Published in final edited form as:
Curr Protoc Cytom. 2013 July ; 0 1: Unit–1.2. doi:10.1002/0471142956.cy0102s65.

Fluidics

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Abstract

The use of fluidics is implicit in a technology named “flow cytometry”, which flows a cell or particle through a sensing volume to obtain serial analysis of particles on a one by one basis. This flow of particles enables flow cytometry to collect information on multiple particle populations, giving it a distinct advantage over bulk analysis approaches. Moreover, flow cytometers can analyze thousands of particles per second in a single flowing stream. Additionally, use of volumetric sample delivery makes it possible for flow cytometers to accurately count cells and particles. Furthermore, the analysis results can be coupled with a fluidic diversion mechanism to sort and collect particles based on desired properties. Finally, when high throughput sampling technologies are employed to rapidly change the input of the sample stream, a flow cytometer can become an integral tool for high throughput screening. The above properties have made flow cytometry useful in a wide range of biomedical applications. In this unit we will present an overview of fluidic systems that make flow cytometry possible. This will introduce historical approaches, explanations of the commonly implemented current fluidics, and brief discussions of potential future fluidics where appropriate.

INTRODUCTION

The first purpose for the fluidics of a flow cytometer is to deliver a sample containing suspended cells or particles to the flow cytometer. This has historically been done using either simple pump, such as a syringe pump, or a pressure head above the sample (Figure 1). Initial flow cytometers delivered single samples to the instrument and were manually operated (Moldavan, 1934). However, improved technologies have resulted in a number of automated sampling approaches that allow a flow cytometer to sample from many samples without operator intervention and at very high sampling rates (Edwards et al., 2001). Most flow cytometers sold today come with a microplate feeder option or an automatic tube delivery system. The fastest sampling systems today can deliver discreet samples at rates as high as two per second (Edwards et al., 2004). Beyond rapid delivery, there have been numerous inline sample preparation approaches to automate reagent addition, rapidly mix samples, and to automate incubation times. These have included the use of conventional fluidics and the use of microfluidic preparation approaches.

The second function for the fluidics is to position the sample stream for optimal analysis (Figure 1). To ensure that only one particle or cell is measured at a time, an optimal fluidic system minimizes coincident events within the analysis volume. This basic principle was

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first suggested as a method to count microscopic cells (Moldavan, 1934). This system used a pressure differential to drive cells suspended in a liquid medium through a glass capillary drawn to narrow dimensions. The capillary was placed under a microscope objective and cells that obscured the field of view could be automatically counted using a photodetector. The fluidics of this effort was improved upon through the use of a fast moving sheath stream to focus the sample stream in the center of the analysis capillary (Figure 2), which allowed the use of wider capillaries that alleviated clogging and retained tight focal positioning of the sample stream. This approach also reduced coincident events in the focal region of the optics as the narrow sample core limited the number of particles that could be present in the analysis volume (Crosland-Taylor, 1953).

These early instruments counted cells in capillaries and did not use refined flow cells. The first effort to collect detailed optical properties from flowing cells used a syringe pump to draw cells through a 100 μm x 100 μm flow channel that had been ultrasonically cut into a glass slide (Kamentsky and Melamed, 1969), which foreshadowed the field of microfluidic flow cytometry. The fluidics of this instrument delivered the cells with enough precision to enable the collection of absorption and scatter properties of cells, as they passed through a tightly focused illumination spot. The eventual coupling of flow cytometry with fluorescence measurements (Bonner et al., 1972) gave new importance to the use of hydrodynamic focusing. Within the flow cell of a typical modern flow cytometer, hydrodynamic focusing constrains the sample stream to a very narrow diameter. This ensures that the particle largely fills the optical interrogation volume, which is defined by the intersection of the sample stream with focused excitation light (Figure 3). Thus particle associated fluorescence normally dominates the optical measurement as solution based fluorophores are largely excluded from the analysis volume. This enables a flow cytometer to resolve fluorophores bound to the surface of a particle vs. those in solution and allows for homogeneous wash free fluorescence assays in general (Sklar et al., 2002). When used solely for analysis, this approach is typically configured to flow upward, which ensures that any air bubble in the system simply floats upward out of the flow cell (Figure 4A). Though hydrodynamic focusing provides many benefits, it also increases the use of fluids, the amount of waste , and the linear velocity of the particles, which has led to many efforts to develop sheathless flow cytometers that retain the benefits of hydrodynamic focusing without the need for large volumes of sheath.

The third fluidic function is to efficiently sort and collect particles of interest based on the results of the particle analysis that occurs just upstream of the sorting process. Sorting has been most commonly performed using electrostatic droplet deflection. Electrostatic deflection of droplets was originally developed for use in conjunction with Coulter volume measurements (Fulwyler, 1965), it was subsequently adapted for use with scatter and fluorescence measurements (Bonner et al., 1972). In this approach the focused sample stream exits the flow cell or nozzle and the exiting “jet-in-air” is broken into droplets via a standing wave imparted upon the flow stream by a piezoelectric drive (Figure 4 B & C). The particles of interest are entrapped wit hin droplets formed by the jet-in-air mechanism, which are charged electrostatically and, based on their fluorescence and scatter properties, which were determined at the point of analysis, deflected by highly charged metal plates to be collected into discreet sorted samples. Analysis of such systems can be accomplished in a

flow cell, which is followed by droplet formation in an exiting jet in air (Figure 4B). Alternatively, the analysis can be performed directly in the stream as it exits a flow nozzle (Figure 4C). As compared to the use of a flow cell, this approach sacrifices optical sensitivity, but allows for closer analysis to the droplet breakoff point and more accurate sorting decisions. Regardless of the approach taken, electrostatic deflection can reach rates of 50,000 per second, and it has become the dominant sorting technology used today in commercial instruments.

However, droplet based sorting does have some drawbacks such as required high pressures that can be damaging to fragile cells and the generation of aerosols, which can be a significant biohazard. Therefore, it is also becoming common to use actuators to selectively divert the sample stream into collection channels to sort particles of interest. The earliest example of this used the same microchannel approach developed above and sorting was accomplished via triggering a second syringe pump to withdraw cells of interest as they passed the intersection of a crossed channel (Kamentsky and Melamed, 1969). Many variations on this theme have been developed including piezo-based diversion of the flow stream (Gray et al., 1989), directed electrosmotic pumping to selectively change flow directions (Fu et al., 1999), and MEMS based valves (Yang et al., 2006).

While, as the above intro suggests, flow cytometry is certainly an established technology, research into optimal fluidic systems for flow cytometry continues. Recently, research has focused on (i) improving sample delivery systems to reduce system cost, retain volumetric delivery, and to increase the sample to sample delivery rates, (ii) developing alternate focusing techniques to eliminate or minimize the need for sheath fluid, and (iii) developing new sorting mechanisms for safe and gentle handling of biological samples. The remainder of this unit discusses in detail the basics of flow cytometry fluidics and introduces recent advances with regards to sample delivery, particle focusing, and sorting. While most of these advances have been implemented in commercial flow cytometers, some are still early stage technologies that are expected to be seen in wider use in the coming years.

Sample delivery

Sample delivery has been accomplished using either pumps or an increase of pressure differential between the sample and the system. The use of pumps adds complexity, but offers control over delivery rates, which enables direct counting of particles per unit volume without the addition of a particle standard of a known concentration. Beyond these two basic approaches, sample delivery systems can be broadly classified into the following types.

1. Traditional single sample methods
2. Rapid mixing systems that quickly process for rapid single sample delivery
3. High throughput sample delivery for serial delivery of multiple samples

Traditional single sample methods

Pressure differential—In this method of sample delivery, a pressure differential is maintained across the reservoirs containing the sample and sheath fluid. In practice, the sample is contained in a tube that is pressed onto the system forming a sealed pressure head

above the sample (Figure 5A). The seal has an inlet to drive an increase of pressure above the sample. Once the pressure of the sample has increased above that of the system, the sample is driven into the system. In these systems, the rate of sample delivery depends upon the pressure gradient as well as the pressure drop that occurs along the length of the capillary tubing. Once they have been carefully calibrated, such systems enable very smooth sample delivery required for most flow cytometers. Another advantage of pressure differential systems is that removal of the sample can reverse the pressure differential between a sheath fluid and the delivery point. This causes sheath fluid to be driven through the sample line, which washes the line and limits sample carryover between samples. Despite these advantages, pressure differential systems are subject to pressure variations over time, which poses challenges in delivering samples at precisely known rates and prevents them from being truly volumetric. Therefore, microsphere standards that contain particles at known concentrations must be added to the samples to precisely measure the particle or cell concentrations within the sample (Stewart and Steinkamp, 1982). A further disadvantage of the pressure differential delivery system is that pressure head at the sample could cause sample to be introduced into the air due to tube failure or operator error, which is a concern for many biological samples.

Syringe pumps—In a typical syringe pump based delivery system, an arrangement of capillary tubes and valves are used to draw the sample into a fill line and then pump the sample to the system (Figure 5B). The precise action of the stepper motor in a syringe pump enables sample delivery to the flow channel at precisely determined rates, which makes these systems truly volumetric. Despite these advantages, syringe pump systems are problematic in that they can induce pulsations in flow, they deliver defined sample volumes that are predetermined by factors such as the size of the fill line, and they are typically more expensive to construct.

Peristaltic pumps—In a peristaltic pump, the sample is drawn from an open sample into a flexible tubing in contact with the rotor. As the rotor spins, the tubing is subject to alternate cycles of compression and relaxation. The compression cycle moves the fluid within the tubing while the relaxation cycle draws a small volume of sample into the tubing (Jaffrin and Shapiro, 1971). The combination of these cycles pumps the sample through the tubing and delivers the sample to the flow cytometer (Figure 5C). Owing to its volumetric nature, the peristaltic pump offers measurement of cell concentration, which further enables accurate measurement of fluorescence and scatter properties at the interrogation point. These advantages have enabled integrating peristaltic pumps in even low cost commercial flow cytometers (Rich and Howes, 2010). A pair of peristaltic pumps, one each for the sheath and sample, has been used in push/pull configuration. One-pump pushes the sheath fluid through the flow cell while the other pump draws both the sheath and sample by suction. The pressure differential so created enables precise focusing of the sample for optical analysis. However, as in the case for all peristaltic systems, tubing wear at the pump head can cause sample delivery rate variation over time. Therefore, care must be taken to maintain the system and ensure accurate and reproducible sample delivery rates. Peristaltic pump based delivery systems also do not require a pressure head to deliver the sample, which again reduces biosafety concerns during sample delivery. Furthermore, they provide variable

sample delivery rates, do not require multiple draws to deliver large volumes of sample, and are relatively inexpensive and simple to implement. The largest issue regarding the use of peristaltic pumps is that care must be taken to avoid pulsations in flow. This can be accomplished in a variety of ways and instruments have been successfully constructed using these pumps.

Rapid mix for rapid single sample delivery

In the sample delivery systems described thus far, the cells are brought to the analysis point only many seconds after injection. Such delays make them unsuitable for studying kinetics of fast reactions, such as ligand-receptor binding, which require subsecond resolution between mixing and analysis. These applications require delivery systems that not only mix the samples rapidly but also deliver them at low flow rates with reduced dead time (time delay between successive cells at the interrogation point). Toward this aim, two types of systems have been developed:

1. Syringe pump driven systems
2. Coaxial flow systems

Syringe pump driven systems—In a syringe pump driven system, samples are loaded into holding lines that are then rapidly driven through a simple mixer into a delay line. The mixed sample is then driven by drive syringe at a controlled rate into the flow cell. This process requires high-speed valves to switch the delivery from waste to the flow cytometer just after mixing. This is a critical step for hydrodynamic systems, as the high fluidic rates that occur during mixing are incompatible with laminar flow. While mixing, the valves in the fluidic line prevent sample flow into the flow cell. The mixed samples are then delivered to the delay line where fluorescence measurements can be made within 120 ms of the reaction (Graves et al., 2002; Nolan et al., 2005). This approach has the advantage of permitting continuous monitoring of reaction kinetics for two or more reactants. However, even with precisely controlled sheath flow and expensive syringe drives, pressure disturbances at the nozzle affect the stability of the focused streams, ie. the sample streams tend to widen resulting in increased background signal (Graves et al., 2002; Seamer et al., 1999; Sklar et al., 1998).

Coaxial flow systems—In these systems, the samples are mixed via a coaxial jet mixer constructed from two or more capillaries. This mixer is directly inserted into a sheath stream, which enables the mixed sample to be immediately focused for analysis. This type of mixing allows studying kinetics within 60 ms of sample injection (Scampavia et al., 1995). Discreet time points are collected by moving the mixing point relative to the analysis point. As such, this approach requires discreet time points to be measured individually, from which the complete time course can be reconstructed, unlike syringe-driven systems (Blankenstein et al., 1998).

High throughput sample delivery for serial analysis of many samples

Traditionally, high throughput screening (HTS) for lead compounds is performed in small volumes (μL) in multi-well plates to identify target compounds. However, the ability of flow

cytometry to both directly analyze cells and perform multiplex microsphere assays has led to the use of flow cytometry in HTS (Edwards et al., 2007). As HTS requires the processing of millions of potential compounds, as such, sample-to-sample throughput is critical.

Traditional high throughput delivery methods—Traditional high throughput sample delivery systems have included tube loaders, which simply feed tubes on the flow cytometer (Shapiro, 2003), and plate feeders that process micro well plates in an automated fashion (Edwards et al., 2007). In plate feeding systems the plates were typically moved so that the microwells make proper contact of the tip of the delivery tube with the sample. The method of sample delivery, once the tip has made contact, is consistent with the manual delivery systems. These systems do ease the burden on the operator but are typically considered too slow for use in large HTS efforts.

Plug flow cytometry—To speed sample delivery, plug flow methods of sample delivery have been developed (Edwards et al., 1999; Edwards and Sklar, 2001). Also see unit 1.17. In these systems, samples were originally drawn from syringes into a one of two fill lines within a reciprocating valve. The valve could change position quickly and allowed simultaneously filling of one sample and pressure based delivery of another sample into a commercial flow cytometer. This resulted in the rapid injection of small volume plugs of samples into the flow cytometer, hence the name plug flow cytometry. About 5μL of the sample arrived at regular intervals at the interrogation point, allowing nearly 9 plugs to be analyzed per minute. Owing to plug flow, these systems allowed precise measurement of sample concentration and the use of the reciprocating valve made it possible to draw sample from unpressurized samples (Edwards et al., 1999; Edwards et al., 2001; Edwards and Sklar, 2001). This approach did not wash the flow cell or the delivery line between plugs. Therefore, carry over between plugs was carefully monitored and found to be only <2% between samples, which is suitable for most HTS applications (Edwards et al., 1999).

The concept of delivering small plugs of sample was refined in the development of the HyperCyt delivery system (Edwards et al., 2001; Edwards et al., 2004). In this system, a computer controlled autosampler is combined with a peristaltic pump to rapidly deliver plugs of samples to the flow cytometer. In this system the autosampler moves between samples as the peristaltic pump continuously pumps sample to the flow cytometer. This results in small plugs of sample (a few μL) that are separated by air bubbles being loaded into capillary tubing that is connected to the flow cytometer. The plugs of sample and the air bubbles are delivered to the flow cytometer and the plugs of sample are analyzed serially. The bubbles have been shown to not significantly affect the fluidics of the flow cell (Edwards et al., 2004). This approach enables samples to be drawn from multi-well plates, deliver samples continuously at rates of up to 40 samples per minute, again with minimal carryover (Edwards et al., 2009). This system has been successfully developed and used in many HTS efforts for therapeutic compounds of interest.

Parallel microchip—High content screening has also been performed in microfluidic formats. A notable device consisting of 384 parallel channels fabricated into a glass plate was used to simultaneously analyze samples delivered from a 384-well micro titer plate. The samples were drawn by suction into these channels by using a syringe pump. The channels

were conical in shape and hemispherical in cross-section, capable of handling as low as 1-3 μL of the sample. Such low volumetric capacity permitted continuous measurement of sample concentration. Periodical cleaning using chlorine bleach prevents clogging while making these channels re-useable for further assays (McKenna et al., 2011; Mckenna et al., 2008). However, this system required complex scanning optics to analyze the 384 channels.

Positioning of cells for analysis

The confinement of the sample into a narrow stream or channel for optical analysis enhances the accuracy and sensitivity of measurements. Precise positioning of particles in flow cytometry can be obtained using a variety of methods that include:

1. Simple micro channels or capillaries
2. Hydrodynamic focusing
3. Acoustic focusing
4. Inertial particle migration and structured microchannels for particle focusing

3.1 No focusing/Microcapillary flow cytometry

Akin to the earliest flow cytometers, many efforts have continued to analyze particles that are simply positioned by the physical constraints of a microchannel. This approach allows simple coupling to the sample delivery system, but the optical interrogation volume is usually quite large as particles can pass anywhere within the channel. Additionally, as the dimensions used typically dictate the development of a parabolic flow profile, the relative linear velocity of particles varies based on their position in the channel, which results in reduced accuracy of optical measurements. Both of these issues can be minimized by the use of smaller channel cross-sections, but as the channels are reduced in size the likelihood of clogging increases. Thus this approach typically must sacrifice accuracy to be usable for any length of time. Furthermore, as the sample comes into direct contact with the optical surfaces, the channels must be routinely cleaned or replaced for long-term use. Regardless of these disadvantages, these systems are simple and inexpensive to construct, and many successful systems are based on this approach (King et al., 2010).

Hydrodynamic focusing

Most modern flow cytometers tightly position the sample for optical analysis via hydrodynamic focusing. Here, a carrier fluid called the sheath fluid is used to position the sample of cells into a single file for optical interrogation. As both the sheath and sample are incompressible liquids, the hydrodynamic pressure of the fast moving sheath fluid simply accelerates and focuses the slow moving sample stream into a tight sample core that can be as narrow as a few microns (Kachel et al., 1990). This approach can be used to axially focus the core by simply inserting a narrow capillary tube into the center of the fast moving sheath, which results in a tightly focused core in two dimensions. This is the traditional approach taken by most flow cytometers and this enables the alignment of particles for single file optical analysis. Alternatively, if sheath fluid is delivered from only the sides of the flow stream – or at disproportionate rates from the top and bottom flows, focusing

occurs primarily in only one dimension, which can provide a ribbon like core stream. For some applications this would be problematic, but for imaging flow cytometers this approach can be desirable as particles can be constrained to a single image plane (Howell Jr et al., 2008). Regardless of the type of sample core desired and the potential disadvantages, many successful flow cytometer systems have been constructed using capillary-based approaches for hydrodynamic focusing or, more recently, the use of modern microfluidics approaches.

Capillary-based hydrodynamic focusing—For decades, commercial flow cytometers have used a nozzle and tubing arrangement to achieve hydrodynamic focusing of the sample. The sheath fluid flows into a tubing of relatively large diameter than the nozzle through which the sample is injected. The channel tapers into a narrow region (Figure 4). At this region, the hydrodynamic pressure of the sheath fluid focuses the sample into a stream of particle-dimension width, which, as described above, provides improved optical positioning and enables the resolution of free vs. bound fluorophore (Figure 3).

This conventional design for hydrodynamic focusing can be modified suitably depending on its application. In an extreme example, a capillary tubing of 100 μm outer diameter was inserted into a 250 μm square flow chamber. The sample was delivered into the capillary tube while the sheath fluid flowed into the square chamber. The sheath flow from the periphery of the capillary tubing focused the single molecules precisely, yielding high sensitivity of detection for DNA molecules (Keller et al., 1996).

Despite being able to focus cells and single molecules, large particles are problematic for this approach. As particles increase in size, the flow channel cross sections must correspondingly increase to prevent clogging. The flow of fluids is characterized by a dimensionless number called the Reynolds number (Re), which is the ratio of inertial force to the viscous force acting on the fluid. The Reynolds number is a function of the linear flow velocity *vf* as well as the physical properties such as density ρ and viscosity μ of the fluid and given by the equation

$$Re = \frac{\rho v_f D}{\mu} \quad (1)$$

Here *D* refers to the diameter for a cylindrical channel. When *Re* is less than 2300, flow is smooth and laminar, but at *Re* greater than 2300 flow can be turbulent. The Reynolds number equation predicts that, as the channel cross-section increases, turbulence will occur at lower linear velocities. The very low linear velocities required for laminar flow has limited the use of hydrodynamic focusing systems at channel cross-sections wider than about 400 microns. Though this is problematic for larger particles, which include Drosophila embryos, tumor microspheroids, and many others, a variety of hydrodynamic focusing flow systems have been designed to both analyze and sort such particles (Pulak, 2006). Of course in all cases the restricted linear velocities dramatically reduce the particle analysis rates.

Microfluidic hydrodynamic focusing—Many groups have been exploring microfluidic flow cytometry to facilitate integration of flow cytometry into lab on a chip applications (Huh et al., 2005). Hydrodynamic focusing in such systems has been developed using a

number of approaches. A brief review of the various microfabrication-based hydrodynamic focusing is discussed below.

Among the various microchannel designs for hydrodynamic focusing, the simplest ones consisted of two inlets for sheath flow. These inlets were located on either side of the sample inlet and were oriented either perpendicular or at an angle. By using high aspect ratio (height/width) channels, the sample was focused into a very narrow stream. Moreover, the parabolic flow profile in these channels provided some degree of focusing of the particles in the vertical direction (Lee et al., 2006). In an attempt to use gas instead of liquid as sheath, Huh et al. pumped air into a microchannel with hydrophobic walls. The sample was well focused at low flow rates, but at higher flow rates, pressure disturbances in flow resulted in drop formation (Huh et al., 2002).

Simmonet et al. designed a device from a single mold of polydimethylsiloxane that was created with three inlet ports for the sheath fluid to hydrodynamically focus the particles. The sample was introduced from the bottom. Varying hydrostatic pressure of the sheath fluid from each inlet resulted in particle diffusion to the geometric center (with respect to width and height) of the channel (Simonnet and Groisman, 2005). Precision of focusing was further improved with introduction of sheath at different depths, which enabled high resolution images to be obtained using a charge-coupled device (CCD). Three-level introduction of sheath not only focused the particles precisely, but also increased throughput and precision, comparable to a commercial flow cytometer. (Simonnet and Groisman, 2006).

Fluorescence activated cell sorting by hydrodynamic focusing on a microscale was demonstrated by Wolff et al. A sample consisting of beads and chicken red blood cells in the ratio of $2.4*10^{-5}$ (depicting a rare event) was focused by introducing buffer solution through the side inlets. While the throughput was much higher than that of a commercial cell sorter, particles were confined only in one dimension ie. the particles were focused only at the sides but not at the top and bottom. However, coaxial sample sheathing precisely focused particles in two dimensions. In this modified cell sorter, the sheath fluid moved the sample introduced from the bottom of the microchannel just like smoke from a chimney. Hence this focusing approach was called the smoking chimney method. Lower variation in fluorescence intensity was observed with a sample of fluorescein coaxially sheathed using phosphate buffered saline (PBS) (Wolff et al., 2003). Sheath fluid injection from as many as seven ports at varying heights in a sandwich type device microfabricated by soft lithography techniques precisely focused particles in both vertical and lateral directions (Sundararajan et al., 2004).

Acoustic focusing

Acoustic focusing uses ultrasound to form an acoustic standing wave inside the flow channel(Ward et al., 2009), which has been detailed for a cylindrical focusing geometry in unit 1.22. In the presence of an acoustic field, the acoustic radiation force acts on the particles and moves them either to the pressure node (pressure minimum) or pressure antinode (pressure maximum) depending on the sign of the acoustic contrast factor which is a function of the density and compressibility of the particle and the surrounding medium. Particles that have positive value of the contrast factor (most cells and particles) migrate to the node while the negative contrast particles migrate to the antinode of the standing wave

(Cushing et al., 2013; Laurell et al., 2007). The acoustic radiation force is also directly proportional to the volume of the particle, which confers a size dependence to this approach. Nonetheless, this approach has been shown to be effective for particles as small as bacteria (Hammarström et al., 2012).

As described in unit 1.22, this approach was first developed for flow cytometry using a cylindrical geometry (Goddard and Kaduchak, 2005; Goddard et al., 2006), In this form, the cylinder forms an axially positioned node, which makes it very analogous to the central positioning provided by hydrodynamic focusing. However, it does not accelerate particles and it also provides a measure of inline particle concentration as the particles are simply moved to the center of the channel. Both of these effects make it possible to analyze samples at slower particle velocities, but with similar overall particle analysis rates.

Acoustic focusing has also been successfully adopted in a planar mode where the acoustic theory is perhaps simpler (Austin Suthanthiraraj et al., 2012; Piyasena et al., 2012). In this single dimensional example, a standing wave can be established in a microchannel whose width equals half the wavelength of the applied ultrasound. Using this relation, either the flow channel can be fabricated to match the driving frequency or a suitable driving frequency be chosen to match the width of the flow channel. Thus, on application of the acoustic field, a pressure node is formed at the center and two pressure antinodes at the walls of the flow cell (Wiklund et al., 2004). The use of higher harmonics of standing wave across the channel also enables the development of multiple streams for parallel flow cytometry (Austin Suthanthiraraj et al., 2012; Nilsson et al., 2004; Piyasena et al., 2012).

As acoustic focusing is a sheathless approach, it does minimize waste and also reduce particle acceleration, thereby increasing particle transit times at the interrogation point. Moreover, the elimination of sheath reduces complexity of fluidics and improves the portability and cost of a commercial flow cytometer. However, direct contact of the sample with the walls of the flow chamber makes them susceptible to fouling. Hence periodical cleaning or the use of a small amount of sheath is required; otherwise fouled surfaces may interfere with optical detection.

Inertial particle migration and structured microchannels for particle focusing

The principle of inertial focusing in microchannels has been well studied and reviewed by the Di Carlo group (Di Carlo, 2009; Di Carlo et al., 2007; Di Carlo et al., 2008). In inertial focusing, particles migrate to precise equilibrium positions based on the principles of fluid dynamics. The particles in flow experience two types of forces namely the inertial lift force and the drag force. The drag force accelerates the particle in the direction of flow, and the lift force acts perpendicular to it and causes the particle to migrate in the lateral direction. The inertial lift force is composed of two components namely the wall lift and the shear-induced lift. The wall lift moves the particle away from the wall while the shear-induced lift acts down the shear gradient. The superposition of these two components gives rise to lateral migration of the particle to a shear region of high inertial lift, which is away from the channel centerline and closer to the wall but a little away from it. Such equilibrium positioning of particles depends on the geometry of the channel and the size of the particle. Furthermore, such migration occurs over a specific time scale and hence over a

characteristic length (Di Carlo, 2009). As the magnitude of lift force depends on particle dimension, it is possible to separate particles of different sizes to different lateral positions within microchannels, thus achieving an effective size-based separation.

With regards to particle positioning, the most important aspect to consider is how the particles reach a predicted position. While this is complex in nature, some basic rules are known. In a rectangular channel, the inertial lift forces result in particle migration into four equilibrium positions near the center of each wall face, of which only two are stable. In cylindrical channels, the particles have been observed to move to an annular region, approximately 0.6 times the radius of the channel. For a square channel, there are eight equilibrium positions, four along the corners and four at the center of the faces of each wall. While multiple positions are potentially valuable, the use of additional microfluidic structures can result in unique and consistent spacing of particles for flow cytometry analysis (see below).

Another important aspect of inertial focusing is that it can induce particle ordering. Under certain conditions, the particles arrange themselves in trains with uniform inter-particle spacing (Hur et al., 2010). This has been shown to eliminate particle coincidences in flow cytometry (Oakey et al., 2010). Uniform ordering of particles observed in inertial focusing has also been used to trap beads and cells within picoliter drops (Edd et al., 2008). Such precise ordering may eventually help in overcoming drawbacks with stochastic particle delivery commonly observed in droplet sorting.

Inertial focusing in curving microchannels is assisted by Dean flow, where mismatch of inertia between the fluid elements at the channel center and the wall results in a vortex at the curving edges (Di Carlo, 2009). Careful design of such inertial microfluidic channels have stabilized focusing to a single equilibrium position with efficiencies comparable to that of a low-end commercial flow cytometer (Di Carlo et al., 2008; Oakey et al., 2010).

Both parallelization of microfluidic channels (Hur et al., 2010) as well as the use of advanced imaging techniques (Goda et al., 2012) have enabled achieving high analysis rates required for the detection of circulating tumor cells.

Despite not using any external field, inertial focusing coupled with Dean flow precisely positions the particles in two dimensions in flow channels of different geometric symmetries. Moreover, uniform interparticle spacing observed in inertially focused streams avoids particle coincidences at the interrogation region. However, such spacing may not be observed in low concentrated samples (Oakey et al., 2010). It also requires cleaning or disposal of the optics surfaces, and the dependence of inertial lift and secondary forces on particle dimensions may not only position particles based on their size, but also limit the range of particle dimensions that can be handled in a specific device.

In addition to Dean flow, structure-induced flow effects using several pairs of grooves and chevrons, with or without sheath fluid, have also been used to position particles to the channel center for flow cytometry applications (Howell Jr et al., 2008). While for the groove design the degree of sheathing depends on the ratio of flow rates of the sheath and sample, the chevrons provide complete sheathing at all flow rates. Furthermore, vertical compression

of the sample stream observed with chevrons find use in image analyzers. Flow induced microvortices, without using the sheath fluid, have also been demonstrated in microfluidic channels patterned with herringbone structures. In these channels, the balance between fluid drag, gravity and buoyancy forces determines equilibrium positioning of particles (Hsu et al., 2008).

Sorting

After positioning and optical analysis, those particles having desired optical parameters can be sorted (den Engh, 2000). The success of a flow cytometer is partly determined by its ability to effectively sort cells of interest at high rates and within the safety envelope required for a specific application. It is very important to emphasize the need to consider biosafety when performing fluidic sorting, as the generation of aerosols and other operator exposure paths can occur (Schmid et al., 2007). While there are many methods of sorting, we will only discuss those that are based on manipulation of the fluid stream, which excludes optical techniques such as laser ablation or laser trapping (Applegate Jr et al., 2004; Applegate Jr et al., 2009; Shen and Price, 2001). Even so, there are many variations on fluidic sorting, which can be broken down into the following subcategories:

1. Jet-in-air sorting
2. Pneumatic sorting
3. Mechanical sorting
4. Piezo diversion sorting
5. Microfluidic mechanisms

Jet-in-air sorting

This topic has been described in detail in Unit 1.7 of this series (Leary, 2001), but it will be summarized here. In this type of cell sorting, the fluid is ejected through a nozzle into air. Just prior to exiting the flow cell or just after exiting a flow nozzle (Figure 4), the optical properties of individual cells are read using a laser excitation source and optical collection system. Droplet generation is made more uniform by vibrating the flow cell using a piezo ceramic transducer coupled externally. The distance between the interrogation point and the droplet break-off point is called the droplet delay. It is determined by the dimensions of the orifice as well as by the flow and physical properties of the sheath fluid and is critical to the success of a flow sorter. Once the droplets are formed, a charge is placed on each droplet depending on the optical parameters of the cell within it. These charged droplets, on entering a static electric field of kV voltage maintained between two plates are attracted towards oppositely charged plates and deflected towards collection tubes or multiwell plates. The strength of the applied electric field depends on the number of populations of interest and hence the magnitude of charge on individual drops and the distance through which they are deflected also vary accordingly. Though the cells are entrained within drops even at high linear velocities, stochastic cell arrival may result in either no cell or more than one cell within droplets, and hence not all cells of interest are sorted. In the event of cell coincidence at the interrogation point, the droplets are not charged and hence collected as waste, due to

the delay in processing such events by the detector. These are termed as hardware aborts. Similarly, when two cells travel too close to be entrained in separate drops, they may be charged as unwanted events. This is called a software abort. However, purity of sorting can be compromised by adjusting the sorting logic suitably. Besides these drawbacks, droplet generation in air renders this type of sorting unsuitable for handling biohazardous substances (Davies, 2007).

Pneumatic sorting

The pneumatic sorting mechanism is with large flow cells that are capable of handling cells up to 1500 μm in size. In this type of sorting mechanism, while the cells of interest are allowed to flow undisturbed into a collection receptacle or a well plate, the unwanted events are gently pushed by a puff of air from a solenoid valve located below the flow cell into the waste collection chamber. Besides cell and tissues, even live organisms have been non-destructively handled in this fashion (Pulak, 2006).

Mechanical sorting

This is another type of commonly used closed sorting mechanism. If the cell crossing the analysis point is identified as an event of interest, it is collected into a catcher tube that moves into and out of the flow cell at rates up to 300 times per second; otherwise it is directed into the waste tank. Despite being safe for handling biohazardous substances, sorting rates are low and the sorter is capable of handling only a single population of interest (Davies, 2007).

Piezo diversion sorting

In this type of cell sorting, upon identification of a cell of interest, the flow cell is actuated by a diamond-shaped piezo that drives acoustic pressure into it and deflects that cell along with some sheath fluid into the collection channel. The unwanted cells flow into the waste chamber. Besides being a closed type of sorting technique that is safe for biohazardous substances, its gentle cell handling has allowed sorting fragile but large cells such as the islets of Langerhans (Gray et al., 1989).

Microfluidic sorting approaches

Due to the breadth of the field of microfluidics, there has been an enormous amount of work towards cellular manipulation (Huh et al., 2005; Patel and Perroud, 2010). In this unit we will just mention two notable efforts that have garnered interest.

Particles have also been sorted in microfluidic channels fabricated using soft lithography. Owing to small sample volumes, these channels offer the ability to handle very small samples and are also less hazardous than droplet approaches, as samples are handled in enclosed structures. Sorting in microfabricated structures has been demonstrated using a T-shaped device consisting of one inlet well and two outlet wells. Cells were sorted by electro-osmosis using three platinum electrodes placed below the inlet and outlet wells. The fluorescence emission from the labeled cells was directed by the microscope into a photomultiplier tube. Those cells emitting fluorescence higher than the threshold limit were selectively diverted to the collection outlet by an automated increase in voltage while the

remaining cells were allowed to flow into the waste outlet well. Using this technique, cells can be sorted both forward and backward. For example, on identification of a cell of interest, the flow may be stopped temporarily to prevent that cell from entering the waste outlet and the flow may be reversed to collect that cell. Despite effective sorting, throughput is limited in electro-osmotic flows. However, pressure-switching mechanisms may also be used, which not only increases throughput but also avoids high voltages required in electro-osmosis. Throughput can also be increased through the use of parallel microchannels (Fu et al., 1999).

Cells have also been sorted using a microfabricated polymer-based cell sorting device. This device consists of serpentine-shaped inlet channels for sheath and sample fluids. These channels provide the necessary hydrodynamic force for focusing the sample. A single electromagnetic valve was used to adjust the pumping rate of the pneumatic micropumps. The width of the focused stream may be adjusted by tuning the control frequency of these micropumps. The labeled cells were excited by a laser diode and the fluorescence signals were read using avalanche photodiode and a CCD camera. Cells of interest were sorted based on fluorescence signals using three microvalves downstream of the detection region to direct those cells to the corresponding outlets (Yang et al., 2006).

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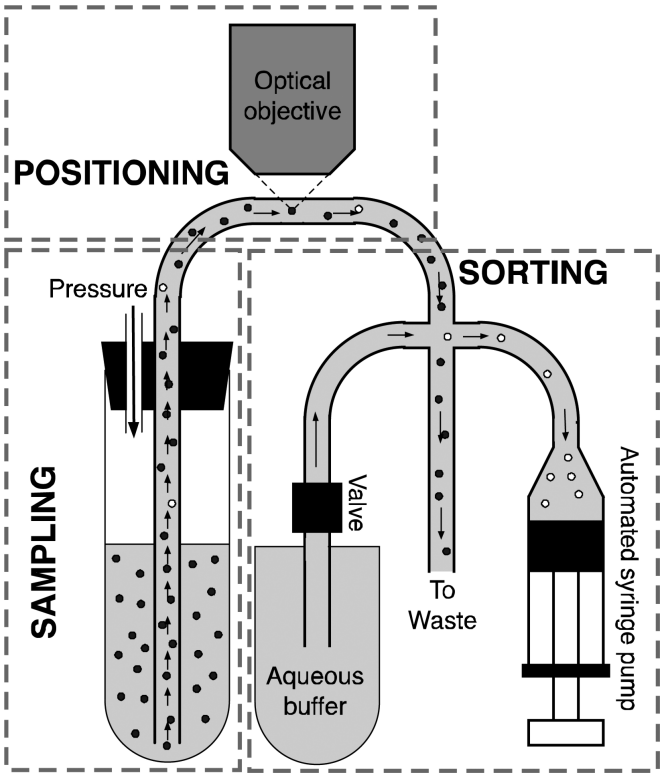

Figure 1.

A conceptual diagram of the three primary functions of the fluidics of a flow cytometer. Many options are possible to accomplish each of these functions. Potential examples are shown.

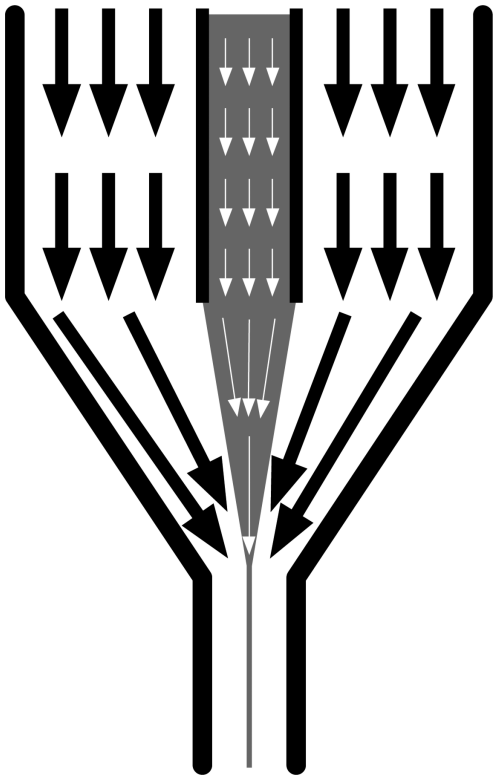

Figure 2.

The principle of hydrodynamic focusing. Sheath fluid (Black arrows) flows at a high linear velocity compared to the slow moving sample stream (Gray with white arrows). The fast moving sheath accelerates and focuses the sample stream to a narrow core as it exits the flow cell or nozzle, or the capillary tapers towards the exit orifice.

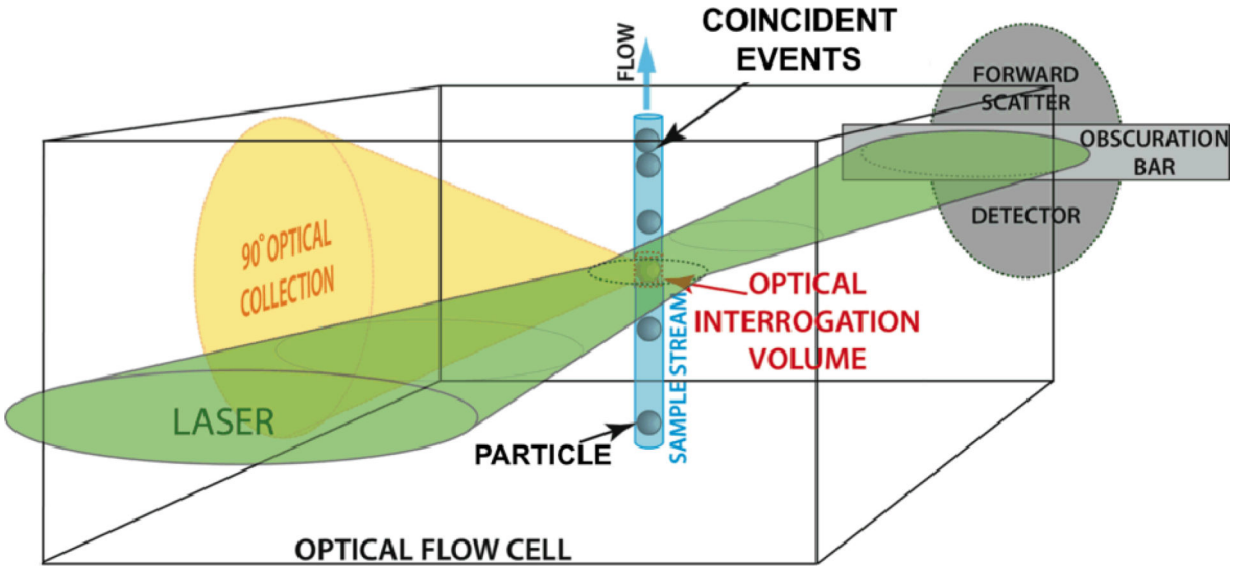

Figure 3.

Optical analysis of a focused sample stream in a typical hydrodynamic focusing flow cytometer. The sample stream is surrounded by a clean sheath fluid that is not pictured.

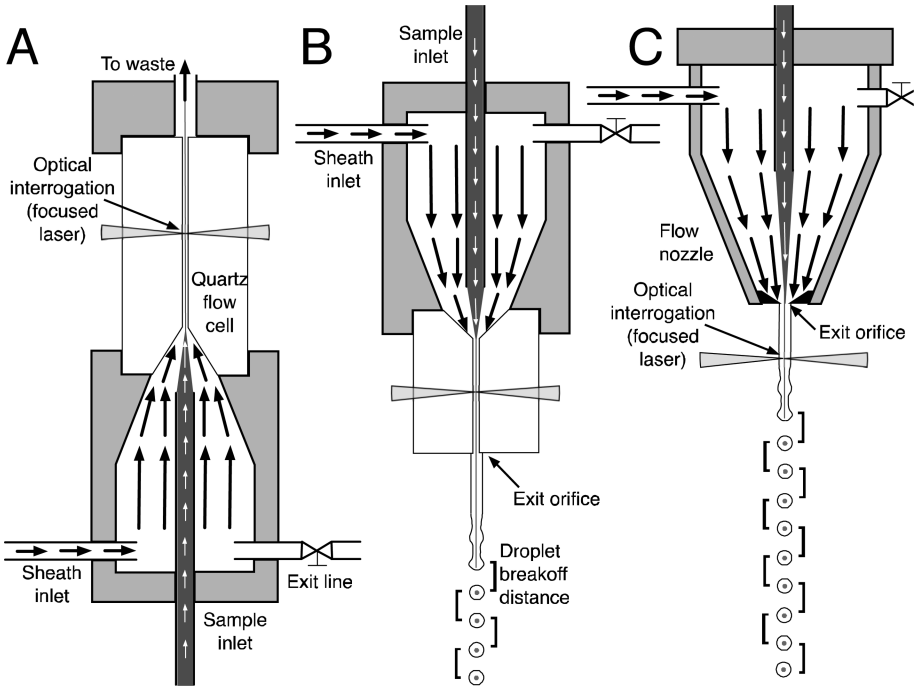

Figure 4.

Typical configurations of flow cytometer flow cells for (A) analysis, (B) electrostatic droplet sorting after analysis in a flow cell, and (C) analysis directly in the jet-inair system after ejection from a nozzle.

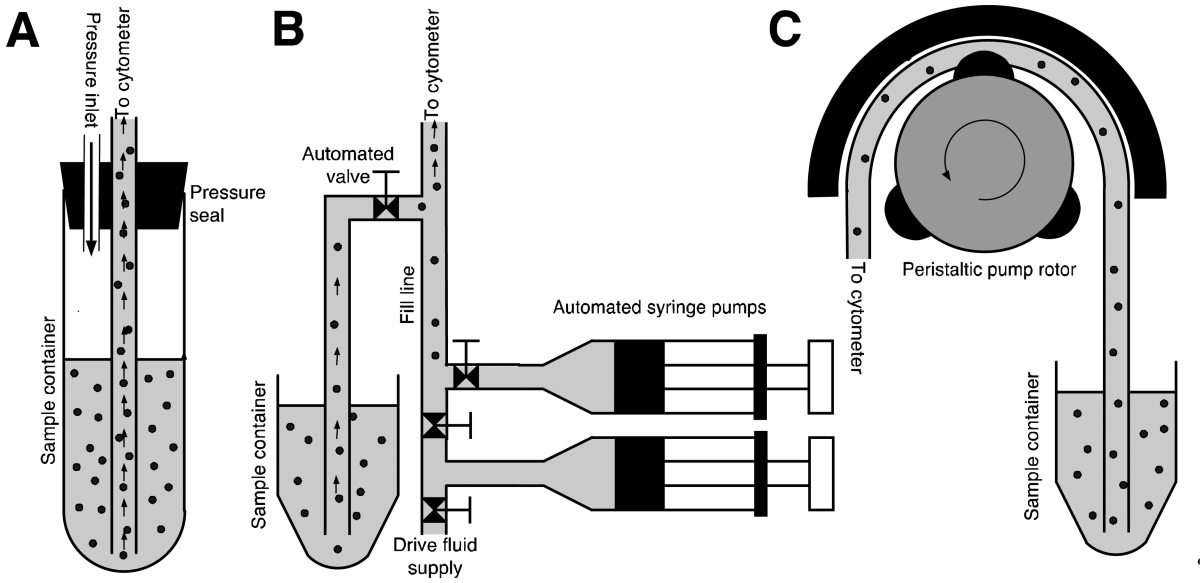

Figure 5.

Traditional delivery methods. (A) Pressure differential, where a pressure head is introduced into a sealed sample tube to drive sample to the flow cytometer. (B) Syringe pumps in combination with automatic valves that can first fill a fill line and then drive the sample to the cytometer. (C) A simple peristaltic pump that pumps an unsealed sample to the flow cytometer.