Microfluidic chips for cell sorting

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1. ABSTRACT

Micro total analysis systems (µTAS) also referred to as "lab-on-a-chip" is one of the fastest progressing fields in biological and chemical analyses. In recent years, µTAS for single cell analysis has drawn the attention of researchers due to its significant advantages over traditional methods for single cell manipulation, fast cell sorting and integration of multiple functions. As the preliminary step for studying cells on chips, cell sorting using microfluidics have been investigated by researchers intensively. This article reviews the most recent advances on microfuidics-based cell sorting techniques including cell sorting principle, strategy, mechanism and procedure with emphases on the sorting mechanism and procedure. Furthermore, evaluation criteria for successful cell sorter are also discussed and future research directions are given.

2. INTRODUCTION

Systems biology was first introduced by Hood et al in 1999 as a research filed to understand biology at system level (1-3). Different from molecular biology that interprets biological problems by analyzing individual molecules, systems biology focuses on the studies of interactions among components of biological systems and how these interactions give rise to the function and behavior of that system. Cells are the smallest units of living organisms or systems with multi-functionality. Therefore, research on cells is of great interest in systems biology. In the past a few years, single cell analysis has increased dramatically especially on cancer cells, stem cells and nerve cells. To study cells individually, it's important to separate them from complex mixtures. Thus, cell sorting

Table 1. Comparison of separation strategies

Strategy	Separation mode	Sorting mechanisms	Procedure of cell sorting	Separation objects	Throughput	Ref
Active multi-channel switch	Active	Optical mechanism; Electric mechanism; Hydrodynamic mechanism	Transportation, focusing, recognition, separation, collection	Single cell one by one	Low/Median /High	7, 15, 16
Field flow fractionation	Passive	Electric mechanism Magnetic mechanism	Transportation, separation, collection	Continued flowing cells	High	19, 20-22
Local nonuniform field fractionation	Passive	Electric mechanism; Magnetic mechanism	Transportation, separation, collection	Continued flowing cells	High	25-27, 70
Specific immobilization	Passive	Magnetic mechanism; Surface Absorption	Transportation, separation, collection	Cells in batches	Low/Median	29, 31, 93
Direct manipulation	Active	Optical mechanism	Transportation, recognition, separation, collection	Single cell one by one	Low/Median	33, 34, 58

Techniques provide necessary approaches for researchers to fulfill this task (4).

Conventional methods for separating cells include gradient centrifugation, magnetic-activated cell sorting (MACS) and fluorescence-activated cell sorting (FACS) (5). Gradient centrifugation operates with a large quantity of cells of various density and sizes, which usually has fairly poor resolution for separation (6). MACS and FACS normally has excellent sorting performances in terms of throughput and purity, however, the requirement for bulky expensive equipments and skillful technicians for operation (7, 8) limits the application of both techniques. Since the advent of Micro total analysis systems (μTAS), miniaturized devices and systems have already been proved significantly advantageous for biological research and biomedical applications (9, 10). Microfluidic chips are one successful example of μTAS which had great impact on the development of cell sorting technologies with advantages such as microchannel networks with dimensions on the order of single cells, laminar flow phenomena on the micro-scale, fast heat transfer (11) and ease of fabrication with minimal cost. Moreover, integration of multiple functions such as cell transport, cell culture, cell sorting and biochemical analysis can also be realized onto one single microfluidic chip (12). Therefore, microfluidic chips have become increasingly studied by researchers for cell sorting purposes.

Recently, there have been several review articles on microfluidics-based cell sorting techniques. Andersson and Berg (11) earlier presented an extensive review of microfluidic devices for cellomics where mechanical sorting, electric sorting and flow cytometry on chips were summarized. In another literature, Toner and Irimia (8) discussed microfluidic applications for blood analyses with focus on cell sorting methods using mechanical forces, dielectrophoresis, optical interactions, magnetic interactions and biochemical interactions. Huh et al (13) then overviewed microfluidics for flow cytometry of cells and micro-scale particles where microfabricated fluorescence-activated cell sorters (μFACS) were described in detail. Lately, Yi *et al.* (14) and El-Ali *et al.* (12) both reviewed the recent progress on micofluidics for cell research on chips with emphases on cell manipulation and cell sorting approaches respectively. In this review, we summarized the most recent advances on cell sorting techniques using microfluidic chips with aspects of cell

sorting principle and strategies, sorting mechanisms, general procedure and evaluation criteria. Challenges and potential research directions are also presented.

3. PRINCIPLE AND STRATEGIES FOR MICROFLUIDIC CELL SORTING

In general, cell sorting is to distinguish and separate cells from complex cell mixtures based on their distinct properties from the others. Intrinsic difference (physically or chemically) is commonly considered the first choice where different cells are separated directly. With no difference intrinsically, extrinsic difference can then be introduced such as fluorescence labeling. In this case, it's necessary to involve certain external recognition mechanism and feedback control for separation such as fluorescence detection and impedance monitoring. Therefore, based on whether real-time recognition and feedback are involved in the procedure, cell sorting can be categorized in to active sorting and passive sorting.

Up to date, a large number of papers have been published on cell sorting methods using microfluidic chips. Various sorting strategies have been reported which can be generally classified as active multi-channel switch, field flow fractionation, local nonuniform field flow fractionation, differential immobilization and direct manipulation. Each strategy will be discussed in detail in this chapter and a brief comparison of them is summarized in Table 1.

3.1. Active multi-channel switch

"Active multi-channel switch" represents a class of methods where external force is employed to actively switch cells into desired channels. The external forces for switching usually include optical force (15), electric force (7) and hydrodynamic force (16). In this strategy, it's necessary to focus cells into lines before the recognition and switching. Thus, it is only suitable for separating focused cells in continuous flow and it is widely employed in μFACS. Moreover, the throughput of this strategy is relatively limited by the response time for switching. To enhance throughput, side switch can then be used before the separating junction to reduce the switching time in electric switch (17) and hydrodynamic switch (18).

3.2. Field flow fractionation

Field flow fractionation (FFF) is based on the fact that particles with different physical properties experience

forces of different amplitudes and directions in a uniform field. Thus, particles can be separated into a number of streams in the presence of a certain field and then be collected according to the space difference. Typical fields employed in this strategy include optical field (19), magnetic field (20), acoustic field (21) and electric field (22). As noted, particles can also be separated by different moving velocities as demonstrated by Gasperis et al (23) with a 2D cell sorting device. However, this velocity dependent approach was rarely employed for cell sorting on microfluidic chips (5).

3.3. Local nonuniform field flow fractionation

Similar to FFF, local nonuniform field flow fractionation also differentiates cells based on their physical properties. However, in this strategy, cells are isolated out of a stream flow by a localized nonuniform field. Movement of the cells depends on the resultant force generated by the local field and the dragging force determined by the flow velocity. Local nonuniform field is usually generated around the micro-elements integrated in the microchannel, such as magnetic stripes (24), magnetized wires (25), insulating blocks (26) and microelectrodes (27). This strategy can be employed for separating a large number of cells simultaneously without focusing cells or cell recognition. However, fabrication for such micro-devices is relatively complex and expensive.

3.4. Differential immobilization

This strategy isolates cells by specific immobilization. There are two selection modes. The positive mode immobilizes the cells of interest and the negative mode eliminates all undesired cells by immobilization (28). Surface adsorption is one example of the positive mode where cells are perfused through microchannels decorated with specific ligands (2). Thus, only desired cells are immobilized by affinity and others are removed by fluid flow. Field based immobilization is another form of the differential immobilization. Cell selection is based on either intrinsic or extrinsic difference of the cells. For instance, positive dielectrophoresis (pDEP) based separation directly takes advantage of the intrinsic difference of cells responding to DEP. Therefore, only cells with stronger attraction in DEP can be remained in a fluid flow of certain velocity. By varying the velocity of fluid flow, different cells can then be isolated. In contrast, immunomagnetic separation is one example where extrinsic difference is introduced by specific binding of magnetic beads to target cells for separation (31). In summary, Differential immobilization can specifically isolate target cells in batch mode with minimal requirement for channel structures.

3.5. Direct manipulation

The idea of direct manipulation is to control cells on chips for various purposes including cell sorting. Traditionally, manipulating individual cells is a labor-intensive job which limits the application of this strategy. With the rapid progress of μTAS technology, direct manipulation of multiple cells simultaneously can be realized such as addressable optical laser array (32), microelectromagnet matrix (33) and microelectrode matrix

(34). These novel devices allow manipulation of more than 100,000 cells at one time, which can be potentially used for high throughput cell sorting devices. However, microfluidic chips using direct manipulation require complex microchannel structures. Therefore, it's currently relatively less employed than the other strategies.

4. SORTING MECHANISMS

Common procedure for microfluidics-based cell sorting normally consists of transportation, focusing, detection, separation and collection. Separation is the key step in the whole procedure. Commonly employed sorting mechanisms include electric mechanism, optical mechanism, magnetic mechanism, hydrodynamic mechanism, acoustic mechanism, filtration, cocurrent extraction and surface adsorption. In this chapter, all sorting mechanisms will be discussed individually in detail and examples of microfluidic chips will be described. To give a brief overview of different sorting mechanisms, characteristics of these mechanisms are summarized in Table 2.

4.1. Electric mechanism

Electric mechanism is a straightforward approach for cell sorting on chips. Microchips employing this mechanism have advantages of fast separation speed, flexibility, controllability, and potential for automation (11). Thus, electric mechanism is the most popular method in microfluidics-based cell sorting. Based on different interactions between the electric field and the targets, electric mechanism can be generally summarized as dielectrophoresis and electrokinetic approaches.

Electrokinetic approaches combines electroosmosis with electrophoresis where electroosmosis is usually the dominant force for cell manipulation on chips such as switching cells into different flow channels upon specific recognition. This mechanism has been widely used for cell sorting purposes. Fu *et al*. (7) earlier reported a μFACS system for separating micron-sized latex beads and bacteria. *E. coli* that express green fluorescent protein (GFP) were isolated from a mixture of non-fluorescent cells with a enrichment of 30-fold and a throughput of 20 cells/s. Similarly, the same mechanism was used for separating cells with different autofluorescence (35) and isolating CD4 cells for HIV diagnosis (36). Johann and Renaud (37) further presented a novel design of microchannels (Figure 1A) for separating particles by electroosmosis combined with pressure driven fluid flow. Different from the literatures reported above where electroosmotic forces were used as the switching power, a faster switch mode was then proposed by Fu *et al*. (17) where electrokinetically focused cells were switched in a cross channel (Figure 1B). Similar approach was also reported by Dittrich *et al*. (38). Instead of electroosmotic switching, Yao *et al*. (9) successfully attempted electrophoretic switching for μFACS where electroosmosis and cell adhesion were significantly reduced by adding hydroxylpropylmethyl cellulose into the buffer solution and the fluid flow was driven by the gravity. However, electrokinetic sorters employ electric field of high potential which is harmful for living cells. In addition, issues like buffer incompatibility, ion depletion, pressure

Table 2. Comparison of separation methods

Sorting mechanism	Method	Strategies	Cell focusing	Separation criterion	Throughput	Separation objects	Ref
Electric mechanism	Electrokinetic switch	Active multi-channel switch	Yes	Labelings	Median	Single cell one by one	27, 39
	AC-Dielectrophoretic separation	Local nonuniform field flow fractionation	Optional	Dielectrophoretic character	High	Continued flowing cells	7, 51
	Travelling-wave dielectrophoretic separation	Field flow fractionation	No	Dielectrophoretic character	High	Continued flowing cells	23, 53
Optical mechanism	Optical tweezers or trappers	Active multi-channel switch / Direct manipulation	Yes	Labelings	Low/Median	Single cell one by one	15, 58
Magnetic mechanism	Uniform field cross separation zone	Field flow fractionation	No	Magnetic character	High	Continued flowing cells	20, 68
	Nonuniform field in separation zone	Local nonuniform field flow fractionation	No	Magnetic character	High	Continued flowing cells	24, 25
	Magnetic switch	Active multi-channel switch	Yes	Magnetic character	Low	Single cell one by one	76, 77
	Immunomagnetic separation	Differential immobilization	No	Labeling magnetic beads	Low/Median	Cells in batches	31, 72
Hydrodynamic mechanism	Hydrodynamic switch	Active multi-channel switch	Yes	Labelings	Median/High	Single cell one by one	16, 40, 79
Acoustic mechanism	Uniform field cross separation zone	Field flow fractionation	No	Density and compressibility of the particles and the medium	Median	Continued cell field flow	21

imbalance and water evaporation limit the application of this mechanism (15, 40).

Dielectrophoresis (DEP) was first discovered by Pohl et al (41) in 1951. It is a phenomenon where particles are manipulated by different polarization effects in a nonuniform electric field (26), which can be either a direct or an alternating field (42). In general, particles with a higher polarizability than the buffer solution exhibit positive DEP moving toward regions of greater field intensity and particles with a lower polarizability move in the opposite direction exhibiting negative DEP (26). In case of cells in such a field, the induced polarization depends on the physiologic condition of the cells and the frequency of the alternating current (AC) field. Thus, cells can be separated by altering the frequency or amplitude of the AC field. Furthermore, DEP devices operate at low voltages and cell-labeling or cell-modification procedure is not necessary for successful separation (8). Hughes et al (43) and Gascoyne *et al*. (44) have previously reviewed DEP methods for separating cells and particles with aspects of theories, strategies and methods. Therefore, in this chapter, only the most recent advances on DEP-based cell sorters are summarized. Different types of exciting fields (direct or AC fields), microelectrode structures and DEP modes (negative DEP, positive DEP) will be discussed separately.

AC induced nonuniform electric field is most frequently used in DEP-based cell sorting without electroosmosis or gas generation (26). Thin-film microelectrodes (5, 30, 45-48) are normally employed for conducting AC fields in microchannels. Li and Bashir (45) have demonstrated the use of AC-DEP to separate live and heat-treated *Listeria innocua* cells with a separating voltage of 1V at 50 kHz. 90% separation efficiency was achieved. Yang *et al*. have also reported successful separation of

human breast cancer MDA-435 cells from normal blood cells (5, 46). Arrays of planar interdigitated microelectrodes were fabricated to generate nDEP force levitating cells at different heights where the cells were transported at different velocities under the control of a parabolic flow profile (Figure 1C). Separations can then be achieved with performance depending on the frequency and voltage of the DEP field and the fluid flow rate. Holmes *et al*. (47) designed two consecutive microelectrode arrays, one of interdigitated electrodes at top and bottom of the microchannel to focus cells into a well-defined layer, and the other to separate the cells with a combination of positive and negative DEP (Figure 1D). In the same way, Huang *et al*. (30) utilized interdigitated microelectrodes to separate *E. coli*, *B. cereus* and *L. monocytogenes* from blood samples individually or simultaneously. Bacteria were attracted to the electrodes by pDEP and blood cells were eluted by fluid flow. Lee *et al*. (48) has also reported a thin-film microelectrode-based flow cytometer to split cells/particles into different outlets by nDEP forces.

3D microelectrode is another important category of designs used for conducting AC field and DEP forces (27, 49-51). In this type of microchips, electrodes can be designed to take full advantage of microchannel geometry. Therefore, wider channels can be used to eliminate potential issues such as cell clogging. However, the flow velocity within this type of devices is fairly limited in order to achieve trapping efficiency (49). Müller *et al*. (49, 50) have demonstrated a microchip consisting of a two-layer microelectrode structure spaced by a flow channel. Electrode elements such as funnel, aligner, cage and switch were designed and powered by AC field for focusing, trapping and sorting purposes of eukaryotic cells and particles with a diameter of 10–30 μm. Park *et al*. (51) developed a 3D-asymmetric microelectrode system with
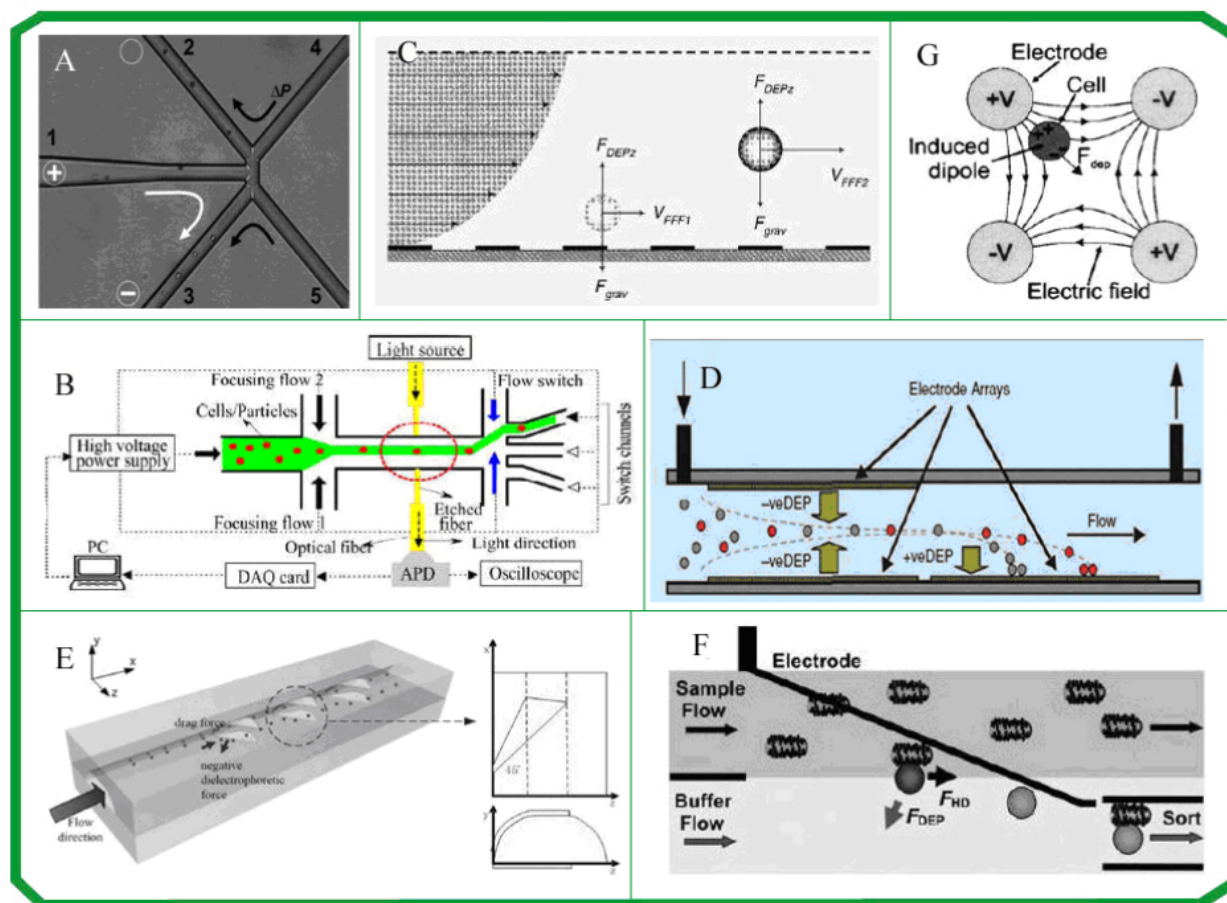
Figure 1. Cell sorting by electric mechanisms. (A) Chip device during operation. From a mixture of red-stained and natural yeast cells entering from channel 1, stained cells are sorted into channel 2, natural ones into channel 3. Electrokinetic flow is indicated by the white arrow, hydrodynamic buffer flows by black arrows. The channels 2–5 are 22 μm wide and 10 μm deep. Reprinted with the permission from Ref (37), © 2004 WILEY-VCH Verlag GmbH & Co. KGaA. (B) Schematic representation of experimental setup for the micro flow cytometer; detection light propagates in the waveguide structure and its intensity changes as particles pass through the detection region.. Reprinted with the permission from Ref (17), © 2003 Elsevier B.V. (C) Schematic drawing of the DEP/G-FFF principle. Cell equilibrium height in the fluid-flow profile is determined by the balance of DEP levitation forces (F_{DEPz}) generated by the interdigitated microelectrodes and the sedimentation force (F_{grav}). Cells that are farthest from the bottom electrode plane are carried faster by the fluid ($V_{FFF2} > V_{FFF1}$) and exit the chamber earlier than those at lower positions. Reprinted with the permission from Ref (5), © 1999 American Chemical Society. (D) Schematic diagram of the dielectrophoretic separator. The first section of the device has two interdigitated electrode arrays at top and bottom of the channel, which focus the particles into the center of the channel by negative DEP. The second section contains a single electrode array, which differentially pulls the focused particles from the fluid flow by positive DEP, separating them into distinct bands.. Reprinted with the permission from Ref (47), © 2003 IEEE. (E) Schematic diagram of a 3D asymmetric microelectrode system. Reprinted with the permission from Ref (51), © The Royal Society of Chemistry 2005. (F) The DACS concept: Cells entering in the sample stream are only deflected into the collection stream if they are labeled with a dielectrophoretically responsive label. Reprinted with the permission from Ref (27), © 2005 The National Academy of Sciences of the USA. (G) Schematic representation of a quadrupole DEP trap, showing the four electrodes of the quadrupole and a cell trapped in the middle. The n-DEP configuration shown induces an effective dipole moment in the cell that is antiparallel to the electric field. This creates a dielectrophoretic force (F_{dep}) that repels the cell from the electric field, causing it to be stably trapped at the quadrupole's field minimum. Reprinted with the permission from Ref (52).

electric fields of continuously varying magnitudes along the transverse direction of a channel owing to the changing widths of the electrodes in the half-circular shaped cross section of the microchannel (Figure 1E). This design greatly enhanced the sorting sensitivities to the dielectric properties of living cells resulting in highly efficient cell separation. Different from previous approaches where intrinsic dielectrophoretic properties of cells ware explored for cell sorting, Hu et al. (27) presented a new method for sorting rare cells using surface dielectrophoretic labeling with DEP. namely DEP-activated cell sorting (DACS) (Figure 1F). As cell mixture entered the channel, the

dielectrophoretically labeled cells were selectively deflected into the collecting stream by nDEP. Labeled and unlabeled *E. coli* were successfully sorted with a throughput of 10000 cells/s and a 250-fold enrichment. Compared to those mechanisms using intrinsic dielectrophoretic properties of cells as the sorting criteria, DACS is relatively labor-intensive for its labeling procedure. However, in case of separating cells of similar intrinsic dielectrophoretic properties, DACS is superior to other methods with no doubt.

Instead of using 3D electrodes or thin-film planar electrodes, Voldman *et al*. (52) demonstrated a microdevice with regular arrays of non-contact single-cell traps using a novel asymmetric extruded quadrupole geometry (Figure 1G) that can be physically arrayed and electrically addressed for use in parallel luminescent single-cell assays. These traps can confine cells in positions by DEP with no disturbing in fluid flow and cells were introduced into the array and sorted according to their responses to dynamic fluorescence.

Traveling-wave dielectrophoresis (twDEP) is an asynchronous motive force generated from the displacement of the induced dipole moment of particles traveling in an electric field. The twDEP force exerted on a particle depends on several factors such as the particle volume, polarizability of the particle and medium, electric field strength and the frequency of the traveling field (53). twDEP is often used to enhance particle differentiation by combining with negative or positive DEP separation (44). Morgan *et al*. (53) earlier reported a large-area twDEP separator constructed using multilayer microelectrode techniques. Components of whole blood were then separated for demonstration. Gascoyne's group (23) developed a 2D microfluidic dielectrophoretic cell sorter as an expansion to their previous work (5, 46). Cells were first separated by DEP resulted in different flow velocity. As they reach the exit point, twDEP was then used to deflect cells to different locations. Thus, 2D separations can be achieved. Furthermore, the same group has also demonstrated isolation of malaria-infected and uninfected blood cells on two different types of microelectrode arrays using twDEP (54), one with interdigitated electrodes (5, 46) and the other with spiral electrodes. In the spiral design, four phase signals were applied to the electrode trapping erythrocytes at the edge of the electrode while levitating parasitized cells to the centre of the spiral.

Fuchs *et al*. (34) developed an original cell sorting and recovery technology based on a microelectronic complementary metal-oxide-semiconductor silicon chip integrating an array of over 100,000 independent electrodes and sensors which allowed individual and parallel single cell manipulation of up to 10,000 cells, while maintaining viability and proliferation capabilities. 3D dynamic dielectrophoretic traps can then be configured at any position on this chip by simply applying a sinusoidal waveform to the indium tin oxide /polycarbonate lid and to a center electrode and a counterphase waveform to all eight surrounding electrodes. As a result, individual fluorescent

K562 cells can be isolated and recovered from a bulk of unlabeled cells.

Compared to AC-DEP, DC-DEP was much less concerned for cell sorting purposes. However, the recent emergence of insulator-based dielectrophoresis (iDEP) brought many applications of DC-DEP for separating cells. Insulators have advantages of being less prone to fouling, more robust, chemically inert and easy for fabrication. In addition, transportation by electrokinetic effects and deflection of particles by DEP can be realized simultaneously by DC fields (26). Encinas *et al*. first reported the application of DC-iDEP for the selective concentration of live and dead bacteria on microfluidic chips (26). A nonuniform electric field was generated by applying a DC electric field across a microchannel filled with insulating posts. Regions of higher field intensity were generated in the narrowest spaces among insulating posts, which were then successfully used to simultaneously concentrate and separate live *E. coli* in the presence of dead *E. coli* and inert particles by modifying their relative responses to DEP with varying magnitude of potentials. Moreover, this group has also demonstrated differential trapping of polystyrene beads of different sizes and selective dielectrophoretic trapping of *B. subtilis* vegetative cells from spores (55) using the same DC-iDEP microchips. It's the first polymer-based iDEP devices that can deifferentiate vegetative cells and spores. Li et al (56) further proved that iDEP can be used to separate microparticles by sizes with high efficiency. By using electrokinetic flow in a microchannel with an insulating block, a mixture of microparticles was continuously separated by adjusting the voltages at the ends of different branches. Furthermore, the same group attempted oil droplet as the insulating obstacles between two electrodes (57). Particles experienced different nDEP forces according to their sizes when they enter the non-uniform DC field locally generated by the droplet. Thus, separations of particles can be achieved in a size dependent manner. Since the size of the droplet can be dynamically changed, the electric field gradient, and hence DEP force, becomes easily controllable and adjustable to various separation parameters.

Electric field can also be used for microparticle sorting in the case of isoelectric focusing (IEF). In IEF, a pH gradient is created by electrolysis of water at the electrodes and stabilized by ampholytes-amphoteric molecules with a range of isoelectric points in most commercial buffers. Lu *et al*. (22) have fabricated a microfluidic device that can separate and concentrate organelles by micro isoelectric focusing. This microdevice realized fast separation of samples of very small volume without use of large voltages or experiencing heating effects typically associated with conventional electrophoresis-based devices. The principle of the separation is the presence of membrane proteins that give rise to the effective isoelectric points of the organelles. Separations of mitochondria from whole cell extracts and from mitochondria mixture were both successfully attempted.

4.2. Optical mechanism

Optical cell sorting on microfluidic chips is also an attractive approach due to its advantages of non-physical contact and minimal contamination in manipulation processes. Radiation pressure forces (generally considered as a gradient and scattering force) of a focused optical beam can be employed to manipulate cells or particles in solution. The optical force exerted on a cell is related to the optical power and the optical properties of the cells and its surrounding fluidic medium (15). By different effective spaces of the light sources, optical forces for microfluidic applications can be summarized as point force (15, 58-61), multipoint force (32, 62), line force (63, 64), surface force (65, 66) and field force (19).

Optical tweezer employing point forces of optical lasers is one of the most popular approaches for manipulating single cells on chips. Arai et al (58) developed a high-speed separation system for random selection of single microorganisms by integrating laser-trapping forces with dielectrophoretic forces. An arbitrary single microbe can then be isolated from the extraction port into the main stream by a laser manipulator with other objects surrounding the target remained intact within the extraction port by DEP. Furthermore, the same group has also demonstrated a microdevice combining optical tweezers with thermosensitive hydrogel for cell culture and cell separation (59). The state of the thermosensitive hydrogel can switch between sol and gel depending on the temperature controlled by a micro heater. Target cells were isolated by the optical tweezer and immobilized by the hydrogel while other cells were eluted by fluid flow in the microchannel. Xie *et al*. (60) then presented a compact laser tweezers Raman spectroscopy (LTRS) system which combined near-infrared Raman spectroscopy with optical tweezers for unique identification and manipulation of single biological cells with a low-power-diode laser. Raman spectroscopy can provide fingerprints for identifying different biological cells. Therefore, the LTRS system has great potential for future microfluidic applications of universal label-free cell sorting. By taking full advantage of the inherent laminar nature of microscale fluid flow, Oakey *et al*. (61) utilized optical trap to switch target cells individually between laminar streams upon recognition through an optical imaging system. Furthermore, Wang *et al*. (15) realized a µFACS system to recover unstressed living mammalian cells by optical switching (Figure 2A) with high throughput. Flynn *et al*. (32, 37) employed addressable vertical cavity surface emitting laser (VCSEL) arrays as optical tweezers for parallel manipulation of cells and microparticles in microfluidic systems. Both the attractive gradient force and the scattering force of optical beam have been used for cell sorting in "T","Y" and multi-layered "X" shape fluidic channels. For example, scattering force acting as an elevator switched cells between two fluidic channels at different layers (Figure 2B).

Line force can focus cells in lines, which is sometimes more efficient than the point force. Applegate et al (63) demonstrated the use of a diode laser to trap, manipulate and sort cells and microparticles of different sizes and refractive indexes in a microfluidic system. The diode laser can control a large trapping zone from 1 to 100 µm. Transmission of the trap can be simply controlled by an amplitude mask. Consequently, objects of interest could be positioned into target streamlines by tilting the traps with respect to the microchannel and by blocking/unblocking different sections of the laser beam. Moreover, they also expanded above work by using femtosecond pulsed laser to fabricate monolithic optical waveguide networks that could provide precise spatial control over the localization of fluorescence labeled particles and cells for tracking and sorting purposes (64) (See Figure 2C).

Recently, waveguide's evanescent field has also been employed for particle sorting in microfluidic chips. A novel approach for particle manipulation and sorting was presented by Grujic and his coworkers (Figure 2D) (5). Polystyrene microspheres can be reliably sorted above a Y-shaped optical waveguide by simply switching the power distribution between the two branches. A similar approach was followed by Gaugiran *et al*. (66), where optical manipulation of cells and dielectric particles on the surface of silicon nitride waveguides was demonstrated. In general, this type of system can easily incorporate various optical structures and has potentials for future development of highly integrated optics microfluidic cell sorters.

Different form the active sorting methods discussed above, MacDonald *et al*. (19) proposed a passive optical sorter where separation was based on the interactions between microparticles and an interlinked dynamically reconfigurable 3D optical lattice (Figure 2E). As a mixture of particles within the lattice, selected particles were deflected from the fluid flow while others passing straight through. Particle sorting by both sizes and refractive indexes were both demonstrated with separation efficiency of nearly 100%. Although there hasn't been passive optical sorter so far for separating living cells, applications of this approach for size-dependent and refractive index-based cell sorting are highly expected.

4.3. Magnetic mechanism

It's been a long history in using magnetic forces for isolating magnetic particles from nonmagnetic materials and for separating magnetic particles from one another (68). Šafařík and Šafaříková (28) have previously reviewed the use of magnetic techniques for isolating cells where various methodologies, strategies and materials that can be employed for separation in magnetic fields were summarized with special emphasis on immunomagnetic separation. However, the elements conducting magnetic fields were relatively less employed for microfluidic application for the reason that it's difficult for them to miniaturize. With the rapid progress on microfabrication technology, increasing amount of magnetic elements have been developed and brought into use on microfluidic chips. Numbers of literatures have then been reported on cell sorting approaches using magnetic mechanisms. Pamme et al (69) have reviewed applications of magnetism in microfluidics-based cell sorting devices. Lately, Inglis *et al*. (70) also briefly summarized cell separation approaches
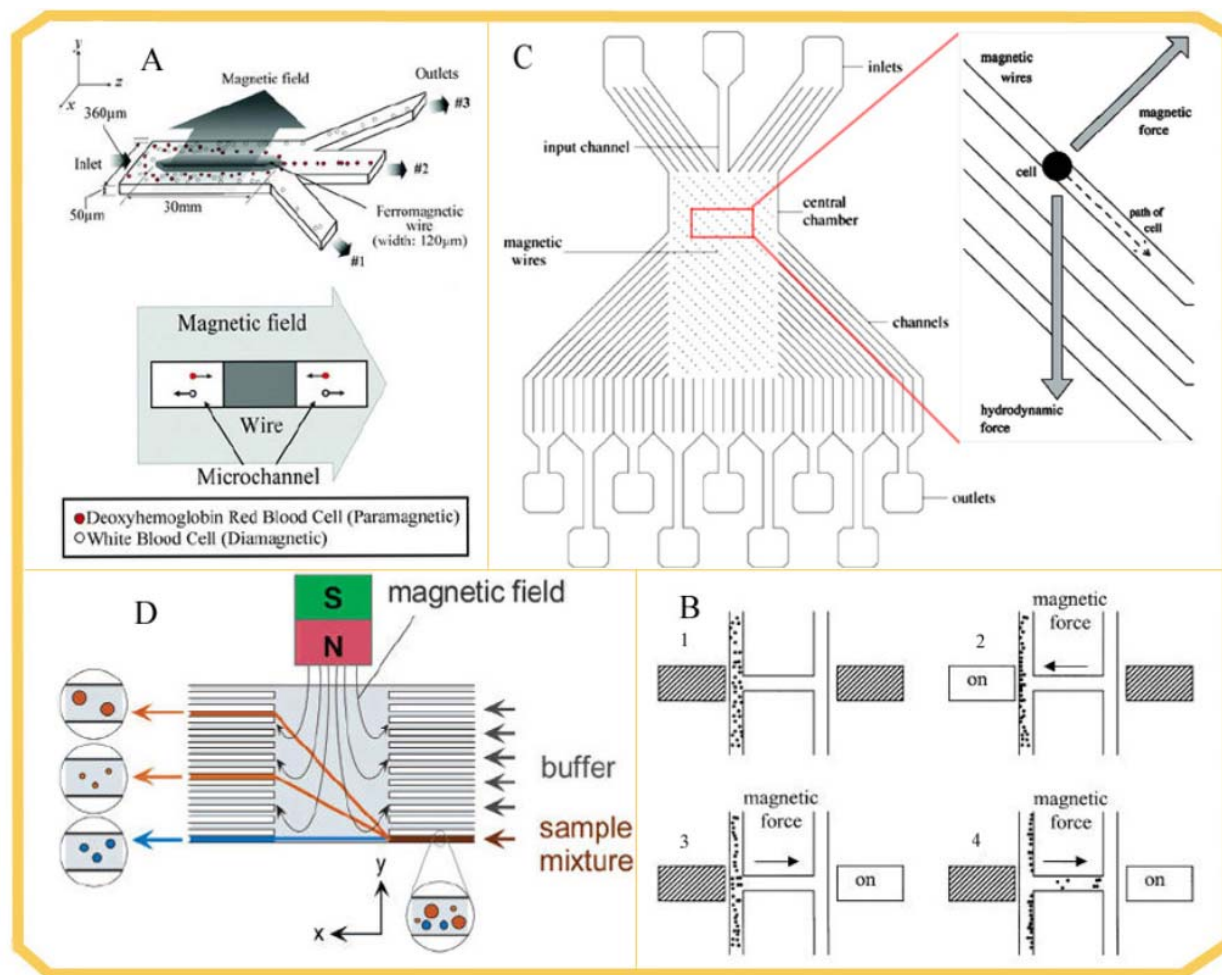
Figure 2. Cell sorting by optical mechanisms. (A) Layout of the microfluidic sorting junction and the optical switch. After being aligned to the center of the channel by flow focusing, cells are analyzed and then switched based on their detected fluorescence. Target cells are directed by the laser to the collection output while all other cells flow to the waste output. Reprinted with the permission from Ref (15), © 2005 Nature Publishing Group. (B) The scattering force from an optical beam transfers an object between two fluidic channels at different levels. Reprinted with the permission from Ref (67), © 2003 Kluwer Academic Publishers. (C) Composite image of tracer particles flowing in actual device combined with the 100 µm diode laser bar and waveguide outputs. Also used for scale. The two outside channels are waste, while the central two channels are used for fluorescing and nofluorescing sorted particles. Reprinted with the permission from Ref (64), © The Royal Society of Chemistry 2006. (D) Experimental setup used for particle sorting by evanescent field. Reprinted with the permission from Ref (65), © 2004 Optical Society of America. (E) The concept of optical fractionation. Low Reynolds number flows will be laminar: without an actuator all particles from chamber 2 would flow into chamber 3. Chamber 1 would typically introduce a 'blank' flow stream, although this could be any stream into which the selected particles are to be introduced. By introducing a three-dimensional optical lattice—in this case a body-centred tetragonal (b.c.t.) lattice—into the fractionation chamber (FC), one species of particle is selectively pushed into the upper flow field. The reconfigurability of the optical lattice allows for dynamic updating of selection criteria. For weakly segregated species, the analyte can be either recirculated through the optical lattice or directed through cascaded separation chambers. This latter option also allows the use of multiple selection criteria in a single integrated chip. The flow volume in our current sample cells is 100 µm thick; scale bar, 40 µm. Reprinted with the permission from Ref (19).

using native susceptibility of cells and specific attachment of magnetic beads with detailed discussions on principles for generating magnetic forces via the susceptibility of an particle and how to combine microfluidics with magnetic fields for better performance in separations. In this section, magnetic cell sorting will be discussed as two main categories, native magnetic susceptibility based sorting and magnetic labeling based sorting according to the sources of magnetism.

In the first category, native magnetic susceptibility of cells is sufficient for magnetic separation without additional modification. Currently, only two types of cells, red blood cells and magnetotactic bacteria, have
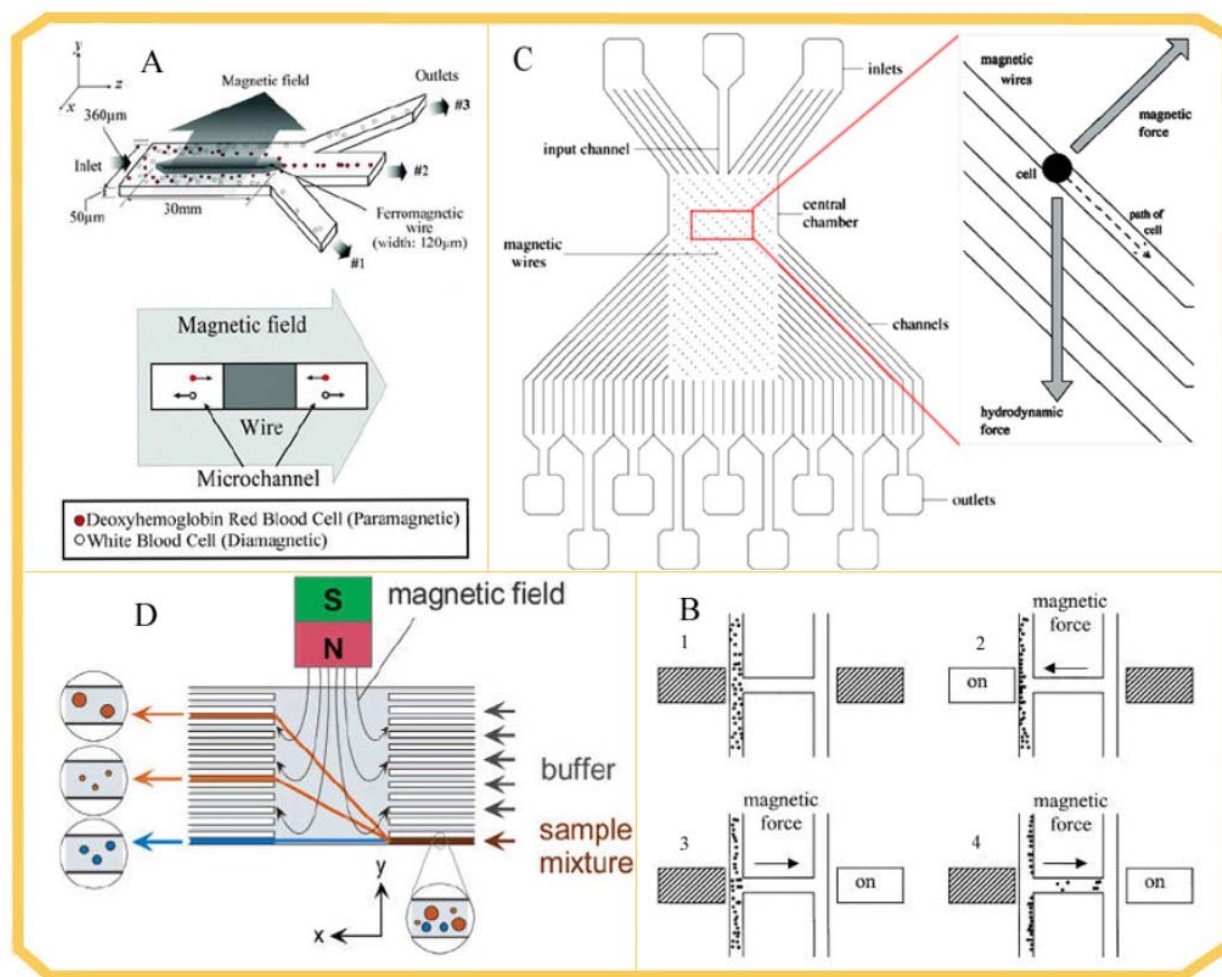
Figure 3. Cell sorting by magnetic mechanisms. (A) Illustrations of the single-stage PMC magnetophoretic microseparator with a rectangular ferromagnetic wire. Reprinted with the permission from Ref (20), © The Royal Society of Chemistry 2006. (B) The procedure of magnetic manipulation. Picture 1: the storage compartment is filled with particle suspension, picture 2: the alignment electromagnet aligns the particles with the left channel wall, picture 3: the launch electromagnet releases the particles from the channel wall, picture 4: the particles are pulled through the sample compartment and are detected when they enter the detection compartment. Reprinted with the permission from Ref (76), © 1999 Elsevier Science B.V. (C) The basic schematic of the microfabricated magnetic cell fractionation chip. Reprinted with the permission from Ref (25), © 2001 WILEY-VCH Verlag GmbH. (D) Concept of free-flow magnetophoresis. Magnetic particles are pumped into a laminar flow chamber; a magnetic field is applied perpendicular to the direction of flow. Particles deviate from the direction of laminar flow according to their size and magnetic susceptibility and are thus separated from each other and from nonmagnetic material. Reprinted with the permission from Ref (68).

been found in nature with native magnetic susceptibility (28). Han and Frazier (20) demonstrated continuous single-stage and three-stage cascade paramagnetic capture (PMC) mode magnetophoretic microseparators (Figure 3A) for high efficiency separation of red and white blood cells from diluted whole blood sample. The sorting mechanism for both PMC microseparators was high gradient magnetic separation (HGMS) approach. In another work (71), a single-stage diamagnetic capture (DMC) mode magnetophoretic microseparator was investigated. Successful separation of blood cells was achieved similarly.

In the second category, selective magnetic labeling is employed to introduce magnetic susceptibility to target cells. By modifying the surface of the magnetic labels with antibodies, peptides or lectins, they can then be attached to or engulfed by cells of interest with high selectivity (33). Thus, separations of target cells can be realized. Among magnetic labeling methods, immunomagnetic separation is the most frequently used one. Cells attached with antibody-coated magnetic beads are trapped by magnetic field. After eluting other cells by fluid flow, the trapped cells can then be released by removing the magnetic field and collected for other purposes. Grodzinski *et al.* (72) developed an integrated microfluidic device consisting of a chaotic mixer, an incubation channel and a capture channel. Immunomagnetic separation of *E. coli* from PBS and whole

blood, was demonstrated with a separation efficiency of 53% and 37% respectively. In a similar way, Furdui et al (31, 73) demonstrated immunomagnetic separation of Jurkat cells on microfluidic chips. Furthermore, on-chip magnetic bead separators with different magnetic patterns and structures were developed by Ramadan *et al*. (74) and Choi *et al*. (75) for immunomagnetic cell sorting.

Østergaard *et al*. (76) fabricated a magnetic switch based separation microchip with an H-shaped channel networks (Figure 3B). Electromagnets were positioned at each end of the connecting channel with opposite magnetic states for switching magnetic particles to one of the parallel channels. Berger *et al*. (25) and Inglis *et al*. (24) developed a novel magnetic cell sorter consisting of magnetized wires or stripe arrays laying at an angle to the hydrodynamic field flow (Figure 3C). The resultant hydrodynamic force and the magnetic force could deflect the cells attached with magnetic beads from the main stream. This device was successfully used for separating leukocytes from human whole blood samples.

Unlike the above qualitative separation methods that can only isolate magnetic particles, Pamme and Manz (68) developed a quantitative separation method, namely on-chip free flow magnetophoresis, which was capable of separating different magnetic particles from one another. In continuous flow, magnetic particles were deflected from the laminar flow by a perpendicular magnetic field depending on the size and magnetic susceptibility of the particles and the fluid flow rate (Figure 3D). Thus, magnetic particles could be separated from nonmagnetic materials. An original approach was proposed by Lee *et al*. (33) where a microelectromagnet matrix was used to trap, move and position biological cells at desired locations inside a microfluidic channel. The matrix consisted of two layers of straight gold wires aligned perpendicular to each other with insulations for protection. By varying the current in each independent wire, the microelectromagnet matrix can then create diverse magnetic field patterns to control the movement of individual cells in fluid. As a result, multiple yeasts could be manipulated simultaneously along different paths for cell-sorting or cell-assay purposes. Another more universal magnetic separation method was presented by Wang *et al*. (77) where cells without magnetic properties can be differentiated using magnetohydrodynamic (MHD) micropumps on chips. MHD pumps couple electric current with magnetic field to generate Lorenz force (pressure) on conductive fluids. MHD pumps can generate bidirectional pumping force and switch the flow direction of each channel by controlling the amplitude of either current or the electromagnetic field (or phase difference between two fields in case of AC MHD pumps). Separations of mouse neural stem cells and neuroblastoma cells were achieved in a "Y" shape microfluidic chip using this method.

4.4. Hydrodynamic mechanism
Hydrodynamic mechanism for cell sorting is based on the ability of cells to follow fluid flow due to the dragging forces (78). By controlling the hydraulic pressure difference of the microchannels via pumps, valves or dampers, cells can then be transported to desired locations on the chip. The fluid flow is usually laminar in microscale. Therefore, turbulence has minimal influence on controlling the flow. Both on-chip and off-chip control of fluid flow have been reported for cell sorting. However, the off-chip setting is more widely employed due to its simpler chip fabrication processes. Krüger *et al*. (79) constructed a pressure driven µFACS, in which cells can be individually isolated by hydrodynamic switching or valve switching. Remarkably, this device employed the latest photonic components including semiconductor laser, ultra bright LED sources, highly sensitive avalanche photodiodes, micro-prism, holographic diffraction gratings and optic fibers for activation and detection. Similarly, Chen *et al*. (78) proposed hydrodynamic switch for embryo and cell sorting, where an off-chip pressure control technique was used to switch the fluid flow between different micro-channels with a response time of 5 ms. Wolff *et al*. (16) developed a pressure-driven µFACS with highly integrated functions including a chimney structure for sheathing and focusing cells, a on-chip micro-chamber for cell culture and integrated optics for detecting cells. Hydrodynamic force was used to sort fluorescent latex beads from chicken red blood cells with a throughput as high as 12000 cells/s and a 100-fold enrichment was achieved. Different from the above switching modes that was realized by controlling the pressure difference between channels, a faster switching mode was developed by Bang et al (18) where an actuation channel was designed before the bifurcate junction (Figure 4A). At normal state, the actuation channel was blocked and fluid flow directly into the waste channel by the asymmetry of the channel geometry. On actuation, check valve opened with an actuation plug flowing out of the actuation channel. Since the disturbance required for switching the fluid flow was minimal, this design resulted in short response time.

Compared to the off-chip control of fluid flow, on-chip control has more compact structures that usually integrate microvalves, micropumps and microdampers using multilayer microfabrication techniques. A microfabricated cell sorter incorporating peristaltic pumps, dampers and switch valves was demonstrated by Fu *et al*. (40). Fluorescence labeled *E. coli* were controlled by peristaltic pump and then sorted into selected channels by opening\closing the dampers (Figure 4B). Furthermore, sophisticated sorting modes such as reversing sorting can be achieved by designing corresponding sorting algorithms. Studer *et al*. (80) developed a novel cell sorter for isolating fluorescence tagged rare objects diluted in a concentrated solution of non-fluorescent objects. This device consisted of several active pumps pneumatically actuated by monolithic soft microvalves (Figure 4C). To the best of our knowledge, it was the first demonstration of close loop sorting devices, which can peristaltically pump cells to move circularly around the sorting loops. Once target objects were positioned between the two arms of the recovery channel, valve would be opened with target objects transported towards the end of the channel.

4.5. Acoustic mechanism
It is well-known that particles suspended in solution can be enriched at certain regions by forces
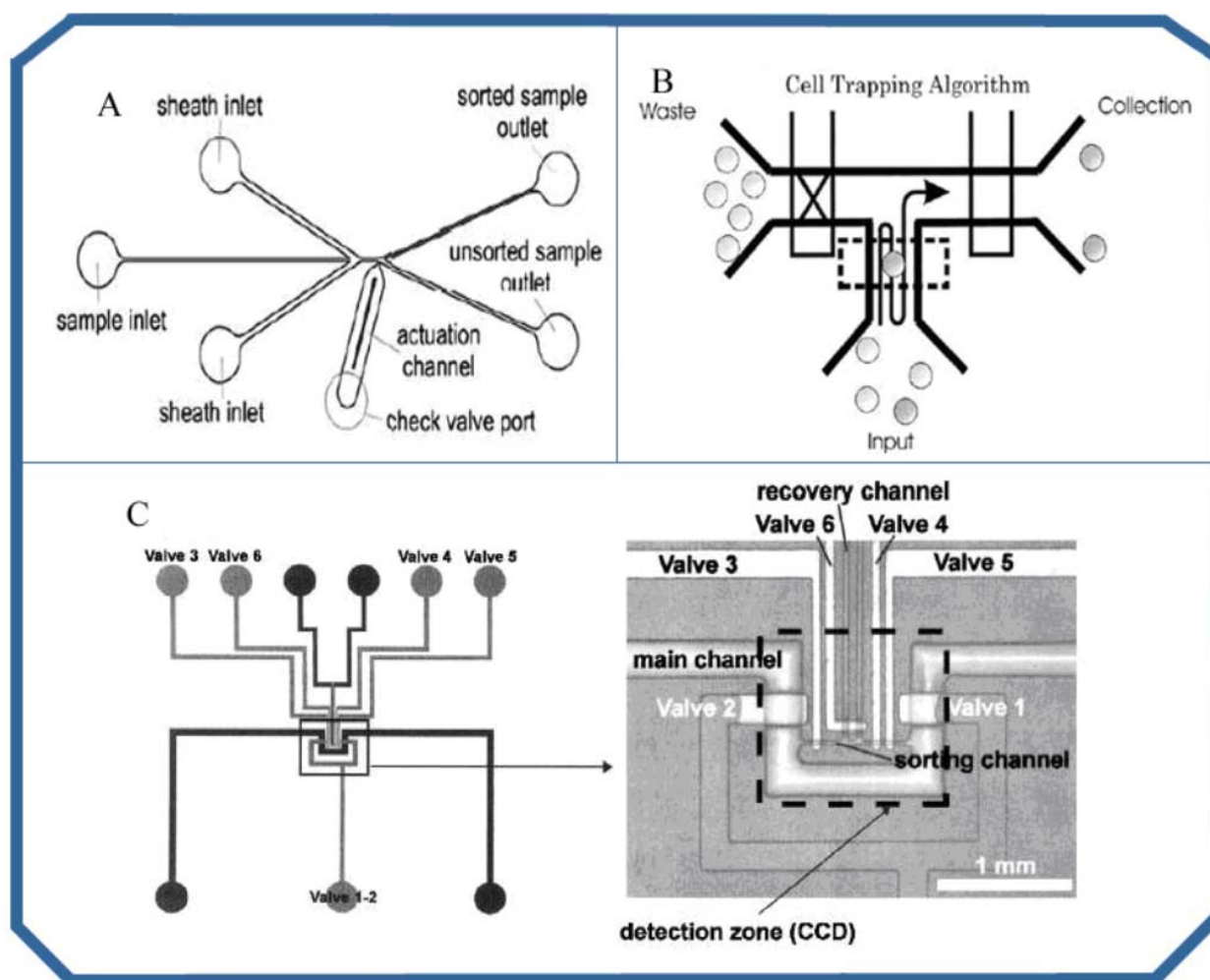
Figure 4. Cell sorting by hydrodynamic mechanisms. (A) Channel configuration of hydrodynamic switch by actuation channel. Reprinted with the permission from Ref (18), © 2006 Springer-Verlag. (B) Schematic of the cell trapping algorithm. A cell can be trapped within the detection region (dashed box) by reversing the flow at each detection. Reprinted with the permission from Ref (40), © 2002 American Chemical Society. (C) Left: Layout of the cell sorter. The fluidic channels appear in dark, the control channels controlling each valves (1–6) appear in grey. Right: Optical micrograph of the active area of the cell sorter chip (bottom view). The channels are named. The main U shaped fluidic channel is 300 µm wide, the control channel controlling valves 1 and 2 is being dead end filled with orange dye. A dashed box shows the detection area which corresponds to the field of view of the CCD camera. Reprinted with the permission from Ref (80).

generated from acoustic standing wave fields (81). Suspended particles exposed to an ultrasonic standing wave field experience acoustic radiation forces which can control the movement of particles towards either the pressure nodes or antinodes depending on the sizes, density and compressibility of the particle (21, 82). This mechanism has been used for continuous separation of cells or particles in microfluidic channels. Araz *et al*. (82) constructed an actuator by bonding glass capillaries to laser-cut lead zirconate titanate oxide (PZT) plate for ultrasonic control of microparticles or cells inside microfluidic channels. The high velocity generated by the actuator focused the sample at the node and antinode of the bending waves along the channel. Due to the nonlinear effects of PZT/Capillary actuator, particles of same density and acoustic impedance but different sizes can be differentiated by frequency

hopping. Separation of red and white blood cells and microparticles of different sizes were demonstrated. For comparison, Nilsson *et al*. (21) proposed cell sorting by exerting acoustic field across the microchannel. Ultrasonic excitation at the first harmonic resonance mode, namely matching the ultrasound wavelength to the channel width, enabled the focusing of particles into different lines. Thus, separation of particles can then be achieved by splitting the fluid flow. Furthermore, they have also expanded this work by successful isolation of erythrocytes from lipid microemboli in whole blood samples (81, 83). A separation efficiency of more than 70% was achieved.

4.6. Filtration

Filtration is a traditional passive method for separating particles of different sizes. Filtration-based

sorting exploits the geometrical difference (shape and size) among cells and sometimes the difference in deformability. Filtration usually requires external driving forces (84). Fabrication of chips for filtration is relatively easy by using standard microfabrication technologies. However filtration doesn't accommodate to the changes of the sample sizes. Therefore, different samples often require different designs of microchips. In addition, the separation efficiency of filtration is fairly limited especially in cases of separating similar-sized cells or particles (8). According to the position of filters, microfluidics-based filtration can be categorized as the "On the channel" mode (84-86) and the "Beside the channel" mode (87, 88). Wilding *et al.* (85) fabricated several types of micro-filters in microchannels such as the "coiled-bed weir-type", the "Comb-type" and the "tortuous-type" to isolate white blood cells from whole blood samples. Mohamed *et al.* (86) developed a series of massively parallel microfabricated sieving devices, which were constructed with four successively narrower regions of channels numbering 1800 per region. Mixtures of neuroblastoma cells and whole blood cells were then successfully separated for demonstration. Multi-step microchannels with decreasing depth were fabricated by Vankrunkelsven *et al.* (89) for separating particles of different sizes. Spherical particles were transported by shear-driven flow and then arrested at the step gap that was shorter than the diameter of the particles. As a result, particles of different sizes were concentrated in different bands. Separation of binary mixtures of *S. aureus* and *S. cerevisiae* cells, and of *S. cerevisiae* and *E. coli* cells were demonstrated using this approach. Shelby *et al.* (90) constructed microfluidic chips with narrow capillary blockages linked to two wide channels for separating malaria infected erythrocytes from normal cells. Infected erythrocytes lost their cell deformability, thus can not pass through the capillary. Instead of using microfabrication techniques, Moorthy and Beebe (84) utilized in situ emulsion photo-polymerization to fabricate porous filters in microchannels with advantages of constructing filters at specific locations.

In the "On the channel" mode, cells accumulated at the filters can easily clog the filter (84). To resolve this issue, "Beside the channel" mode of filtration was introduced recently. Crowley and Pizziconi (87) constructed microfluidic chips with transverse flow microfilters connected to a main flow channel for isolating nanoliter volume plasma from a single drop of blood. Sethu *et al.* (88) demonstrated the use of diffusive filter for separating leukocytes from whole blood samples. Approximately 50% erythrocytes were isolated at a flow rate of 5ul/min. Sieves were positioned on the side of the channels allowing the passage of biconcave erythrocytes while blocking spherical leukocytes of larger sizes. Diffusers were designed in a flared geometry in order to ensure equal volume of fluid flow through each sieve (Figure 5A).

4.7. Surface absorption

Surface absorption has also been used in microfluidic cell sorting, which utilizes interactions between cell membranes and channel surface to immobilize cells or decrease the mobility of the cells to achieve separation. The affinity interactions between cell-surface receptors and specific ligands were usually employed for this purpose. Toner et al (29, 91, 92) used PEG microwell arrays with microchambers decorated of specific antibodies to selectively capture and concentrate T-lymphocytes and B-lymphocytes. Influence of the fluid flow and the surface conditions of the microchannels on lymphocyte isolation was also investigated (29, 91). In addition, laser-mediated cell retrieval technology was employed to remove individual cells of interest from the cell array (92). Chang *et al.* (6) then demonstrated a protein adhesion method for cell sorting. White cells were captured by immobilized proteins in the microchannel transiently and then dissociated readily. Different types of cells adhere to different adhesion proteins resulting in different transit speeds under a given fluid shear. Therefore, mixture of cells can be separated based on the differences in adhesion-mediated cell transit. By using this method, HL-60 cells and U-937 cells were successfully fractionated in microchannels filled of pillars coated with E-selectin IgG chimera. Furthermore, adsorption by hydrophobic or other interactions has also been used for cell sorting. Horsman et al (93) reported successful separation of sperms from biological mixtures of epithelial cells based on their different sedimentation rates and different adsorption to the glass surface.

4.8. Cocurrent extraction

Cocurrent extraction uses the affinity difference of particles between the two immiscible flows of an aqueous two-phase flow to continuously separate and fractionate different biomaterials. Cocurrent extraction has the advantage of biocompatibility and selectivity. However, its application in macroscale has drawbacks such as brittle interfaces between the two flows, sedimentation of samples and long separation time. In microscale, the Reynolds number becomes very low ensuring a stable interface between the two phases (62). Thus, the surface to volume ratio becomes very high (94). In addition, influence of gravity in microsacle is neglectable. Yamada *et al.* (94) first demonstrated the use of cocurrent extraction in microfluidic chips to continuously aggregate plant cells of diameters approximately 37-96 μm. Nam *et al.* (62) have also attempted cocurrent extraction method for isolating living CHO-K1 cells from dead cells using polyethylene glycol 8000 (PEG 8000, 4%) and dextran T500 (5%). Cell samples were focused by two fluid flows of dextran T500 so that a relatively high surface to volume ration could be realized. Living cells were collected in the PEG-rich phase with a recovery efficiency of ～100% and a fractionation efficiency of ～97%.

4.9. Other sorting mechanisms

Like the nonuniform electric field separating cells by differential forces, nonuniform fluidic velocity field can also be used for cell sorting purposes (95-97). Due to the boundary effect of the channel walls, the velocity field of flow is nonuniform in cross section of the channel, which can then be further amplified in winding channels (an effect similar to centrifugation). Particles with different density and volumes distribute in different flow streams as a result
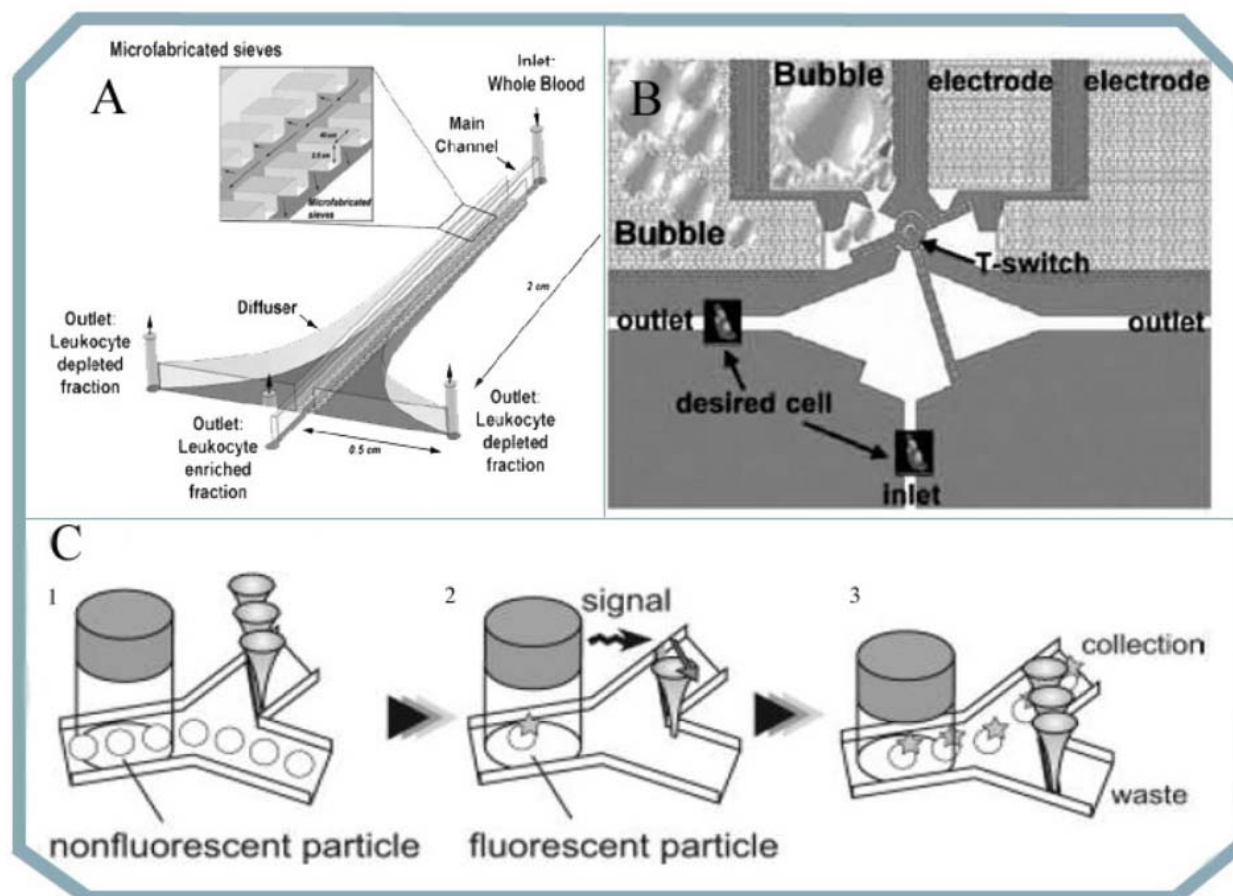
Figure 5. Other sorting mechanisms. (A) Schematic of the diffusive filter for size based continuous flow fractionation of erythrocytes from whole blood. Insert shows the 40 μm 6 2.5 μm sieve structure and the arrangement connecting the main channel to the diffuser. Reprinted with the permission from Ref (88), © 2006 The Royal Society of Chemistry. (B) Illustration of the structure design and the sorting mechanisms of mechanical micro-T-switch cell sorter chip. The desired cells are sorted out to the left outlet channel by actuating the micro-T-switch counterclockwise Reprinted with the permission from Ref (99), © 2005 The Royal Society of Chemistry. (C) Principle of the cell sorting method. Picture 1: The window for detecting the fluorescence signal is located upstream of the junction. In the absence of a fluorescence signal, the collection channel continues to be plugged and flow directed to the waste channel. Picture 2 and 3: Upon detection of a fluorescence signal, the entrance to the waste channel is plugged by switching the position of laser illumination, directing flow to the collection channel. Reprinted with the permission from Ref (100).

of the nonuniform field. Therefore particles can be separated by splitting the fluid flow. Blattert *et al*. (95) successfully separated blood cells and plasma in bending channels using this mechanism. Similarly, an S-shaped microchannel connected to a consecutive cavity of increasing width was used for continuous amplification and separation of particles by Zhang et al (96). Based on the phenomena that erythrocytes tend to flow at the center of the blood vessels while leukocytes distribute near the sidewalls, Shevkoplyas *et al*. (97) utilized this intrinsic nature of blood flow to isolate leukocytes from whole blood sample in microchannel networks. 34-fold enrichment of leukocytes to erythrocyte was achieved in a single run of sample.

Mechanical switch and multi-way valve can also be used for direct cell sorting. It usually has relatively low switch rates, however, it's still suitable for separating less concentrated cells. Terray et al (98) developed a passive colloidal valve and an actuated three-way valve to configure flow path for microparticles by controlling hydrodynamic flow. The actuated valve has great potential for cell sorting. Recently, active binary micro-mechanical-switch cell sorters with a double T-structure design were developed by Ho and his coworker (Figure 5B) (99) where switches were actuated and controlled by electrolysis-bubbles. Separation of human hepatoma cells with ～84.1% cell viability was demonstrated.

Shirasaki et al. (100) developed a μFACS system with valves that rely on sol-gel transformation of a thermoreversible gelation polymer (TGP). Fluorescence labeled cells in solutions containing TGP were introduced into a Y-shaped microchannel. The sol-gel transformation
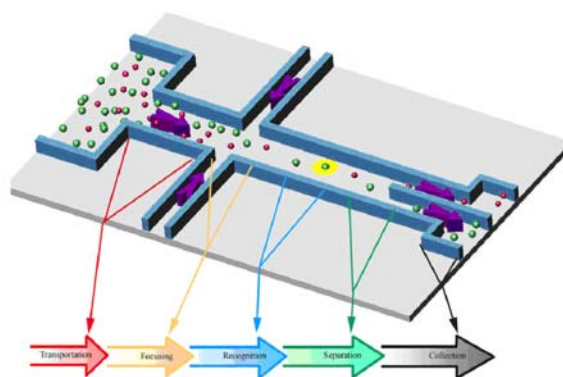
Figure 6. Illustration of steps of cell sorting. General steps of cell sorting include transportation, focusing, recognition, separation and collection.

of the TGP functioning as in-channel valves was locally induced by a site-directed infrared laser at the junction of the two outlets (Figure 5C). One of the two microchannel outlets is plugged according to fluorescence signal. Compared to other microfluidic cell sorters, this system had good performance in maintaining cell viability.

Yamada et al. (101-104) proposed a microfluidics-based particle sorting method that adopted flow splitting and recombining. By continuously splitting and recombining mainstream through the side channels, particles were concentrated and aligned along the sidewalls which can then be collected by flowing through branch channels of different sizes. A mixture of 1.0-5.0 mm particles were successfully separated and isolation of erythrocytes from blood samples was demonstrated.

Cho *et al.* (106) described a self-contained microfluidic system that can identify and isolate motile sperm from non-motile sperms and other cellular debris based on the ability of motile sperm to cross streamlines in a laminar fluid flow. This method has great potential for intracytoplasmic sperm injection.

5. PROCEDURE OF CELL SORTING

A complete process for cell sorting usually involves steps of transportation, focusing, recognition, separation and collection (Figure 6). In the following section, each step will be briefly discussed according to their applications in microfluidic cell sorting.

5.1. Cell transportation

Cell transportation is an initial step for cell sorting. Hydrodynamic and electric transportations are two most widely employed methods. In hydrodynamic transportation, fluid is driven by the pressure difference generated by syringe pump (16, 38, 48), pneumatic pump (15, 40), peristaltic pump (18) or the gravity (39, 105). A wide range of velocity with a relative higher limit can be easily achieved. In electric transportation, an external power source is required. Electroosmosis (7, 35, 36) usually has a high transporation velocity. Together with

electrophoresis (107), dielectrophoresis generated by electric field (53) were used to transport cells. In addition, optical transportation by evanescent field and magnetic transportation (65) by magnetohydrodynamic micropumps (77) were also presented in MCS, but both methods require complex chip designs and increase the cost of MCS.

5.2. Cell focusing

Cell focusing is an important step in the strategy of "active multi-channel switch". In addition, cell focusing is widely employed in µFACS and microfluidic flow cytometry. Cell focusing can be generally categorized passive focusing and active focusing (107). In passive focusing, channels of sizes wider than the diameter of a single cell are used for lining cells without additional power (7, 40, 77). However, shallow and narrow channels increase the risk of clogging (16). In active focusing, external power is utilized for cell focusing by hydrodynamic mechanism (15, 38, 48) or electric mechanism (17, 36, 107). In conventional hydrodynamic or electrokinetic focusing, channels are wide but shallow, because the sample is sheathed on two sides. Therefore clogging of samples may still be an issue. Wolff *et al.* (16) developed a chimney type hydrodynamic focusing structure, which was embedded in the center of the sheathing channel for sample injection. This method allowed focusing of cells from four sides without clogging issues.

5.3. Cell recognition and separation

Cell recognition, as the basis for cell separation, is a necessary step for the strategies of both "active multi-channel switch" and "Direct manipulation". Cell recognition involves detection, data collection and data analysis. On chip or off chip detectors and collectors are utilized for collecting the morphological, physical and chemical information of cells which is then used as the basis for differentiation. Upon successful recognition, separation can then be realized by employing external forces. As noted, the validity of recognition determines the purity and recovery rates of the cell sorting. Therefore, the recognition rate is an important factor related to the separation throughput. For the other three strategies, cell recognition step is not necessary. However, separation is completed by differential forces according to different physical or chemical properties of the cells.

In general, cells are distinguished for separation by their intrinsic or extrinsic difference. Intrinsic difference such as cell color, cell morphology (34, 58, 107) and autofluorescence (35) were used for recognition while dielectrophoretic characteristics (23, 48, 58), optical polarizability (19), native magnetic property (20, 71), surface antibodies of cell membrane (29, 31, 92), density and sizes (21, 95, 96) and deformability (85, 86, 87) were used for separation. In cases that the intrinsic difference is not sufficient for cell differentiation, extrinsic difference can then be introduced by specific labeling such as fluorescence labeling (7, 15, 40) and magnetic labeling (24, 33, 68). In cell recognition, CCD (34, 58, 107), PMT (7, 15, 40) and ADP (17, 38, 79) were frequently utilized for imaging recognition or fluorescence detection.

Table 3. Typical microfluidic cell sorter with detailed criterions

Objects	Throughput (cells/s)	Purity (%)	Recovery rate (%)	Enrichment (fold)	Strategy	Separation method	Ref
GFP tagged E. coli from non-tagged	20	30.7	20	30	Active multi-channel switch	Electroosmosis	7
dielectric particle tagged E. coli from non-tagged	10,000	NA	95	>200	Local nonuniform field flow Fractionation	Dieletrophoresis	27
particles with different optical polarizability	25	NA	96	NA	Field Flow Fractionation	Optical	19
GFP tagged HeLa cells from non-tagged	20~100	82~98	>85	63~71	Active multi-channel switch	Optical	15
EGFP tagged E. coli from non-tagged	up to 44	3.6~34	16~50	7~83	Active multi-channel switch	Hydrodynamic	40
green fluorescent beads from red blood cells	12,000	2.4	NA	100	Active multi-channel switch	Hydrodynamic	16

5.4. Cell collection

The purpose of MCS is to purify cells for further cells analyses. Cell collection can be categorized as space differential collection (19) and time differential collection (5) according to the separation methods. In space collection, cells in different flow streams enter different collecting channels, while in time collection, different cells flow in the channel with different velocities thus reaching the collecting point at different time. In general, local nonuniform FFF and FFF in field across channel mode belong to space collection and differential immobilization and FFF in field along channel mode belong to time collection. Active multi-channel switch and direct manipulation employ both collection methods.

6. EVALUATION CRITERIONS FOR MICROFLUIDIC CELL SORTER

Although a variety of different microfluidic cell sorters have been proposed, most of these devices were presented as "proof of concept" and only a few quantitative criteria were given. Therefore, it's important to employ some uniform criteria for evaluating the performance of microfluidic cell sorting. According to several important literatures in this field (7, 15, 16, 27), throughput, purity and recovery rate can be adopted as the main quantitative criteria for evaluating microfluidic cell sorters. In some cases, other criteria such as cell viability and the enrichment factor are also worth mentioning. To quantitatively compare the previous work in the field of MCS, cell sorters with detailed criterions are listed in Table 3. In the following sections, each criterion will be discussed in detail.

6.1. Throughput

Throughput represents the number of cells that pass through the separation zone per unit of time or the average number of cells that are executed in the duration of a single run in case of differential immobilization. Throughput mainly depends on the flow velocity that is controlled by the hydrodynamic pressure or the electric potential. However, purity and recovery rate would be dramatically decreased with increasing velocity of the fluid flow. Thus, balance between throughput and purity and recovery rate should be considered. Switching time and recognition time also influence throughput in case of the "Active multi-channel switch" strategy. For the "Differential immobilization" strategy, throughput also

depends on the immobilization rate and the number of cells immobilized in each run. Up to date, the highest throughput achieved by MCS is ～12,000 cells per second in a single channel with relatively low separation purity (16) (Table 3).

6.2. Purity

Purity is defined as the fraction of the target cells in the collection wells (27). Inaccurate recognitions result in decreased purity. Purity depends on the resolution of differential forces in cases of "field flow fractionation", "local nonuniform field flow fractionation" and "field caused immobilization". Specific extrinsic labeling can be used to improve the resolution of differential forces in separating cells of similar physical and chemical properties. In "active multi-channel switch" and "direct manipulation", purity is closely related to successful recognitions that can be improved at the cost of time and expense by using multi-parameter recognition. Purity depends on the specificity of adsorption in case of surface adsorption.

6.3. Recovery rate

Recovery rate also referred to as separation efficiency is the ratio of successfully isolated cells to the total number of input target cells (27). Inaccurate recognition of target cells decreases the recovery rate. Therefore, recovery rate is in contradiction with purity if a single threshold is used for cell recognition (15).

6.4. Other Criteria

Cell viability is an important criterion for many cell sorting systems in which living cells are collected for cell culture or analyses. In electric mechanism based cell sorting, electric field intensity is often investigated for their influence on cells since cells can be easily damaged by an electric field with intensity above certain value. Similarly, laser power and flow velocity are also evaluated in the optical mechanism and the hydrodynamic driven cells sorters respectively. For MCS, a universal and quantitative criterion (ratio of living cells to the total number of target cells in the collecting well) is used for evaluating cell viability (15, 99).

Enrichment is another criterion in common use for cell sorting when cell sorting is employed as the pre-step to concentrate specific cells for further applications such as PCR (31, 72, 85) and clinical assays (97). Enrichment can be calculated by the ratio of target cells to

other cells in the collecting chamber divided by ratio of desired cells to undesired cells in original sample (97). Hu *et al*. (27) have reported the highest enrichment of more than 200 fold with good throughput and recovery rate (Table 3).

7. FUTURE CHALLENGE AND DIRECTION

Microfluidic chips for cell sorting have been overviewed with aspects of principles, strategies, sorting mechanisms, procedures and criteria for evaluation. Compared to macroscopic cell sorting techniques, microfluidic chips operate with small amount of cells with prospect of being a necessary preliminary step in the field of cell research, clinical assays, diagnosis, drug discovery and pharmacology. A variety of methods have been employed in microfluidics-based cell sorting and some of them have achieved good performance. However, there are still many challenges requiring further investigation. First of all, the performance of MCS methods such as purity, recovery rate and cell viability need to be improved. Normally, high throughput was achieved at the cost of purity and recovery rate. To achieve both, multi-stage separation is suggested as well as parallel multi-channel integration with reduced throughput in a single channel. Secondly, miniaturization is important for on site assays and detections especially when MCS is operated in the open air. Without cumbersome power sources, pumps and detectors, most of the above microfluidic cell sorters can not function. Besides integrated electronic devices, integrated optical devices such as on-chip laser (108), waveguide (65), microlenses (109), micro optical attenuator (110) and avalanche photodiode (APD) (79) have potential prospect for applications of cell sorting on chips. In addition, universalization and specialization will be the long-term goals of MCS development. For universalization, MCS is designed to handle cells of various types. A simple parameter for recognition would result in poor purity and recovery rate while multi-parameter recognition would greatly increase the recognition time and system cost. Therefore, it's important to find a condition that can compromise all the factors. Impedance spectroscopy (111) and Raman spectroscopy (60) may be good candidates for this purpose. For most cell sorters, specialization is a rational choice. In consequence, cell sorters integrated with multiple functions can resolve specific problems. Given certain target, the performance of microfluidic cell sorters can then be highly improved according to characteristics of the cells. Undoubtedly, microfluidic cell sorting has highly potential prospect of applications in various fields of biosciences and biotechnologies.

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Abbreviations: μFACS: microfabricated fluorescence-activated cell sorters; GFP: green fluorescent protein; *E. coli*: *Escherichia coli*; *B. cereus*: *Bacillus cereus*; *B. subtilis*: *Bacillus subtilis*; *L. monocytogenes*: *Listeria monocytogenes*; *S. aureus*: *Staphylococcus aureus*; *S. cerevisiae*: *Saccharomyces cerevisiae*; HIV: human immunodeficiency virus; DEP: Dielectrophoresis; 3D: three-dimensional; 2D: two-dimensional; AC-DEP: nonuniform AC electric field induced dielectrophoresis; nDEP: negative dielectrophoresis; pDEP: positive dielectrophoresis; twDEP: traveling-wave dielectrophoresis; iDEP: insulator-based dielectrophoresis; IEF: isoelectric focusing; PMC: paramagnetic capture; HGMS: high gradient magnetic separation; MHD: magnetohydrodynamic; IgG: immunoglobulin; CHO-K1: Chinese hamster ovary; FFF: Field Flow Fractionation; MCS: microfluidic cell sorting; LTRS: laser tweezers Raman spectroscopy; AC-DEP: alternating electric field

induced dielectrophoresis; DC-DEP: direct electric field induced dielectrophoresis; DC-iDEP: direct electric field induced insulator-based dielectrophoresis; RBCs: red blood cells; AC: Alternating current; avalanche photodiode (APD)

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