of the lens, they are able to effectively collect backscattering data over the range 140–180°.

Excitation sources

Light-emitting diodes (LEDs) and laser diodes can both be integrated directly into a microflow cytometer or can be used in packaged form in the near vicinity of the fluidic device. Compared with lasers, LEDs suffer from the disadvantage of non-collimated emission. However, they are still used in compact optical devices due to their low cost. As an example, Novak and co-workers demonstrate the integration of a blue LED and collimating optics, photodiode, and amplifier into a compact package for detection of fluorescence in liquids [75] (Fig. 5).

Other integrated light sources are possible. Balslev and co-workers describe the development of an integrated device for optical measurement in fluids that contains a microfluidic optically pumped dye laser, SU-8 waveguides, and integrated photodiodes [76]. In this work, the dye (rhodamine 6G) was introduced into a 1 mm wide fluidic channel and replaced at a rate of 1–10 $\mu\text{L h}^{-1}$. The dye was pumped with a pulsed external light source. Five SU-8 waveguides collected light emitted from the laser and guided it to different locations in the integrated cuvette. Finally, SU-8 waveguides guided the emitted light from the cuvette directly to integrated photodiodes, through leaky waveguide mode coupling.

Optical filters

Very high quality commercial fluorescence filters are typically composite structures; because of these laminated layers, they can be quite difficult to machine and are usually used only in manufacturer-supplied geometries. Furthermore, they are expensive and tend not to be disposable. However, other materials have been suggested that are easily machined or directly incorporated into the fabrication of a device. For example, Hofmann and coworkers demonstrate the fabrication of a disposable long-pass optical filter by the addition of organic dyes to PDMS [77]. Other potential solutions include microfabrication of interference filters on to silicon-based devices [78,79], the use of thin-film coatings [80], and the use of colored thin sheets of polycarbonate.

Cytometer electronics

Creative electronics design and data-processing algorithms can have a significant effect on both the acquisition and analysis speed of a microflow cytometer and the quality of the data obtained. As an example, perhaps the most important data parameter in both fluorescence and light-scattering measurements is the intensity (maximum) value of the peak, followed by the area under the peak. The typical way to obtain this information in a classical data acquisition system is to sample each peak with many points, with the hope that one of those samples will be near the true peak intensity, and that there are enough points on the peak to reproduce its shape so that the area underneath can be accurately obtained. As microflow cytometers are run at faster and faster speeds, sampling in this way becomes very demanding, especially in systems that require continuous operation or data processing in real time. As an alternative, the integration of nested sample and hold amplifiers allows the creation of a “peak detector”, which enables the system to very accurately capture the maximum value of the peak, at the expense of time resolution. These types of electronics allow the collection of very accurate data even when sampling just a few points per peak, greatly easing the burden on data acquisition operations and at the same time potentially reducing the coefficient of variation on the data collected. In addition, these types of electronics are typically added to the analog side of the system, reducing the number of (expensive) CPU cycles required for system operation on the digital side. Simultaneous peak integration, using analog electronics, can accurately obtain peak areas with little software processing.

There are many other possible improvements to the electronics of simple data acquisition systems, including filtering, the introduction of thresholding circuits to limit the number of points that pass through software, and the introduction of logic gates for quick comparisons between channels. Many other improvements exist. In most cases, the commercial availability of electronics parts in integrated circuit packaging, coupled with the widespread availability of inexpensive microcontrollers and microprocessors, allow the creation of compact, low-power data collection and analysis systems without the need to send designs to a foundry for microfabrication. We cannot emphasize strongly enough the impact of a well-designed electronics and data-processing package in microcytometer development. However, a thorough discussion of recent innovations in cytometer electronics and data processing algorithms is beyond the scope of this manuscript. A good place for an interested reader to start is the excellent review of flow cytometer electronics that has been published by Snow [81].

Electrical detection methods

As an alternative to standard optical methods that utilize light-scattering information in a flow cytometer, several groups have demonstrated the use of electrical detection methods to extract a variety of useful information from these systems. The methods are amenable to portable cytometric applications, eliminating bulky optical components by use of metal electrodes directly embedded in a microfluidic channel. Because cell membranes act as insulators at low measurement frequencies, electrical detection methods can be extremely useful for cytometry applications. In fact, this is the basis for the Coulter counter which has become a ubiquitous analytical tool in biological laboratories in the last half century. While specificity is limited with these label-free methods, electrical impedance measurements have been used to detect the presence, size, and dielectric properties of particles as they flow through a detection region in a microchannel. In recent years, these methods have been utilized with increasing functionality in microflow cytometric devices. Impedance spectroscopic techniques have been used on-chip to demonstrate differentiation between erythrocytes in various states, and polystyrene and latex beads [30,82]. By interrogating with both low and high frequencies in these studies, cells and particles could be differentiated by both size and electrical properties. Chun et al. used polyelectrolytic salt bridge electrodes to maintain DC currents which are ideal

for electrical cell detection. [83] They report detection of 10- μm beads, at rates of up to 1000 beads s^{-1} , and demonstrate the ability to distinguish between red and white blood cells based on the signal amplitude–cell size correlation.

Integrated systems and applications

A number of microflow cytometer devices have been described above to illustrate strides made in individual areas of fluidics, optics, and detection systems. While most reports appearing in the literature have focused on technical gains in one of these areas, a few stand out as examples of well-designed packages that make a serious attempt to duplicate the capabilities of benchtop instruments. These include cytometers with excellent detection efficiency over multiple channels, cytometers with very high sample throughput, or studies targeted toward specific assays. Furthermore, a number of commercial microfluidic platforms are currently available and worthy of note, although admittedly none is fully integrated. A small selection of these papers is described below. Other excellent manuscripts are not described here [48,84].

Given the potential advantages of reduction of sample consumption, assay time, and power consumption, several groups have developed sample-preparation schemes that may be incorporated into microflow cytometer systems; complete integration has not yet been demonstrated, however. A few recent developments include microfluidic devices for separation and/or manipulation of blood cells [85,86]. Other examples that may be applicable to flow cytometry, such as automated fluorescence labeling of samples, have been highlighted in another review [84].

Integrated prototype systems

Chang et al. [87] and Yang et al. [19] have presented systems integrating two or more microfluidic components. In the first device the flow cytometer consists of a PDMS flow channel integrated with pneumatic serpentine-shape (S-shape) micropumps, embedded optical fibers for multi-wavelength fluorescence detection, and a microflow switching device, composed of pneumatic micro-valves capable of automated cell injection, counting, and switching. Experimental results showed that the developed flow cytometer can distinguish specific cells with different dye-labeling from a mixture of oocytes (15–20 μm) and lung cancer cells (10 μm) labeled by fluorescent dyes in a single process. In the second device the PDMS flow cytometer chip is integrated with similar microfluidic components for a cell counting/sorting system. In addition, the device contains an optical detection system and a data analysis and control system. Fluorescently-labeled human lung cells have been detected and collected (120 cells min^{-1}) using this portable system that has dimensions of 37 cm $\times$ 16 cm $\times$ 18 cm and weighs 3.5 kg. These two devices are capable of sorting 120 cells min^{-1} . A microfabricated cell sorter, integrated with peristaltic pumps, pulse dampeners, switch valves, and input and output wells has been developed by Fu et al. [18]. The device was used continuously for six months with millions of actuations of each valve and pump. *Escherichia coli* cells were sorted and recovered at a rate of 44 cells s^{-1} . Wolff et al. [9] reported a pressure-driven microfabricated fluorescence-activated cell sorter with integrated chambers on the chip for holding and culturing sorted cells. Using this sorter, fluorescent latex beads were sorted from chicken red blood cells, achieving substantial enrichments at a sample throughput of 12,000 cells s^{-1} . Sorted yeast cells collected in the culturing chamber were supplied with a flow of fresh cell media and grew and divided for several days on-chip.

Commercial systems

A few commercial systems that use microfluidic components are currently available. One such system, the Agilent 2100 Bioanalyzer [88], is capable of flow cytometric analysis of cells, and electrophoretic separation and analysis of DNA, RNA, and proteins. The Bioanalyzer was used

by Chan et al. [89] for analysis of protein expression and apoptosis in human primary cells. In the microfluidics chips, the cells are moved by pressure-driven flow through glass-based channels and are analyzed by two-channel fluorescence detection. Approximately 500–1000 cells can be analyzed in four minutes, and up to six cell samples can be handled by one microchip. Staining reactions can be performed on-chip and the analysis can be done without washing steps. The reported results correlated with data obtained using a standard flow cytometer. The Agilent 2100 Bioanalyzer was also used to detect small amounts (10 cfu mL^{-1}) of milk-spoiling bacteria [90] that were undetectable using the standard culture method and to analyze fluorescence in-situ hybridization in natural marine pelagic bacterial communities [91].

Another commercial system has been developed by Micronics that uses a microfluidic laboratory-on-a-card structure. The plastic card, made using a laminate prototyping method, consists of a 30- μL sample loop, a 400- μL reagent reservoir, a sample injector, labeling loop, view port, and a sorting slit for removal of tagged cells or particles. As discussed earlier, Lancaster et al. [12] demonstrated the detection and sorting of rare cancer cells from blood using the Micronics device. As the sample was injected, a thin ribbon monolayer of cells was produced that enabled multiple cells to be viewed and sorted as a whole cell row or section of the ribbon at a time. The cell injector was also capable of antibody labeling within 20 s. Within 30–60 min, target cells could be detected at sensitivity levels 1,000 to 10,000 times those of existing flow cytometers.

Applications

As more flow cytometer components are miniaturized and integrated with microfluidic devices, more microflow cytometers will be used for applications in fields including gene diagnosis, transfusion medicine, bacteria analysis, clinical hematology diagnosis, DNA molecular sizing, and environmental microbial sensing. A few examples of applications are presented below.

In a paper discussed earlier, Simonnet et al. [17], describe two PDMS microfluidic devices fabricated for two microflow cytometers. The first system analyzed up to 17,000 particles s^{-1} and the fluorescence detection accuracy was comparable with that of a conventional flow cytometer. The second system design focused the sample stream to a flow layer of submicrometer thickness that enabled imaging of particles with a resolution comparable with that of still micrographs. To the best of our knowledge, this is the highest throughput microflow cytometer reported to date.

In addition to the need for high throughput in some applications, the viability of sorted and recovered cells is also important. Wang et al. [92] developed a fluorescence-activated microfluidic cell sorter that used optical forces for the rapid (2–4 ms) and active control of 20–100 cells s^{-1} on a microfluidic chip. Transfected HeLa cells, detected and sorted using the device, were shown to be viable and unstressed following analysis.

Several bacteria studies have been performed by groups using microflow cytometers. In two other examples using PDMS channels, the devices were used for counting bacteria in potable water [93] and discrimination of live from dead bacteria [10] with counts of 10^4 – 10^5 cells mL^{-1} and 1000 cells min^{-1} , respectively. Cell and particle-based assays have also been analyzed in microflow cytometer systems. Using a cytometer based on silicon hollow-core antiresonant reflecting optical waveguides, Bernini et al. [13] differentiated between two main populations of Jurkat cells at different stages in the cell cycle, on the basis of fluorescent labeling of DNA; the results showed the same trend as those produced using a bench-top flow cytometer.

A PMMA device has been used to count and size particles and particle agglomerates on the basis of laser-light scattering [45]. Light scattering by 2 to 9- μm polymer beads at two different angles, 15 and 45 degrees, was detected with a throughput of 150 particles s^{-1} . Size discrimination of particles with a diameter ratio of 1:2 was also achieved. The device was used to detect C-reactive protein (CRP) in a particle-enhanced immunoassay, with a limit of detection of 100 ng mL^{-1} .

The way forward

Clearly, there is widespread excitement generated by the concept of transforming flow cytometry capabilities into a portable, microfluidic format. The ideal microflow cytometer will be small, automated, and robust. Components are being optimized, but systems integration efforts are relatively immature.

As the heart of a flow cytometer is the generation of a sample stream with a diameter of the order of the particles to be measured, it is no surprise that flow focusing has received the most attention to date. The “perfect” flow focusing system should be:

1. easily manufacturable,
2. easily replaceable, and
3. resistant to clogging or fouling.

In other words, the device needs:

1. to be as simple as possible, with as few layers as possible,
2. to have a minimum number of fluidic and electronic interconnects, and
3. to allow no contact between the channel walls and the sample stream wherever the diameter of that stream approaches the diameter of the particles to be analyzed.

This goal has not yet been achieved, but the scientific community is approaching it asymptotically.

For reliable data, the position of the sample stream within the sheath flow must be very well aligned with regard to the excitation and collection optical paths. Pumps amenable to on-chip integration are under intense development for a multitude of microfluidic applications and will become available in the near future. On-chip valves are also being widely investigated for other applications and should be available as problems with the reliability of materials are solved.

The minimal requirement for robust, highly sensitive optics will be on-chip waveguides and lenses to deliver the light and collect the signal from a region of a channel probably less than 20 μm^2 . For many applications, incorporation of the excitation and collection devices on-chip will be important to construct a continuous monitoring system in a very small footprint. The microelectronic materials community has developed the necessary tools to manufacture integrated optics, and the required components are becoming commercially available. In addition to more traditional laser diodes and APDs, polymer components such as OLEDs and organic detectors could be fabricated on-chip.

Applications will drive the incorporation of on-chip sample processing and sorting. For point-of-care and field applications, automated sample preparation is essential. In addition to requiring small pumps and valves under automated control, microfluidic mixers must be incorporated into the systems—perhaps even on the same chip as the analytical device. Sorters that respond to the analytical information and collect targets of interest will provide capability that complements the flow cytometry analysis—saving viable samples for additional analysis such as confirmatory culture, genetic identification, or mass spectroscopy. The first sorters will

employ electrophoretic, electrokinetic, or magnetic-bead-based methods. However, eventually sorting methods based on optical forces may prevail because they are less likely to affect fragile cells.

More attention must be paid to system integration, including user-friendly control and data-analysis software. Analytical software has largely been the purview of the cytometry industry. As microflow cytometers move out of research and into commercialization, the software issues will be addressed. However, developers of new microflow cytometers should take the time to understand the software and electronics of existing commercial systems to anticipate the need to mesh the fluidics and optics with the level of data sophistication required by various users. Furthermore, a design process that gives equal consideration to fluidics, optics, biochemistry, electronics, and the intended application scenario will yield a truly effective microflow cytometer.

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Fig. 1. Schematic diagram of the detection region in a common flow cytometer. A particle is detected in the interrogation volume which is defined by the union of the sample stream, the volume illuminated by the excitation light, and the volume from which the collection optics detect light

Fig. 2. Schematic illustration of the hydrodynamic focusing device developed by Chang et al. [50]. (a) 3-D view, (b) top view, (c) side view, and (d) cross-section perspective depicting core stream centered in channel. Reproduced with permission from the Institute of Physics Publishing Limited

Fig. 3. Schematic diagram of the operating principles for sample focusing. (a) Top view and (b) side view. Cells or particles are focused at the center of the sample stream using DEP and hydrodynamic forces. Reproduced from Lin et al. [55] with permission from IEEE

Fig. 4.
A 15 mm² microfluidic dielectrophoretic cell sorter. The device combines dual digital cytometer sensors, in-channel electrokinetic electrodes, and hydrodynamic fluid focusing. Reproduced from Hartley et al. [70] with permission from the IEEE

Fig. 5. Photograph of a lab-on-a-chip device with integrated microfluidic dye laser, optical waveguides, microfluidic network, and photodiodes. The metallic contact pads for the photodiodes are seen on the far right. The chip footprint is 15 mm by 20 mm. The photograph, which was taken before a lid was bonded to the structures, is reproduced from Balslev et al. [75] with permission from the Royal Society of Chemistry