



Review

Microfluidic chip: Next-generation platform for systems biology

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ABSTRACT

Systems biology advocates the understanding of biology at the systems-level, which requires massive information of correlations among individual components in complex biological systems. Such comprehensive investigation entails the use of high-throughput analytical tools. Microfluidic technology holds high promise to facilitate the progress of biology by enabling miniaturization and upgrading of current biological research tools due to its advantages such as low sample consumption, reduced analysis time, high-throughput and compatible sizes with most biological samples. In this article, we documented the recent applications of microfluidic chips in biological researches at the molecular level, cellular level and organism level, serving the purpose for systems-level understanding of biology.

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1. Introduction

Since large-scale sequencing projects yielded complete genomes for several species, such as *Escherichia coli* (*E. coli*), *Caenorhabditis elegans* (*C. elegans*) and human, biological science

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has entered the post genomic era. Tremendous information at the molecular level has been generated up to date. However, little is known about biology at the systems-level.

Currently, systems biology holds a high promise for fulfilling such a challenging goal [1–7], which seeks to develop a complete understanding of complex biological systems by both qualitatively and quantitatively studying the interactions and correlations among individual elements. Such a complex system can be a molecular network, cell, tissue, organ, or even entire organism. Instead of reduction methods advocated by molecular biology, systems biology requires a shift in our philosophy to integration of dynamic components for systematic interpretation of biology.

Due to the complexity of biological systems, systems-level understanding of biology requires the use of high-throughput and accurate analytical tools to gather massive amount of information, goals that are perhaps beyond the scope of current experimental practices [5]. Thus, technical innovations in experimental devices are an urgent task in systems biology. Currently, many novel technologies have been developed for fulfilling such demanding requirements of systems biology, such as microfluidic systems, nanotechnology and femto-second technology.

The advent of microfluidic systems [8] has revolutionized the methodology for biological researches. Since microfluidic systems have compatible sizes with most biomacromolecules, cells and small organisms, microfluidic technologies have then been proposed for the miniaturization of existing biological tools to address issues like speed, throughput, and sample cost. For example, miniaturization of capillary array electrophoresis significantly accelerated the progress of large-scale sequencing projects. Microfluidic cell sorters not only speed up the separation and measurements of live cells, but also encourage automation for high-throughput analysis at the cellular level.

Applications of microfluidic chips in biology are growing fast. In this article, the recent applications of microfluidic chips in biological researches at the molecular, cellular and organism levels were overviewed from the perspective of systems biology. As the next-generation platform, microfluidic chips will certainly open up new avenues for high-throughput biological analysis, facilitating the understanding of biology at the systems-level.

2. Analysis at molecular level

2.1. Genomics

Since the discovery of DNA double-helix structure by Watson and Crick [9], molecular level understanding of biology started emerging. The well-known human genome project resulted in successful discovery of ~32,000 human genes, which allowed further understanding of functional genomics at systems-level. Microfluidic chip based methods certainly play an important role in high-throughput genomic studies.

2.1.1. Sequence analysis

Capillary array electrophoresis (CAE) [10–12] has been the golden standard for genome sequencing purposes, where multiple capillaries are used in parallel for high-throughput sequencing of target DNAs. With the development of microfabrication technology, enhanced speed and parallelism of electrophoretic analysis can be readily achieved by system miniaturization. Microfabricated CAE device (μ CAE) was first introduced by Woolley and Mathies [13,14] for DNA sequencing using microfluidic channel arrays. With a four-color labeling and detection system, 97% accuracy and single-base resolution to ~150 bases was achieved in 540 s [14]. In a subsequent work [15], 12 separation channels were integrated on a CAE chip, which allowed parallel analysis of 12 samples in less than 160 s. To

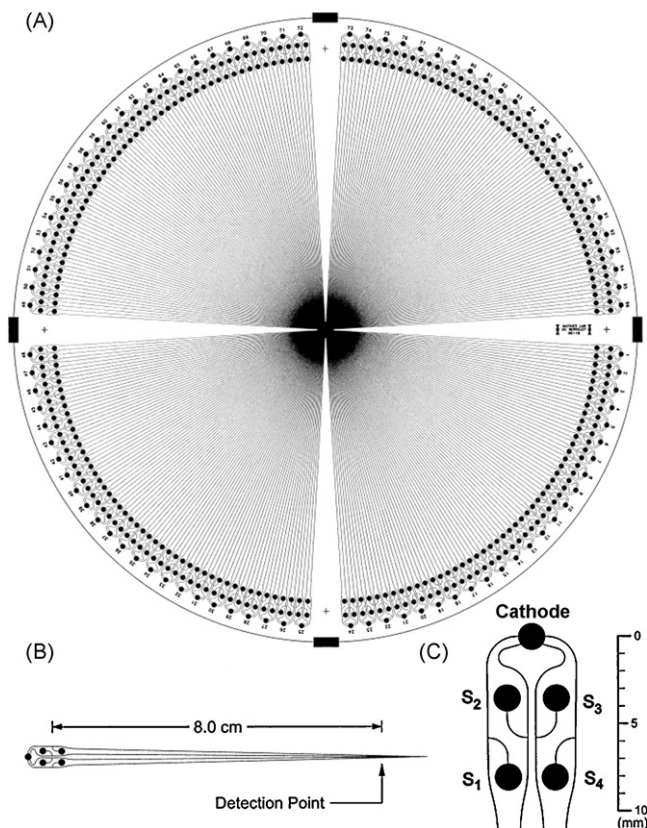


Fig. 1. (A) Layout of the 384-channel μ CAE device on a 200-mm-diameter wafer. Channels are 60 μ m wide and 30 μ m deep, and the effective separation length is 8.0 cm. (B) Expanded view of a single quartet of channels with their injectors. (C) All channels in a quartet share a common cathode reservoir located closest to the edge of the wafer. The injector design for every channel is shown at right. S1–S4 indicate individual sample reservoirs. (Reprinted from Emrich et al. [23], with permission of American Chemical Society, © 2002).

further enhance the separation speed and throughput of the on-chip CAE assay, the separation channel density was significantly increased [16–19]. Liu et al. [18] reported automated DNA sequencing in 16-channel microchips with an integrated four-color confocal fluorescent detector, which yielded more than 450 bases in 15 min with a 99% accuracy. Simpson et al. [19] realized integration of 48 separation channels with an injection unit that permitted the serial analysis of two different samples on each capillary. In consequence, 96 samples can be efficiently analyzed by this CAE microchip in less than 8 min. A 96-channel radial capillary array was subsequently developed by the research group of Mathies detected by a rotary confocal fluorescence scanner [20–22], resulting in a rapid sequencing rate of 1.7 kilobase pairs (kbp) per minute. They further pushed the limits of channel density for high-throughput DNA sequencing by creating the 384-channel radial CAE device [23] (Fig. 1). A 20-fold increase in sequencing performance was readily accomplished over the conventional 96-capillary instrumentation.

In addition to μ CAE, many other miniaturized systems have also been reported. A disposable plastic electrophoresis system was demonstrated earlier by Shi and Anderson for high-resolution single-stranded DNA analysis [24]. Plastic microchannels with a 4.5 cm effective separation length were fabricated from an etched glass master. Four-color separation of a 320-base terminator sequencing standard was achieved in 13 min with an accuracy of 99.1%. Kartalov and Quake [25] reported a fully integrated PDMS (polydimethylsiloxane) microfluidic system for DNA sequencing-by-synthesis, using a heterogeneous assay that combined active plumbing, specific surface chemistry and parallelism.

Reliable sequencing of up to four consecutive bps was demonstrated. Pyrosequencing is a nonelectrophoretic bioluminescent DNA sequencing method that uses a four-enzyme mixture reaction to monitor nucleotide incorporation in real time. Russom et al. [26] presented a nanoliter-volume microfluidic platform for the pyrosequencing of DNAs by trapping the DNA on microbeads in an on-chip filter chamber and monitoring flow-through of the pyrosequencing reagents at the same time. Applications of miniaturized systems for DNA sequencing are still growing, several reviews [27–32] on this fast progressing field have also been reported lately.

2.1.2. PCR and other applications

Besides DNA sequencing, other applications of microfluidic chips include DNA separation, analysis and polymerase chain reaction (PCR). Riehn and et al. [33] reported restriction mapping of DNA molecules in 100–200 nm channels. With the restriction enzymes *Sma*I, *Sac*I and *Pac*I, the position of restriction sites was successfully measured with a precision of ~ 1.5 kbp in 1 min using single DNA molecules. Heule and Manz [34] demonstrated a passive mixing microsystem for sequential DNA hybridization assays. By injecting the sample solutions one after the other and mixing them rapidly on the chip, the kinetics of hybridization can be investigated. Using this method, sequence dependent signals at single nucleotide polymorphisms (SNP) level were successfully recorded. Wang et al. [35] developed a microfluidic chip capable of quantitative detection of low-abundance nucleic acids by incorporating confocal fluorescence spectroscopy, molecular beacons and a molecular-confinement microfluidic reactor. Kaji et al. [36] developed a nanofabrication technique for constructing high aspect ratio nanopillars as DNA sieving matrixes for the separation of large DNA fragments in microchannels. With the optimal chip design, DNA fragments ranging from 1 to 38 kbp were successfully separated as clear bands. Separation of λ DNA and T4 DNA has also been demonstrated using a 380 μ m long nanopillar channel within 10 s. Zeng and Harrison [37] reported self-assembled colloidal arrays as three-dimensional nanofluidic sieves for the separation of double-stranded DNA fragments between 50 bp and 50 kbp (Fig. 2). Li et al. [38] constructed gold nanopatterns on silicon surface for the electrophoretic separation of linear, relaxed and supercoiled DNAs based on different dielectrophoretic forces between DNA segments and gold nanopatterns.

Lagally et al. [39] earlier demonstrated an integrated microfluidic device, combining submicroliter PCR chambers with a microfabricated capillary electrophoresis (CE) system for the stochastic PCR amplification of single DNA template molecules and subsequent CE analysis. Recently, Wang et al. [40] reported a polycarbonate-based spiral continuous flow thermal cycler microchip for sanger cycle sequencing using terminator chemistry. A polycarbonate microchip was fabricated by Chen et al. [41] for the PCR amplification of DNA templates using an electrokinetically driven synchronized continuous flow PCR configuration. A bidirectional flow DNA amplification microreactor was reported by Chen et al. [42] for ultrasensitive PCR and real-time detection of human genomic samples. An RNase P gene detection limit of 24 human genome copies with a reaction efficiency of 77% was achieved. Marcus et al. [43] demonstrated a microfluidic device for parallel reverse transcriptase-PCR (RT-PCR) assays. With integrated micromechanical valves, this microchip was able to perform 72,450 pL RT-PCRs in parallel. Ottesen et al. [44] reported a microfluidic digital PCR platform with 12 panels each containing 1176 PCR chambers for the multigene analysis of single bacterial cells harvested from nature. As a result, previously unknown ribosomal RNA-based species identity of several symbionts was successfully discovered.

2.2. Proteomics

Proteome is directly derived from the genome and it in turn regulates both the gene expression and cellular metabolism. Thus, quantitative investigation of the proteome is essential for understanding biology at the systems-level. Here, we discuss the recent applications of microfluidic chips in proteomics with aspect of pre-concentration, on-chip separation, single-cell proteomics and mass spectrometry coupling.

2.2.1. Preconcentration

Preparation of proteins is usually a prerequisite step in proteomic studies. When working with natural low-abundance proteins, preconcentration is necessary to bring target proteins into detectable range. Various methods have been reported for protein preconcentration on microfluidic chips.

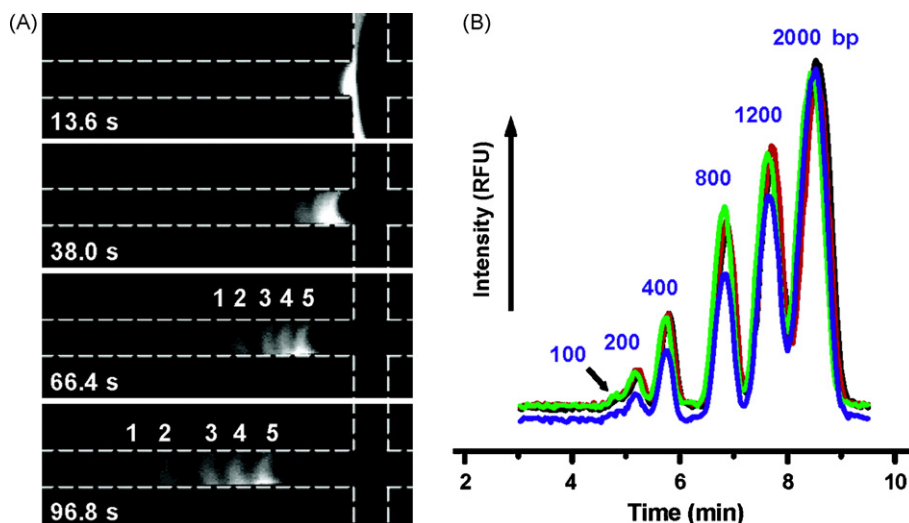


Fig. 2. (A) Sequence of fluorescent images of the electrophoretic separation (15 V cm^{-1}) of a low DNA mass ladder in a microfluidic chip packed with $0.9\text{-}\mu\text{m}$ silica beads. In this case, the microchip has $100\text{-}\mu\text{m}$ -wide and $20\text{-}\mu\text{m}$ -deep microchannels with a cross-injection design. Dashed lines were added to aid visualization of the microchannels. Frames are time-stamped in seconds. DNA fragments 1–5 are assigned as 200, 400, 800, 1200 and 2000 bp, respectively. The 100-bp band was not detected due to the very low concentration. (B) Four consecutive runs of the same ladder obtained using $0.9\text{-}\mu\text{m}$ silica beads in a device that had been operated for 5 h ($E = 19.2 \text{ V cm}^{-1}$, separation length $L = 5 \text{ mm}$). (Reprinted from Zeng and Harrison [37], with permission of American Chemical Society, © 2007).

Song et al. [45] reported laser-patterned nanoporous membrane at the junction of a cross channel on a microfluidic chip. Upon application of voltage, linear electrophoretic concentration of charged proteins can be achieved at the membrane surface with a concentration increase of 2–4 orders of magnitude. Using phase-changing sacrificial layers, Kelley and co-workers [46] demonstrated miniaturization of electric field gradient focusing on microfluidic chips for protein preconcentration. Successful enrichment of fluorescently labeled peptides were realized with a >150-fold increase. With a similar method, a membrane-based protein preconcentration microchip was further fabricated, which allowed a >10,000-fold enrichment of protein samples.

Recently, Wang et al. [47] reported a microfluidic sample preconcentration device based on electrokinetic trapping. A flat nanofluidic filter was fabricated within a microchannel, functioning as an ion-selective membrane for electrokinetic trapping of charged biomolecules. As a result, the electrokinetic trapping of proteins can be maintained at the nanofluidic filter region for several hours with a concentration factor as high as 10^6 – 10^8 . A PDMS microfluidic chip was reported by Kim et al. [48] for the enrichment of proteins. A thin-walled PDMS section ($\sim 20\ \mu\text{m}$) was fabricated between two microchannels. Under electric field, preconcentration of negatively charged proteins was readily achieved on the anode side of the section with a 10^3 – 10^6 -fold increase.

Affinity and immunoaffinity based preconcentration methods have also been extensively used on microfluidic chips. Guzman [49] previously reported an improved solid-phase extraction (SPE) device for use in on-line immunoaffinity CE. A four-part cross-shaped configuration was designed with an analyte concentrator-microreactor at the intersection, which contained semipermeable membranes allowing the confinement of beads

or suitable microstructures. The molecular recognition adsorbing chemicals or affinity ligand materials on the surface of the beads enabled trapping, enrichment and elution of specific analyte from biological fluids or tissue samples. Lectin affinity chromatography was miniaturized onto a microfluidic chip by Mao et al. [50]. A lectin affinity monolith column was fabricated in the microchannel by UV-initiated polymerization of ethylene dimethacrylate and glycidyl methacrylate in the presence of porogenities, followed by immobilization of pisum sativum agglutinin on the monolith matrix. Separation of three glycoproteins, turkey ovalbumin, chicken ovalbumin and ovomucoid was then successfully demonstrated with enhanced separation performance and reduced separation time.

An integrated microfluidic device coupling SPE with micellar electrokinetic chromatography was reported by Ramsey and Collins [51], where automated sequence of extraction, elution, injection, separation and detection were performed in less than 5 min. Another coupling of SPE with microchip electrophoresis was demonstrated by Long et al. [52], where a nanoporous membrane sandwiched between two PDMS substrates allowed SPE preconcentration and electrophoretic separation in upper and lower microfluidic layer sequentially.

Olvecka et al. [53] reported protein separations by an on-line combination of zone electrophoresis and isotachopheresis (ITP) preconcentration on a PDMS column-coupling chip with integrated conductivity detection. Huang et al. [54] integrated ITP preconcentration with gel electrophoresis (GE) on a microfluidic chip to lower the detection limit of SDS-proteins by 40-fold. Lin et al. [55] integrated ITP and GE onto a plastic microchip, where 50 nL of samples were hydrodynamically or electrokinetically injected and enriched by ITP into narrow bands before introduced into a homogeneous GE channel for separation and detection.

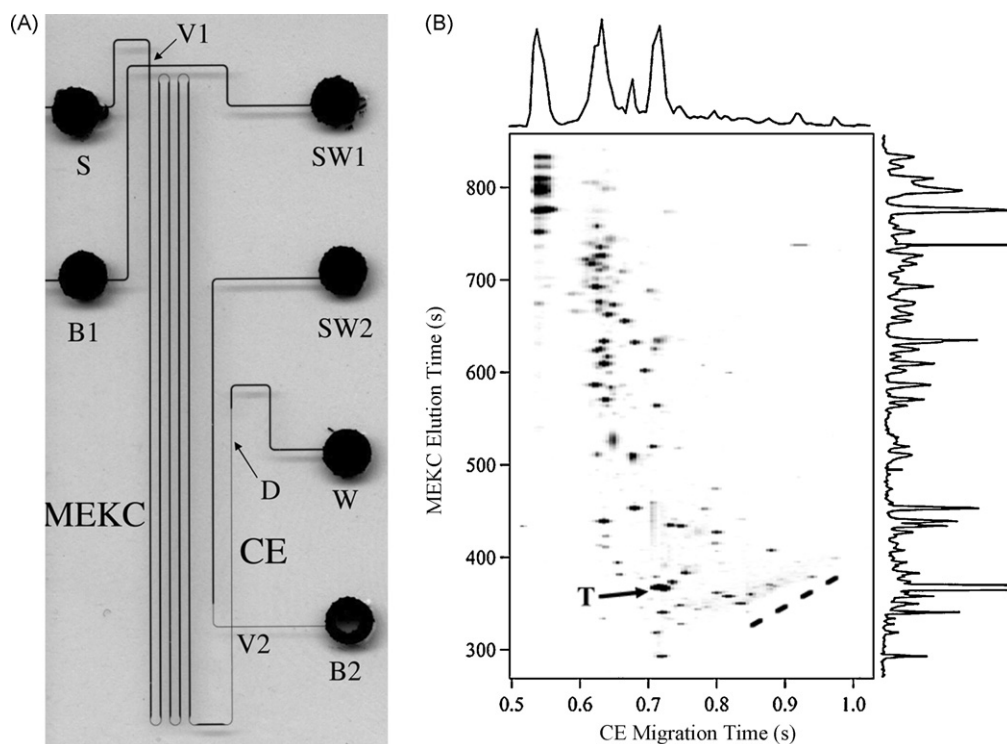


Fig. 3. (A) Image of a microchip with a serpentine channel for two-dimensional separations. Injections were made at valve 1 (V1) for the first-dimension MEKC separation and at valve 2 (V2) for the second-dimension CE separation. The sample was detected 1 cm downstream from V2 at point D using laser-induced fluorescence. The reservoirs are labeled sample (S), buffer 1 (B1), sample waste 1 (SW1), buffer 2 (B2), sample waste (SW2) and waste (W). The channels and reservoirs were filled with black ink for contrast. (B) Two-dimensional separation of a bovine serum albumin tryptic digest. The projections of the two-dimensional data onto each axis are shown. Spots marked with a "T" represent unreacted dye. The dotted line illustrates correlation in the separation mechanisms. (Reprinted from Ramsey et al. [81], with permission of American Chemical Society, © 2003).

2.2.2. On-chip separation

To study biology at the protein level, it is necessary to know the total number of proteins that are present in a given system before further evaluation of their dynamic interactions. Among many protein separation approaches, CE is one of the most commonly used methods. Micro-CE (μ CE) that takes advantages of system miniaturization has become a highly focused area since its first introduction by Manz and co-workers [56–59].

There are many different separation modes in CE, such as capillary zone electrophoresis (CZE) [60], micellar electrokinetic chromatography (MEKC) [61], capillary isoelectric focus (CIEF) [62], capillary gel electrophoresis (CGE) [63] and capillary electrochromatography (CEC) [64]. So far, most separation modes for conventional CE have been validated for μ CE.

Duffy et al. [65] reported a rapid prototyping method for microfluidic systems in PDMS. Using this method, a PDMS-based μ CE system was fabricated in less than 24 h. Separations of amino acids, charge ladders of positively and negatively charged proteins, and DNA fragments in aqueous solutions were realized with resolutions comparable to those obtained using fused silica capillaries. Hofmann et al. [66] reported the first miniaturization of CIEF on a glass microchip. After comparison of three common mobilization methods, electroosmotic flow (EOF)-driven mobilization was most suitable for CIEF miniaturization. Separation of a mixture of Cy5-labeled peptides revealed a maximum peak capacity of 30–40 within 30 s. The research group of Yager and co-workers reported electrochemical formation of pH gradients in microfluidic channel for CIEF in both static [67] and flowing [68] conditions. In a subsequent work [69], they applied this method to perform transverse

isoelectric focusing (IEF) in microfluidic channels for continuous concentration and fractionation of proteins. More examples for CIEF miniaturization [70–73] can be found in recent years. Throckmorton et al. [74] developed a microfabricated glass chip containing microfluidic channels filled with polymer monolith for reversed-phase electrochromatography. Separation of bioactive peptides and amino acids labeled with a fluorogenic dye was realized in 45 s with a plate number of 600,000, outperforming the capillary-based separations in both speed and efficiency.

In addition to one-dimensional CE, two-dimensional capillary electrophoresis (2D-CE) can also be achieved by coupling two separation modes orthogonally for enhanced separation resolution [75]. Good examples of 2D-CE were given by the research group of Dovichi and co-workers [75–78]. However, it usually took several hours to complete a two-dimensional (2D) electropherogram by 2D-CE. Besides, the interface of the two capillaries was relatively complex. Thus, system miniaturization using microfluidic technologies was then proposed for 2D-CE.

The research group of Ramsey and co-workers [79] earlier reported a microfluidic device coupling MEKC with high-speed open channel electrophoresis for automated 2D analysis of peptides. Second-dimension separation was performed by sampling the effluent from the first-dimension. 2D separation of tryptic peptides was then demonstrated with peak capacity of 500–1000 and enhanced separation resolution compared to either dimension alone. In a subsequent work [80], they further demonstrated 2D separation of β -casein tryptic digests by using open-channel electrochromatography as the first-dimension and capillary electrophoresis as the second. By using an optimized glass microchip

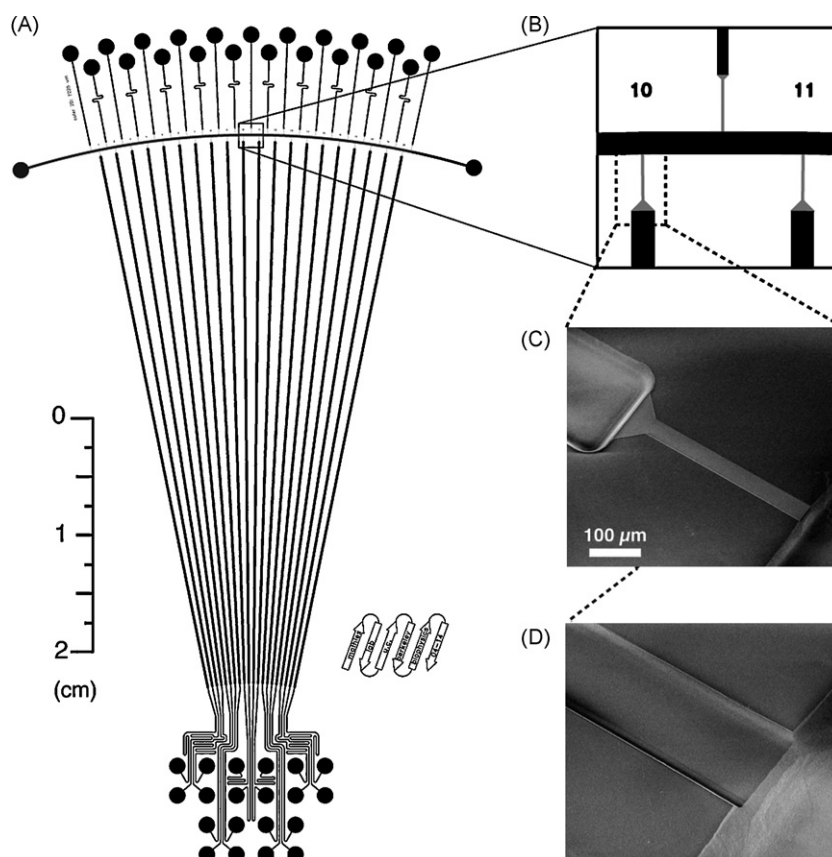


Fig. 4. Design of the microfluidic two-dimensional separation system. (A) The overall design comprises an arched, 3.75 cm long horizontal channel for first-dimension IEF that is punctuated with 20 6.8 cm long vertical channels through which focused proteins are separated in the second dimension by native gel electrophoresis. (B) The separation channels of the two dimensions are joined by much smaller channels forming a microfluidic interface that fluidically decouples the contents of the two separation dimensions, seen more clearly in electron micrographs C and D. The microfluidic interface channels are 25 μ m wide, 400 μ m long and 4 μ m deep. (Reprinted from Emrich et al. [84], with permission of American Chemical Society, © 2007).

with asymmetrically tapered turns in the serpentine channels, Ramsey et al. [81] enhanced the resolving power for MEKC-CZE 2D separation of tryptic digests. Results indicated a peak capacity of 4200 and an analysis time less than 15 min (Fig. 3).

Griebel et al. [82] reported a microfluidic 2D separation system employing a different separation mechanism combining IEF with GE. The microchip integrated a first channel for IEF and a second specially designed transfer channel, 300 parallel microchannels, each having a cross section of $50\text{ }\mu\text{m} \times 50\text{ }\mu\text{m}$, and buffer reservoirs. As a result, efficient separation of proteins can be achieved within 1.5 h. Li et al. [83] developed an integrated protein concentration/separation system, combining non-native IEF with SDS GE on a polycarbonate microfluidic chip. Comprehensive 2D protein separations were achieved in less than 10 min with an overall peak capacity of ~ 1700 . Another example of 2D IEF-GE separation was demonstrated by Emrich et al. [84] using a two-layer borosilicate glass microchip (Fig. 4), consisting of a single 3.75 cm long channel for the first-dimension IEF, which is sampled in parallel by 20 second-dimension channels for native GE. Reproducible 2D separations of both purified proteins and complex protein mixtures were realized with high repeatability.

Herr et al. [85] developed an acrylic microfluidic device that sequentially couples IEF with free solution capillary electrophoresis, resulting in efficient 2D separation with a peak capacity of ~ 1300 . A comprehensive 2D analysis of a fluid volume spanning 15% of the total IEF channel length was completed in less than 5 min. By using on-chip microvalves, Wang et al. [86] realized a 2D microseparation device coupling IEF with high ionic strength electrophoretic separation.

2.2.3. Single-cell proteomics

Single-cell proteomics allows the investigation of protein expression in individual cells, providing a valuable insight of that expression within a heterogeneous cellular population. Different methods have been reported for realizing single-cell proteomic studies. For example, the research group of Ewing and co-workers [87–90] demonstrated mapping of cellular and subcellular contents from individual cells by time-of-flight secondary ion mass spectrometry. Recently, microfluidic systems have drawn attentions of researchers as an attractive platform for single-cell proteomics [6,91], due to their compatible sizes with cells and potential for automation.

The research group of Ramsey and co-workers [92] demonstrated a microfluidic device, integrating cell handling, cell lysis, electrophoretic separation and fluorescent detection. The loaded

cells were hydrodynamically transported from the cell-containing reservoir to a region where they were focused and rapidly lysed using a pulse electric field. Cell lysis was completed within 33 ms. The total separation time was 2.2 s with absolute migration time reproducibility better than 1%. Wu et al. [93] reported an integrated microfluidic device, which allowed cell handling, drug delivery, cell lysis, chemical derivatization, separation and detection. By using three-state microvalves, a volume of $\sim 70\text{ pL}$ is achieved for the lysis and derivatization of the contents of a single Jurkat T cell. Electropherograms of amino acids from individual Jurkat T cells are recorded as a demonstration of this microfluidic platform. A microfluidic device was fabricated by Huang et al. [94], in which they can manipulate, lyse, label, separate and quantify the protein contents of a single cell using single-molecule fluorescence counting. Using this device, Huang et al. successfully quantified β_2 adrenergic receptors expressed in insect cells. They further demonstrated analysis of phycobiliprotein contents in individual cyanobacterial cells and observation of marked differences in the levels of specific complexes in cell populations that were grown under nitrogen-depleted conditions. More examples for on-chip single-cell proteomic analysis can be found recently. The research group of Fang and co-workers [95] used a simple cross-shaped microchannel design and electrical pulse for the manipulation and lysis of individual cells. Glutathione was used as a model intracellular component in single human erythrocyte cells detected after cell lysis. With a similar method, they further demonstrated on-chip cell lysis and simultaneous determination of released reactive oxygen species and reduced glutathione from individual human erythrocyte cells [96]. Ros et al. [97] fabricated micropost structures at the intersection of cross-shaped microchannels for the docking of single cells. After individual cells are manipulated into the docking area by optical tweezers, cells are lysed by an electrical pulse and the released protein contents are subsequently separated by electrophoresis and detected by laser induced fluorescence detection.

2.2.4. Mass spectrometry coupling

Mass spectrometry (MS) based methods have been extensively used for proteomic studies [7,98], such as peptide mapping, post-translational modification and protein–protein interactions. With the rapid progress of microfabrication technology, microfluidic chips have been coupled to MS analysis to address issues like speed, throughput and cost efficiency.

Coupling of μCE with electrospray ionization (ESI)-MS was demonstrated by Li et al. [99]. Disposable nano-electrospray emit-

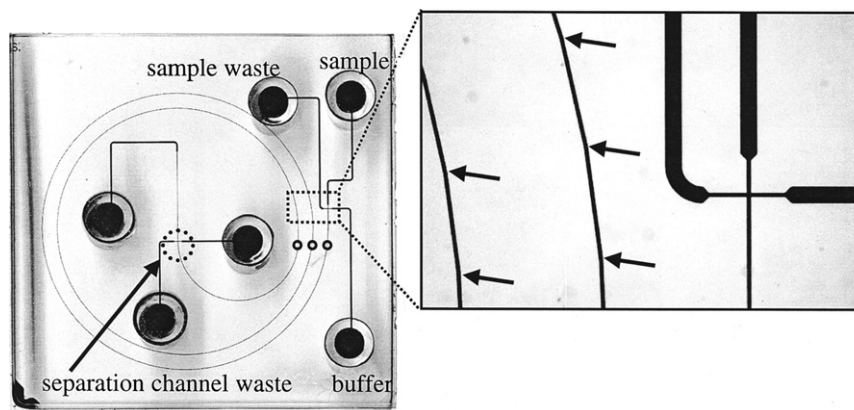


Fig. 5. Image of a microchip with a spiral separation channel. The reservoirs on the interior of the spiral were replaced by a single reservoir located at the injection cross (dashed circle). The small circles are the locations of the detection points: from outside to inside 0.25, 11.9 and 22.2 cm. The inset is a magnification of the injection cross and parts of the spiral channel. The buffer, sample, and sample waste channels are $219\text{ }\mu\text{m}$ wide (half-depth) for all of their length except for the last $500\text{ }\mu\text{m}$ before the injection cross where they narrow to only $40\text{ }\mu\text{m}$ (half-depth). The separation channel was 24.9 cm long and $40\text{ }\mu\text{m}$ wide (half-depth). The black arrows on the inset indicate the vertexes of the polygonally shaped channels. (Reprinted from Culbertson et al. [86], with permission of American Chemical Society, © 2000).

ters were fabricated and directly coupled to the microfluidic device as the sample introduction interface to MS. Identification of membrane proteins that comprise a subset of the *Haemophilus influenzae* proteome was demonstrated. In a subsequent work [100], an autosampler device and a separation channel array were further developed as updates to the previous system. As a result, a throughput up to 30 samples per hour and a maximum sample carryover of less than 3% was realized. By integrating an additional channel immobilized with affinity media on the microfluidic chip, enrichment of trace-level proteins prior to μ CE and MS analysis was successfully demonstrated [101].

Recently, Gustafsson et al. [102] developed a high-throughput microfluidic compact disk (CD) for protein preparation and matrix-assisted laser desorption/ionization (MALDI) mass spectrometry. This microfluidic CD was designed with an array of 96 identical microchannels, enabling parallel preparation of samples with volumes ranging from one to several microliters. Detection of proteolytic peptides down to the 50-amol level was demonstrated. Electrowetting-on-dielectric-based technique for digital microfluidics was used by Wheeler et al. [103] to perform inline sample purification for proteomic analysis with MALDI-MS. A PDMS microfluidic device was demonstrated by Dodge et al. [104], which combined on-line protein electrophoretic separation, selection and protein digestion for subsequent MALDI MS analysis. Liu et al. [105] reported a microfluidic enzymatic reactor that allowed rapid digestion of 16 fmol proteins for each MS analysis. Identification of cytoplasm of the human liver tissue was successfully demonstrated using this microfluidic reactor in combination with ESI-MS/MS.

2.3. Metabolomics

Metabolome is a highly dynamic system, where the level of each metabolite such as amino acids and hormones, are strictly dependent on the physiological conditions of the cell. Thus, quantitative

investigation of metabolome provides important information for systems-level understanding of biology.

Microfluidic chips have been extensively employed in amino acid analysis. Manz and co-workers pioneered the miniaturization of CE [56,57], where CZE was adopted as the separation mechanism, resulting in efficient separation of FITC-labeled amino acids. Culbertson et al. [106] fabricated a 25-cm-long spiral-shaped separation channel on a glass microchip (Fig. 5) for MEKC separation of amino acid. As a result, 19 tetramethylrhodamine-labeled amino acids were efficiently separated in 165 s with an average plate number of 280,000. Recently, Skelley et al. [107] developed the Mars Organic Analyzer, a μ CE system for sensitive amino acid biomarker analysis with a detection limit as low as 0.1 nM.

Hutt et al. [108] explored chiral separations of fluorescein isothiocyanate-labeled amino acids using μ CE. With a sodium dodecyl sulfate (SDS)/ β -cyclodextrin carbonate electrophoresis buffer (pH 10.0) and a separation voltage of 550 V cm^{-1} , baseline resolution was readily achieved for Val, Ala, Glu and Asp enantiomers and Gly in 4 min. Ro and Hahn [109] reported an integrated PDMS microchip for chiral separation of DL-amino acids based on diastereomerization. After diastereomerization in a microfabricated precolumn reactor, amino acid enantiomers were separated by MEKC without chiral selectors in the running buffer. Simultaneous separation of five chiral amino acids was successfully performed in a single run in less than 100 s.

In addition to amino acid analysis, microfluidic systems have also been applied to the investigation of other types of metabolites such as hormones. The research group of Kennedy and co-workers earlier [110] reported a microfluidic device for the determination of insulin secreted from islets of Langerhans by a capillary electrophoresis competitive immunoassay. In a subsequent work [111], they further developed a high-throughput microfluidic chip that allowed continuous monitoring of cellular secretions from multiple independent living samples in parallel. As a result, 1450 electrophoresis-based

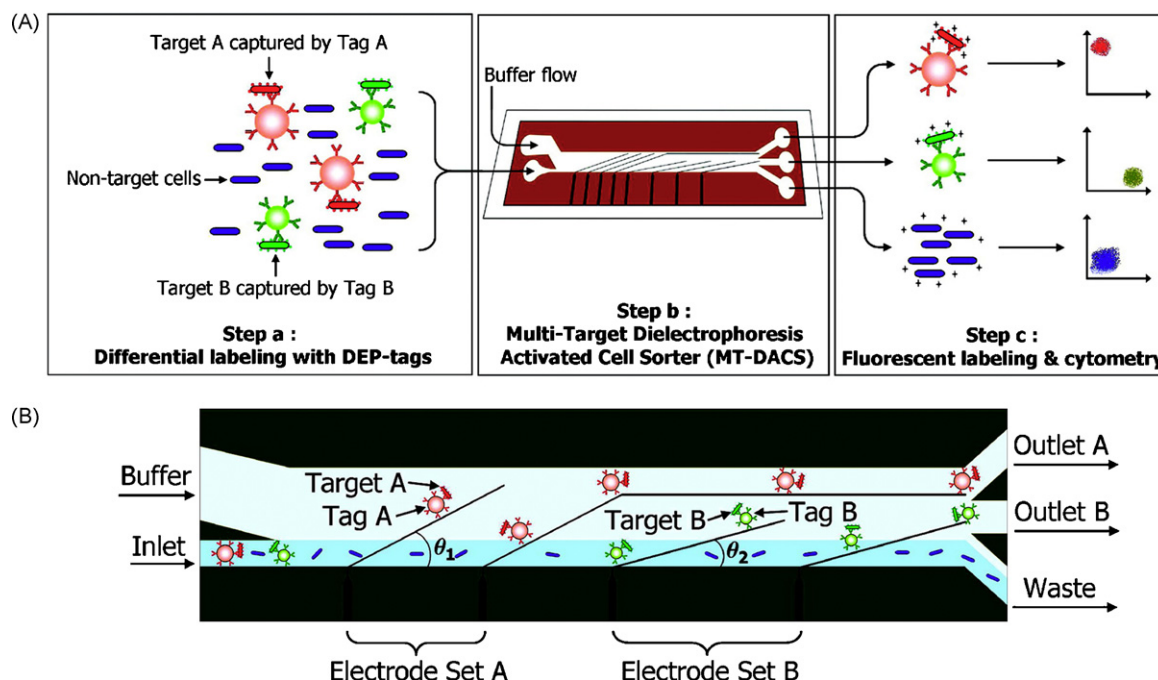


Fig. 6. Multiple bacterial cell sorting procedure using the multitarget DACS device. (A) The experimental scheme. Step a: target cells (target A and target B) are labeled with DEP tags (tag A and tag B, respectively) via their respective surface markers. Step b: cells are dielectrophoretically sorted and eluted through independent outlets. Step c: collected cells from the outlets are analyzed via flow cytometry to quantify the sorting performance. (B) The physics of multitarget separation via multitarget DACS. Two sets of electrodes are positioned at different glancing angles (θ_1 and θ_2) to select for two different targets. Target A cells labeled with tag A are selected at electrode set A ($\theta_1 = 10^\circ$) and elute through outlet A. Target B cells labeled with tag B are sorted at electrode set B ($\theta_2 = 8^\circ$) and elute through outlet B. The unlabeled, nontarget cells are not deflected by either electrode set, and they are eluted through the waste outlet. (Reprinted from Kim et al. [123], © 2008).

immunoassays of biological samples were achieved in less than 40 min.

Liu et al. [112] reported a PDMS microfluidic system that integrated μ CE with bioluminescence detection for ATP-conjugated metabolic analysis. As a result, assays of galactose in human urine samples were successfully investigated. Urbanski et al. [113] developed a microfluidic device that can perform microfluorometric enzymatic assays in an automated fashion. After loading of samples and enzyme cocktail, this microfluidic system allowed automated aliquotting, mixing of reagents, data acquisition and data analysis. Serial measurements of glucose, pyruvate and lactate in triplicate with submicroliter sample volumes were achieved within 5 min.

3. Analysis at cellular level

Bioanalysis at cellular level gives direct information of a single cell or a group of cells, which plays essential role in understanding each cellular element and their correlations within a complex biological system. Due to their compatible sizes with cells, microfluidic systems are ideal platforms for single or multiple cell manipulation and analysis in vitro.

3.1. Cell sorting

Cell sorting is a preliminary step for cellular level investigation of biology. Microfluidic chip based cell sorters that take advantages of system miniaturization have been widely studied in recent years. A large variety of active or passive sorting mechanisms have been employed for microfluidic cell sorters, such as electric, magnetic, hydrodynamic and optical mechanisms [114].

Electric sorting mechanism is the most popular method employed by microfluidic cell sorters, which have advantages

of flexibility, integration and potential for automation. Dittrich and Schuille [115] recently presented a microfluidic electrokinetic sorter that allows precise separation of fluorescent cells or particles in solution with respect to their different spectroscopic properties, such as brightness and color. Kang et al. [116] reported a fluorescence-activated particle counting and sorting system. Automatic sorting of fluorescent particles was achieved by electrokinetic switching triggered by a pre-set fluorescent threshold. Cell sorting based on electrokinetic switching is a common approach [115–118]. However, electrokinetic forces are relatively weak for the manipulation of large cells or particles. Recently, Yao et al. [119] combined gravity and electrokinetic force for flow cytometry and fluorescence-activated cell sorting. This system was applied to estimate the necrotic and apoptotic effects of ultraviolet light on HeLa cells.

Dielectrophoresis (DEP) is another common method [120–130], which has advantages of low-voltage operation and selectivity for driving cells by frequency. The research group of Soh and co-workers [122] developed a microfluidic DEP-activated cell sorter (DACS) for the isolation of rare cells by labeling cell with particles that differ in polarization response. When cell mixture entered the channel, the labeled cells were selectively deflected into the collecting stream by DEP. As a result, isolation of rare *E. coli* expressing a specific surface marker was achieved with an enrichment of more than 200 folds and a throughput of 10,000 cells per second. With a similar sorting mechanism, they further reported a microfluidic multitarget DACS [123] (Fig. 6). By specific labeling with unique synthetic DEP tags, multiple bacterial target cell types were efficiently separated in a single run with ~ 1000 -fold enrichment.

For cells that have native magnetic susceptibility, such as red blood cells and magnetotactic bacteria, microfluidic magnetic activated cell sorter (MACS) can be used for efficient separation

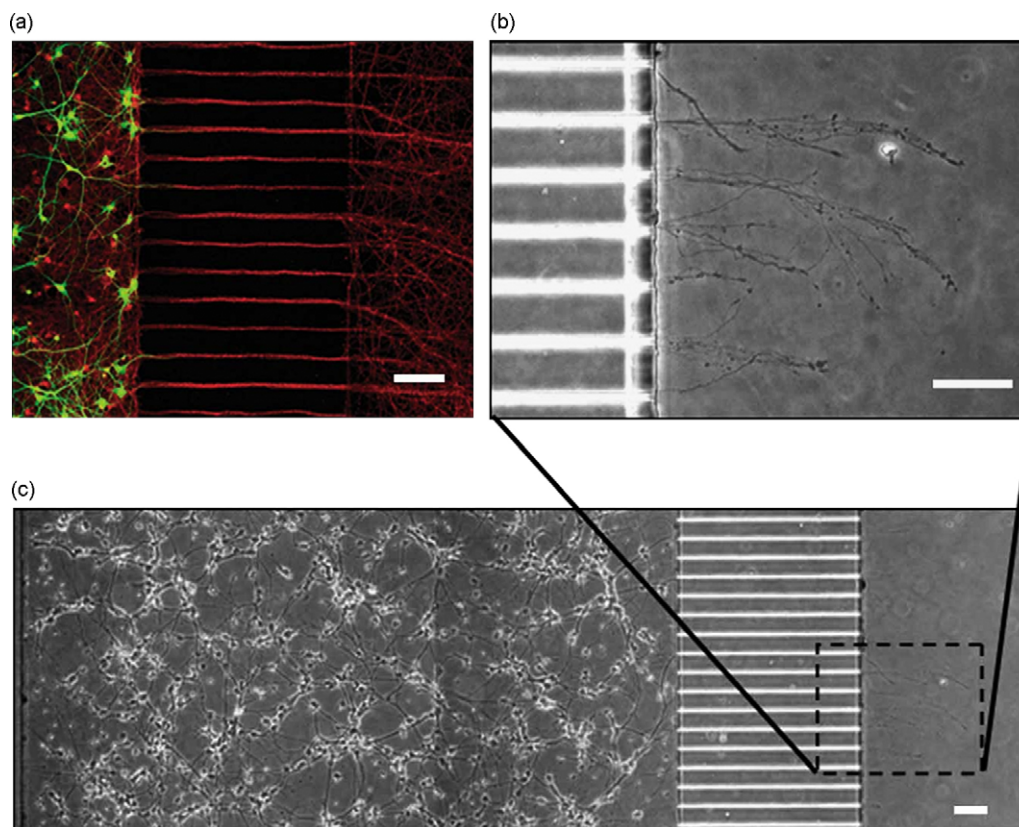


Fig. 7. Confocal micrograph of Tau (green) and MAP5 (red) immunostained neurons (a) and phase micrographs of six DIV neurons (b and c) cultured in the microfluidic device. Immunostain results indicate that all processes that cross the barrier are axons. Scale bars, 100 μ m. (Reprinted from Park et al. [177], with permission of Macmillan Publishers Ltd., © 2006). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

[131,132]. For nonmagnetic cells or particles, selective magnetic labeling can be used to introduce magnetism to the targets for MACS sorting [133–136]. Inglis et al. [134] recently reported a microfluidic device that can isolate cells selectively tagged with magnetic nanoparticles. When cells flow over an array of microfabricated magnetic stripes, a series of high magnetic field gradients trapped the magnetically labeled cells and altered their flow direction.

Hydrodynamic sorting is the most traditional method employed by microfluidic sorters. Generally, cell sorting was realized by active switching of flow directions upon detection of fluorescent signals from cells [137,138]. Recently, Chabert and Viovy [139] reported a high-throughput hydrodynamic cell sorting approach for the high-throughput encapsulation of single cells into picoliter droplets, and spontaneous self-sorting of these droplets. Successful encapsulation and sorting of 70–80% of the injected cell population was demonstrated. Separation of cancerous lymphocytes from a whole blood mixture was further realized with a >10,000-fold enrichment.

Due to its non-physical contact and minimal contamination during manipulation, optical sorting mechanisms such as optical tweezers and optical traps are another attractive type of method for microfluidic sorters [140–146]. Wang et al. [145] demonstrated optical force switching for the separation of fluorescent cells on microfluidic chips. With this method, live stably transfected HeLa cells expressing a fused histone-green fluorescent protein was isolated with an enrichment of 63–71 folds, an 85% recovery and a throughput of ~106 cells per second. Recently, other optical approaches such as optical waveguide [147–149] and optical lattice [150] have also been employed for particle sorting and cell manipulation on microfluidic chips.

In addition to the sorting mechanisms mentioned above, many other type of sorting methods have also been reported lately, such

as acoustic method [151–153], affinity-based selection [154–158], concurrent extraction [159,160], mechanical switching [161] and thermoreversible gelation valves [162].

3.2. Patterning and immobilization

Immobilization of cells is usually necessary before analysis can be effectively conducted on microfluidic chips. One commonly used method is cell patterning in microfluidic channels by soft lithographic techniques [163], such as microcontact printing, patterning using microfluidic channels, and laminar flow patterning.

The research group of Whitesides and co-workers [164] earlier reported cell patterning using multiple laminar fluid flows in microfluidic channels. This novel method provided a way to simultaneously control the surface properties for cell adhesion, the type of cells in vicinity and the media in contact with the cells. In a subsequent work [165], they further developed a three-dimensional microfluidic system for the patterning of proteins and immobilization of mammalian cells. Multiple types of proteins and cells in complex, discontinuous structures on a planar substrate were realized. Recently, Kaji et al. [166] demonstrated patterning of electrically conjugating cardiomyocytes by microcontact printing. Rhee et al. [167] described a plasma-based dry etching method for patterning cell culture inside microfluidic channels. Cell-adhesive and non-adhesive areas on glass and polystyrene substrates were patterned using plasma-based dry etching. The patterned substrates were then successfully used for selective attachment and culturing of human umbilical vein endothelial cells, MDA-MB-231 human breast cancer cells, NIH 3T3 mouse fibroblasts, and primary rat cortical neurons.

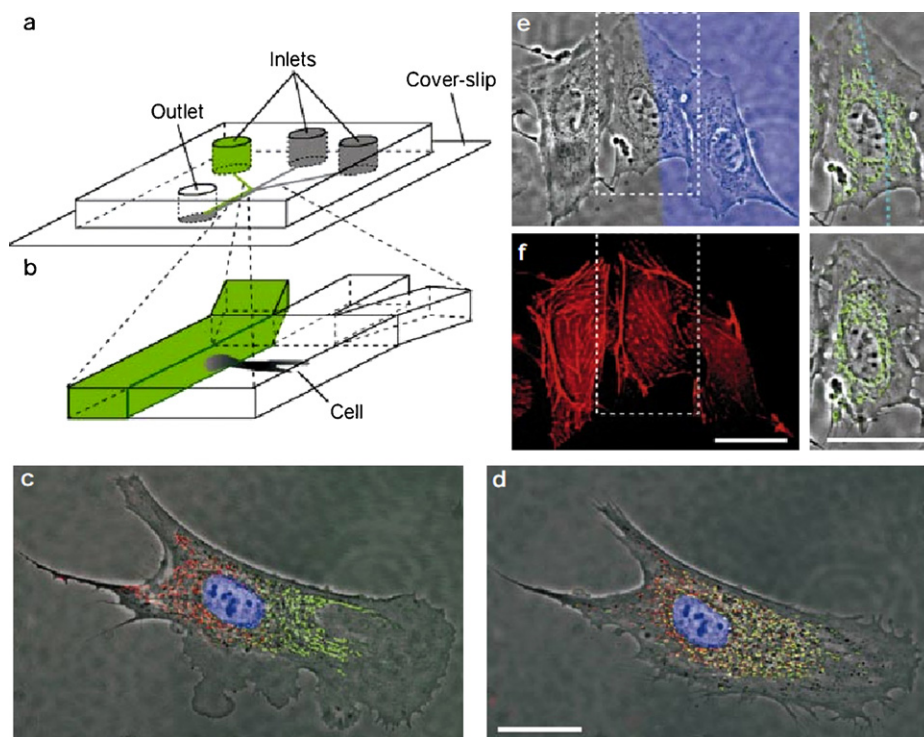


Fig. 8. Differential manipulation of regions of a single bovine capillary endothelial cell using multiple laminar flows. (a and b) Experimental set-up; (b) shows a close-up of the point at which the inlet channels combine into one main channel. (c) Fluorescence images of a single cell after treatment of its right pole with Mitotracker Green FM and its left pole with Mitotracker Red CM-H₂XROS. The entire cell is treated with the DNA-binding dye Hoechst 33342. (d) Image of the same cell as in c but taken 2.5 h later, showing intermixing of the red and green subpopulations of mitochondria. (e) Treatment of a portion of a single cell with latrunculin A. The blue region (Dextran-70,000 Cascade Blue stain) reveals the flow of medium containing latrunculin A. Right, enlarged view of the middle cell and its mitochondria (green). (f) Phalloidin-Alexa 594-labelled image of the same cell immediately after 10 min of treatment with latrunculin A. The cell in the middle, which was only partly in the stream containing latrunculin, shows disruption of actin microfilaments that is limited to this region (scale bars, 25 μ m). (Reprinted from Takayama et al. [178], with permission of Macmillan Publishers Ltd., © 2001). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Other cell immobilization methods have also been reported for microfluidic systems in recent years, such as cell docking [168,169], acoustic trap [170] and optical trap [171].

3.3. Cell culture and development

Stem cells differ from other types of cells due to their special ability to regenerate and to differentiate into multiple cell types. Chung et al. [172] reported a gradient-generating microfluidic platform for optimizing proliferation and differentiation of neural stem cells in culture. By exposing cells to a concentration gradient of growth factors under continuous flow, proliferation and differentiation of neural stem cells into astrocytes could be monitored by time-lapse microscopy and immunocytochemistry. A different method was reported by Park et al. [173], in which an osmosis-driven pump was used to generate a concentration gradient of fetal bovine serum for the culturing of human mesenchymal stem cells. Kim et al. [174] developed a microfluidic device for culturing adherent cells over a logarithmic range of flow rates. Using this device, they demonstrated culture of murine embryonic stem cells in continuous, logarithmically scaled perfusion for 4 days, with flow rates varying $>300\times$ across the array.

Neural development is another hot topic in cell biology. Martinioia et al. [175] earlier reported hydraulically driven deposition of adhesion molecules for the patterning of neurons on silicon micromachined substrates. Entrapment of chick embryo spinal cord neurons in microstructures and formation of patterned neural networks were achieved. Recently, Millet et al. [176] demonstrated culturing of postnatal primary hippocampal neurons from rat in PDMS microfluidic channels. Park et al. [177] reported the use of a microfluidic device to culture central nervous system (CNS) and peripheral nervous system (PNS) neurons for neuroscience applications. CNS and PNS neurons were cultured within the device for 2–3 weeks before further applications (Fig. 7), serving as an *in vitro* model for axonal injury and regeneration in the CNS.

3.4. Localized chemical treatment

After cells are immobilized in microfluidic channels, localized chemical stimulations can be realized using laminar fluid flows. Takayama et al. [178] reported the use of multiple laminar flows in a microfluidic channel to deliver membrane-permeable molecules to selected subcellular microdomains of immobilized cells (Fig. 8). This method was then successfully applied to study the subcellular processes of mitochondrial movement and changes in cytoskeleton structure. With a similar method, Lucchetta et al. [179] studied the robust network in *Drosophila melanogaster* that compensates for variation in the bicoid morphogen gradient. Results indicated that the compensation system counteracted the effects of extremely unnatural environmental conditions in which the anterior and pos-

terior halves of the embryo are developing at different temperatures and thus at different rates (Fig. 9). Sawano et al. [180] used laminar flows to study the lateral propagation of epidermal growth factor signaling in single live COS cells following localized chemical stimulation. Results suggested that ligand-independent propagation of epidermal growth factor signaling occurs only when the receptor density on the plasma membrane is high.

3.5. Patch clamp and electroporation

Patch clamp technique is heavily employed in electrophysiological studies of single or multiple ion channels on cell membrane. However, the conventional patch clamp method is labor intensive and time consuming. System miniaturization is then proposed for patch clamp to address such issues [181]. On-chip patch clamp has been realized using both bottom cell trapping [182–186] and lateral cell tapping [187–191] methods. Recently, Li et al. [185] reported a microfluidic system with disposable PDMS cell interface for simultaneous patch clamp recording. $2\ \mu\text{m}$ apertures were fabricated in PDMS, which was then reversibly sealed to a microfluidic system with microchannels, isolation valves and microfabricated Ag/AgCl electrodes. Gigaseal recordings from RBL-1 cells were successfully achieved with a 24% success rate. Ionescu-Zanetti et al. [188] developed a PDMS microchip that allowed patch clamp based on microfluidic junctions between a main chamber and lateral recording capillaries. Reliable whole-cell recording of activation of the voltage-sensitive potassium channel Kv2.1 in mammalian cells was demonstrated.

Electroporation is a common technique in cell biology for loading exogenous molecules such as fluorescent dye and DNA into target cells. In transition to microfluidic systems, electroporation has become a more versatile technique for single-cell analysis. On a microfluidic chip, polyelectrolytic salt bridges were explored by Kim et al. [192] for cell electroporation at low dc voltage. Wang et al. [193] fabricated elastomeric valves on a microchip for cell electroporation. Lu et al. [194] demonstrated an electroporation chip for cell lysis prior to subcellular analysis. Bao et al. [195] used electroporation for selective release of intracellular molecules at the single-cell level. Wang et al. [196] studied translocation of an enhanced green fluorescent protein tagged tyrosine kinase to the plasma membrane in B cells using microfluidic electroporative flow cytometry, which was further used by the same research group for investigating single-cell biomechanics [197].

3.6. Cell–cell signaling

Cell–cell signaling has also been investigated using microfluidic chip based methods. Klauke et al. [198] recently studied cell–cell signaling between longitudinally linked primary heart cells in a microfluidic system. By exploring the local ionic conditions that

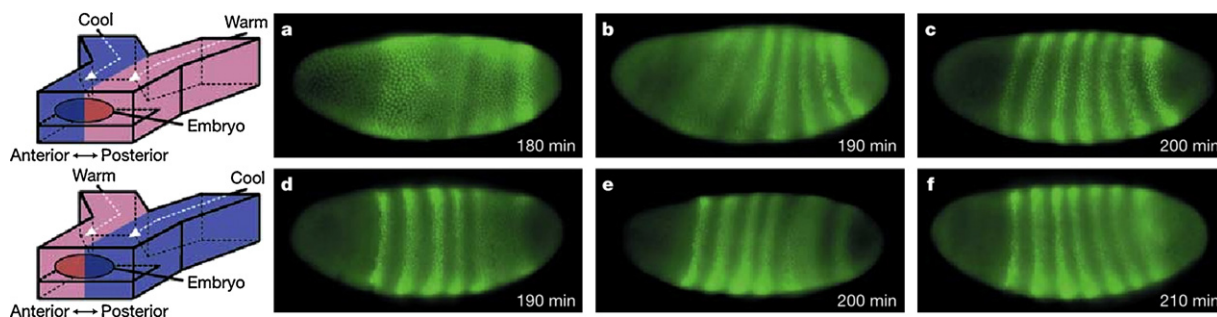


Fig. 9. Even-skipped expression in embryos exposed to a T-step of $20^\circ\text{C}/27^\circ\text{C}$. (a–c) Embryos with a cool anterior half and warm posterior half. (d–f) Embryos with a warm anterior half and cool posterior half. Even-skipped stripes were consistently expressed in the warm half first (a and b, d and e), but resolved in the correct positions (c and f). (Reprinted from Lucchetta et al. [179], with permission of Macmillan Publishers Ltd., © 2005).

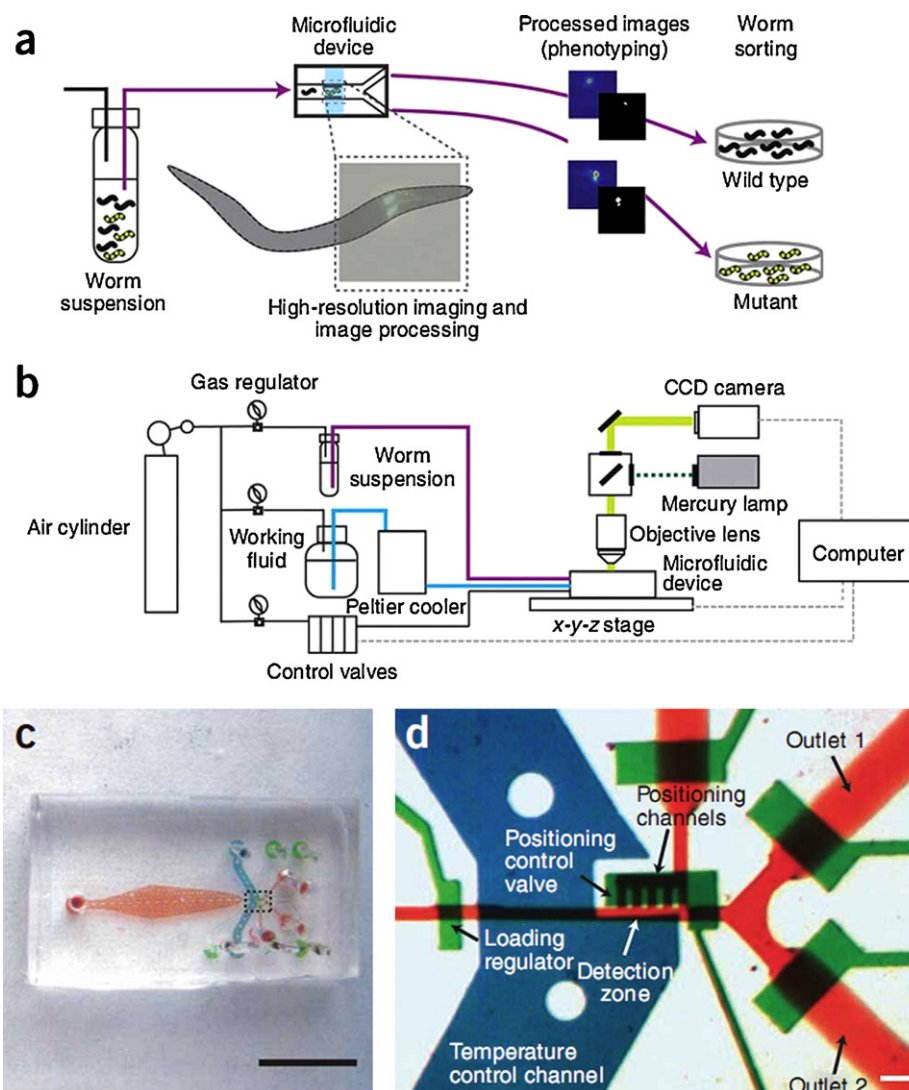


Fig. 10. Schematics of the system and the microchip. (a) A mixed population of worms is injected into the device, worms are imaged, phenotyped and then sorted. (b) System block diagram showing the on-chip and off-chip components. (c) Photograph of the microdevice. (d) Optical micrograph of the microchip's active region (boxed region in c). The channels were filled with dye to show specific features: blue, temperature control channel; green, valves; and red, sample-loading channel. Scale bars, 5 mm (c) and 100 μm (d). (Reprinted from Chung et al. [205], with permission of Macmillan Publishers Ltd., © 2008). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

enabled the propagation of Ca^{2+} waves between two heart cells, they discovered that the ability of intracellular Ca^{2+} waves to traverse the intercalated discs is dependent on the relative concentrations of diastolic Ca^{2+} in the two adjacent cells. Wei et al. [199] fabricated a microfluidic coculture system for investigating cell–cell interaction in distance. In their work, substances released from the up-stream macrophages upon activation generated a linear concentration gradient in down-stream wells by a microfluidic mixing system. Cellular responses and viability of the down-stream osteoblasts were subsequently evaluated. Another microfluidic coculture system was reported by Song et al. [200] for distant cell–cell interaction studies, in which the effect of human embryonic germ (hEG) cells on SKOV3 cells was investigated. By unidirectional perfusion from hEG to SKOV3 cells, the growth inhibition of SKOV3 cells was observed and the apoptosis signals in SKOV3 culture area decreased along the flow of the medium.

4. Analysis at organism level

The nematode *C. elegans* is an excellent animal model, which allows biologist to study the development of individual cells or

a group of cells within a complex biological system. *C. elegans* is a small (about 1 mm long as an adult) free living round worm with bilaterally symmetry. The anatomy of *C. elegans* is invariant from one another. The standard lab strain *C. elegans* is propagated by self-fertilization. Thus, individual worms are considered to be genetically identical. In addition, *C. elegans* has a life cycle as short as a few days. Conventional methods for studying *C. elegans* are usually time-consuming and labor-intensive. Recently, efforts have been made in microfluidic systems to enable automated high-throughput analysis of *C. elegans*.

Immobilization of *C. elegans* is usually a prerequisite step in such studies. Different methods have been reported for the immobilization of *C. elegans* on microfluidic chips, including the use of tapered microfluidic channels [201–203], lateral channel suction [204,205] and microfluidic valves [206,207]. With proper immobilization, subsequent analysis of *C. elegans* can be achieved. Rohde et al. [204] realized high-throughput whole-animal sorting with lateral channel suction. Chung et al. [205] demonstrated an automated microfluidic device for rapid on-chip sample handling, high-resolution microscopy, phenotyping and whole-animal sorting (Fig. 10). Zeng et al. [206] used microfluidic valves to immo-

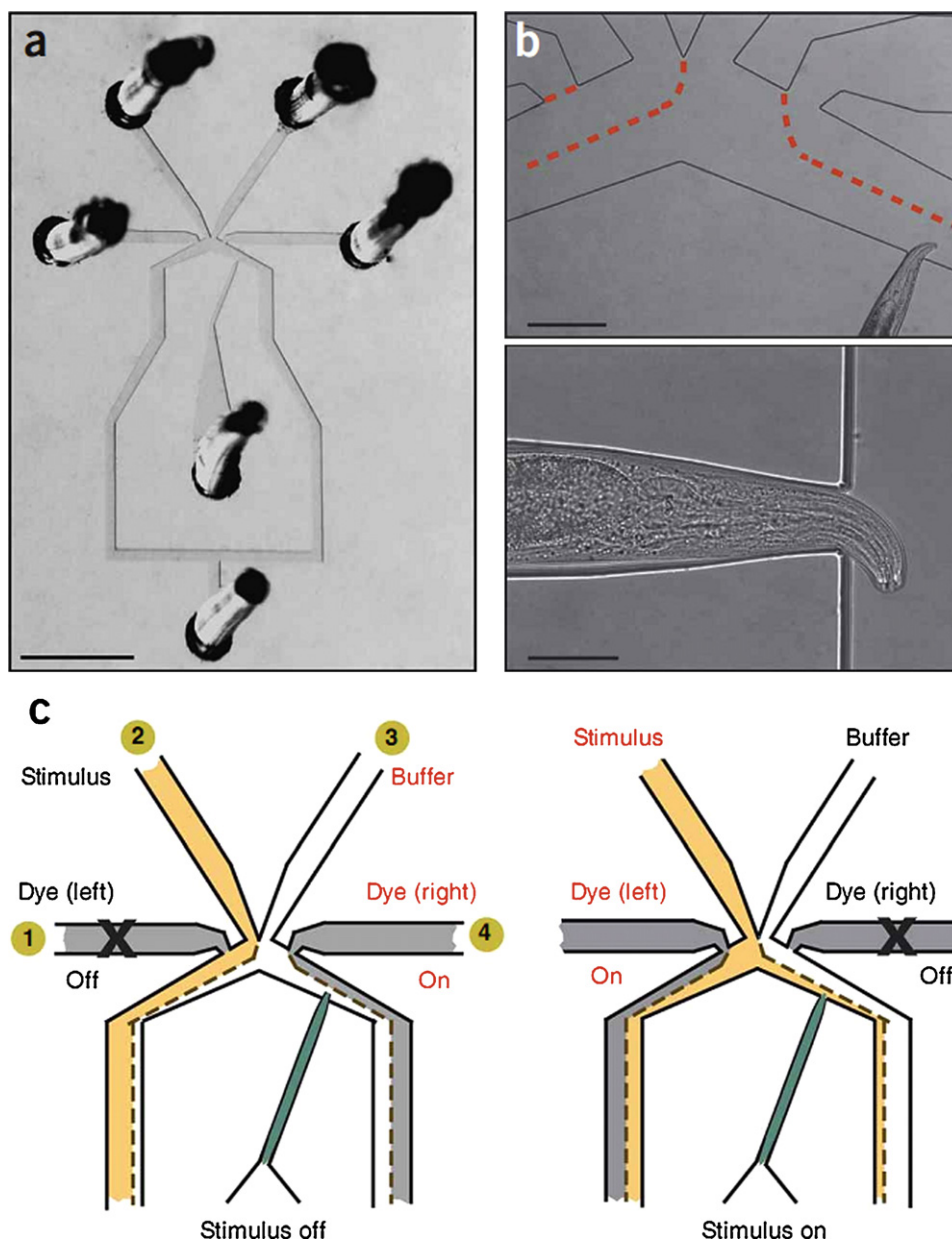


Fig. 11. (a) The microfluidic four-flow device for delivering odor. The microfluidic chip consists of two side channels loaded with fluorescent dye (channels 1 and 4), a stimulus channel (channel 2) and a control, or buffer, channel (channel 3). Scale bar, 2 mm. (b) Higher-magnification photographs of the chip with a trapped worm. The dotted lines mark the interfaces between the fluids. Scale bars, 150 μm (top) and 30 μm (bottom). As shown, the nose is exposed to the buffer stream. (c) The stimulus and buffer streams are directed by the side flows (gray). In the 'off' state (left), the right dye channel (channel 4) is open and pushes the stimulus stream to exit from the left outlet. In the 'on' state (right), the left dye channel (channel 1) is open, and the worm is exposed to stimulus. (Reprinted from Chronis et al. [202], with permission of Macmillan Publishers Ltd., © 2007).

bilize *C. elegans* for observation and manipulation of sub-cellular features using two-photon microscopy and femto-second-laser microsurgery. Imaging of neuronal and behavioral activity in immobilized *C. elegans* was realized by Chronis et al. [202] on microfluidic chips (Fig. 11). With microfluidic valve based immobilization, Guo et al. realized femto-second-laser nanoaxotomy for in vivo nerve regeneration studies of *C. elegans*.

Recently, Heng et al. [208] developed on-chip optofluidic microscopy for high-resolution optical imaging of *C. elegans*. Microfluidic shadow imaging system for studying the behaviors of *C. elegans* was developed by Lange and co-workers [209]. Qin and Wheeler [210] fabricated T-shaped and U-shaped microfluidic mazes for exploring learning of *C. elegans*. An automated microfluidic CD cultivation system was developed by Kim et al. [211,212],

which was further used in studying signal transduction induced by hypergravity in *C. elegans* [212].

5. Conclusions

Systems-level understanding of biology requires massive information of individual components and their correlations within complex biological systems. Microfluidic chip based method certainly plays as one of essential roles in such demanding investigations, revolutionizing the means for biological researches due to advantages such as high-throughput, small sample consumption and reduced analysis time. But it must be noted that there are also disadvantages due to system miniaturization. For exam-

ple, reduced sample consumption can result in the requirement for highly sensitive detection schemes due to reduced detection volumes. Micropumps are usually employed for sample injection, which are difficult to be integrated on microfluidic chips, thus complicating the analysis system. But without doubt, microfluidic technology holds high promise to facilitate biological researches in generating enormous amount of information for systems-level understanding of biology. In this review, we documented the recent applications of microfluidic chips in biological researches. At the molecular level, microfluidic chip based methods have been extensively employed for the analysis of DNA, proteins and metabolites. Miniaturization of CE has been a main focus in such investigations. The 384-channel radial μ CAE chip is one good example, which achieves a 20-fold increase in sequencing performance over the conventional 96-capillary instrumentation. At the cellular level, a wide range of applications of microfluidic chips have been reported, which include cell sorting, cell patterning and immobilization, cell culture and development, localized chemical treatment, patch clamp, electroporation and cell–cell signaling. Microfluidic applications at the organism level are just emerging, which have been mainly focused on the model animal, *C. elegans*. More applications of microfluidic chips at the organism level can be expected in near future.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.aca.2009.04.051.

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Microfluidic chip: Next-generation platform for systems biology

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Abstract

Systems biology advocates the understanding of biology at the systems-level, which requires massive information of correlations among individual components in complex biological systems. Such comprehensive investigation entails the use of high-throughput analytical tools. Microfluidic technology holds high promise to facilitate the progress of biology by enabling miniaturization and upgrading of current biological research tools due to its advantages such as low sample consumption, reduced analysis time, high-throughput and compatible sizes with most biological samples. In this article, we documented the recent applications of microfluidic chips in biological researches at the molecular level, cellular level and organism level, serving the purpose for systems-level understanding of biology.

Keywords: microfluidic chip; systems biology; omics; cell; *C. elegans*

1. Introduction

Since large-scale sequencing projects yielded complete genomes for a number of species, such as *Escherichia coli* (*E. coli*), *Caenorhabditis elegans* (*C. elegans*) and human, biological science has entered the post genomic era. Tremendous information at the molecular level has been generated up to date. However, little is known about biology at the systems-level. 

Currently, systems biology holds a high promise for fulfilling such a challenging goal [1-7], which seeks to develop a complete understanding of complex biological systems by both qualitatively and quantitatively studying the interactions and correlations among individual elements. Such a complex system can be a molecular network, cell, tissue, organ, or even entire organism. Instead of reduction methods advocated by molecular biology, systems biology requires a shift in our philosophy to integration of dynamic components for systematic interpretation of biology.

Due to the complexity of biological systems, systems-level understanding of biology requires the use of high-throughput and accurate analytical tools in order to gather massive amount of information, goals that are perhaps beyond the scope of current experimental practices [5]. Thus, technical innovations in experimental devices are an urgent task in systems biology. So far, many novel technologies have been developed for fulfilling such demanding requirements of systems biology, such as microfluidic systems, nanotechnology and femto-second technology. 


The advent of microfluidic systems [8] has revolutionized the methodology for biological researches. Since microfluidic systems have compatible sizes with most biomacromolecules, cells and small organisms, microfluidic technologies have then been proposed for the miniaturization of existing biological tools to address issues like speed, throughput, and sample cost. For example, miniaturization of capillary array electrophoresis significantly accelerated the progress of large-scale sequencing projects. Microfluidic cell sorters not only speed up the separation and measurements of live cells, but also encourage automation for high-throughput analysis at the cellular level.

Applications of microfluidic chips in biology are growing fast. In this article, the recent applications of microfluidic chips in biological researches at the molecular, cellular and organism levels were overviewed from the perspective of systems biology. As the next-generation platform, microfluidic chips will certainly open up new avenues for high-throughput biological analysis, facilitating the understanding of biology at the systems-level.

2. Analysis at molecular level

2.1. Genomics

Since the discovery of DNA double-helix structure by Watson and Crick [9], molecular level understanding of biology started emerging. The well-known human genome project resulted in successful discovery of ~32,000 human genes, which allowed further understanding of functional genomics at systems-level. Microfluidic chip based methods certainly play an important role in high-throughput genomic studies.

2.1.1. Sequence analysis

Capillary array electrophoresis (CAE) [10-12] has been the golden standard for genome sequencing purposes, where multiple capillaries are used in parallel for high-throughput sequencing of target DNAs. With the development of microfabrication technology, enhanced speed and parallelism of electrophoretic analysis can be readily achieved by system miniaturization. Microfabricated CAE device (μ CAE) was first introduced by Woolley and Mathies [13, 14] for DNA sequencing using microfluidic channel arrays. With a four-color labeling and detection system, 97% accuracy and single-base resolution to ~ 150 bases was achieved in 540 seconds [14]. In a subsequent work [15], 12 separation channels were integrated on a CAE chip, which allowed parallel analysis of 12 samples in less than 160 seconds. To further enhance the separation speed and throughput of the on-chip CAE assay, the separation channel density was significantly increased [16-19]. Liu and co-workers [18] reported automated DNA sequencing in 16-channel microchips with an integrated four-color confocal fluorescent detector, which yielded more than 450 bases in 15 min with a 99% accuracy. Simpson et al. [19] realized integration of 48 separation channels with an injection unit that permitted the serial analysis of two different samples on each capillary. In consequence, 96 samples can be efficiently analyzed by this CAE microchip in less than 8 min. A 96-channel radial capillary array was subsequently developed by the research group of Mathies detected by a rotary confocal fluorescence scanner [20-22], resulting in a rapid sequencing rate of 1.7 kilobase pairs (kbp) per minute. They further pushed the limits of channel density for high-throughput DNA sequencing by creating the 384-channel radial CAE device [23] (Figure 1). A 20-fold increase in sequencing performance was readily accomplished over the conventional 96-capillary instrumentation.

In addition to μ CAE, many other miniaturized systems have also been reported. A disposable plastic electrophoresis system was demonstrated earlier by Shi and Anderson for high-resolution single-stranded DNA analysis [24]. Plastic microchannels with a 4.5 cm effective separation length were fabricated from an etched glass master. Four-color separation of a 320-base terminator sequencing standard was achieved in 13 min with an accuracy of 99.1%. Kartalov and Quake [25] reported the a fully integrated PDMS (polydimethylsiloxane) microfluidic system for DNA sequencing-by-synthesis, using a heterogeneous assay that combined active plumbing, specific surface chemistry and parallelism. Reliable sequencing of up to 4 consecutive bps was demonstrated. Pyrosequencing is a nonelectrophoretic bioluminometric DNA sequencing method that uses a four-enzyme mixture reaction to monitor nucleotide incorporation in real time. Russom and co-workers [26] presented a nanoliter-volume microfluidic platform for the pyrosequencing of DNAs by trapping the DNA on microbeads in an on-chip filter chamber and monitoring flow-through of the pyrosequencing reagents at the same time. Applications of miniaturized systems for DNA sequencing are still growing, several reviews [27-32] on this fast progressing field have also been reported lately.

2.1.2. PCR and other applications

Besides DNA sequencing, other applications of microfluidic chips include DNA separation, analysis and Polymerase Chain Reaction (PCR). Riehn and co-workers [33] reported restriction mapping of DNA molecules in 100-200 nm channels. With the restriction enzymes SmaI, SacI, and PacI, the position of restriction sites was successfully measured with a precision of ~ 1.5 kbp in 1 min using single DNA molecules. Heule and Manz [34] demonstrated a passive mixing microsystem for sequential DNA hybridization assays. By injecting the sample solutions one after

the other and mixing them rapidly on the chip, the kinetics of hybridization can be investigated. Using this method, sequence dependent signals at single nucleotide polymorphisms (SNP) level were successfully recorded. Wang et al. [35] developed a microfluidic chip capable of quantitative detection of low-abundance nucleic acids by incorporating confocal fluorescence spectroscopy, molecular beacons and a molecular-confinement microfluidic reactor. Kaji and co-workers [36] developed a nanofabrication technique for constructing high aspect ratio nanopillars as DNA sieving matrixes for the separation of large DNA fragments in microchannels. With the optimal chip design, DNA fragments ranging from 1 to 38 kbp were successfully separated as clear bands. Separation of λ DNA and T4 DNA has also been demonstrated using a 380 μm long nanopillar channel within 10 seconds. Zeng and Harrison [37] reported self-assembled colloidal arrays as three dimensional nanofluidic sieves for the separation of double-stranded DNA fragments between 50 bp to 50 kbp (Figure 2). Li et al. [38] constructed gold nanopatterns on silicon surface for the electrophoretic separation of linear, relaxed and supercoiled DNAs based on different dielectrophoretic forces between DNA segments and gold nanopatterns.

Lagally and co-workers [39] earlier demonstrated an integrated microfluidic device, combining submicroliter PCR chambers with a microfabricated capillary electrophoresis (CE) system for the stochastic PCR amplification of single DNA template molecules and subsequent CE analysis. Recently, Wang and co-workers [40] reported a polycarbonate-based spiral continuous flow thermal cycler microchip for sanger cycle sequencing using terminator chemistry. A polycarbonate microchip was fabricated by Chen et al. [41] for the PCR amplification of DNA templates using an electrokinetically driven synchronized continuous flow PCR configuration. A bidirectional flow DNA amplification microreactor was reported by Chen and co-workers [42] for ultrasensitive PCR and real-time detection of human genomic samples. An RNase P gene detection limit of 24 human genome copies with a reaction efficiency of 77% was achieved. Marcus et al. [43] demonstrated a microfluidic device for parallel reverse transcriptase-PCR (RT-PCR) assays. With integrated micromechanical valves, this microchip was able to perform 72 450 pL RT-PCRs in parallel. Ottesen et al. [44] reported a microfluidic digital PCR platform with 12 panels each containing 1176 PCR chambers for the multigene analysis of single bacterial cells harvested from nature. As a result, previously unknown ribosomal RNA-based species identity of several symbionts was successfully discovered.

2.2. Proteomics

Proteome is directly derived from the genome and it in turn regulates both the gene expression and cellular metabolism. Thus, quantitative investigation of the proteome is essential for understanding biology at the systems-level. Here, we discuss the recent applications of microfluidic chips in proteomics with aspect of preconcentration, on-chip separation, single-cell proteomics and mass spectrometry coupling.

2.2.1. Preconcentration

Preparation of proteins is usually a prerequisite step in proteomic studies. When working with natural low-abundance proteins, preconcentration is necessary to bring target proteins into detectable range. So far, various methods have been reported for protein preconcentration on microfluidic chips.



Song and co-workers [45] reported laser-patterned nanoporous membrane at the junction of a cross channel on a microfluidic chip. Upon application of voltage, linear electrophoretic concentration of charged proteins can be achieved at the membrane surface with a concentration increase of 2-4 orders of magnitude. Using phase-changing sacrificial layers, Kelley and co-workers [46] demonstrated miniaturization of electric field gradient focusing on microfluidic chips for protein preconcentration. Successful enrichment of fluorescently labeled peptides were realized with a >150-fold increase. With a similar method, a membrane-based protein preconcentration microchip was further fabricated, which allowed a >10,000-fold enrichment of protein samples.

Recently, Wang et al. [47] reported a microfluidic sample preconcentration device based on electrokinetic trapping. A flat nanofluidic filter was fabricated within a microchannel, functioning as an ion-selective membrane for electrokinetic trapping of charged biomolecules. As a result, the electrokinetic trapping of proteins can be maintained at the nanofluidic filter region for several hours with a concentration factor as high as 10^6 - 10^8 . A PDMS microfluidic chip was reported by Kim and co-workers [48] for the enrichment of proteins. A thin-walled PDMS section ($\sim 20\ \mu\text{m}$) was fabricated between two microchannels. Under electric field, preconcentration of negatively charged proteins was readily achieved on the anode side of the section with a 10^3 - 10^6 -fold increase.

Affinity and immunoaffinity based preconcentration methods have also been extensively used on microfluidic chips. Guzman [49] previously reported an improved solid-phase extraction (SPE) device for use in on-line immunoaffinity CE. A four-part cross-shaped configuration was designed with an analyte concentrator-microreactor at the intersection, which contained semipermeable membranes allowing the confinement of beads or suitable microstructures. The molecular recognition adsorbing chemicals or affinity ligand materials on the surface of the beads enabled trapping, enrichment and elution of specific analyte from biological fluids or tissue samples. Lectin affinity chromatography was miniaturized onto a microfluidic chip by Mao and co-workers [50]. A lectin affinity monolith column was fabricated in the microchannel by UV-initiated polymerization of ethylene dimethacrylate and glycidyl methacrylate in the presence of porogenities, followed by immobilization of pisum sativum agglutinin on the monolith matrix. Separation of three glycoproteins, turkey ovalbumin, chicken ovalbumin and ovomucoid was then successfully demonstrated with enhanced separation performance and reduced separation time.

An integrated microfluidic device coupling SPE with micellar electrokinetic chromatography was reported by Ramsey et al. [51], where automated sequence of extraction, elution, injection, separation, and detection were performed in less than 5 min. Another coupling of SPE with microchip electrophoresis was demonstrated by Long et al. [52], where a nanoporous membrane sandwiched between two PDMS substrates allowed SPE preconcentration and electrophoretic separation in upper and lower microfluidic layer sequentially.

Olvecka and co-workers [53] reported protein separations by an on-line combination of zone electrophoresis and isotachopheresis (ITP) preconcentration on a PDMS column-coupling chip with integrated conductivity detection. Huang et al. [54] integrated ITP preconcentration with gel electrophoresis (GE) on a microfluidic chip to lower the detection limit of SDS-proteins by 40-fold. Lin et al. [55] integrated ITP and GE onto a plastic microchip, where 50 nL of samples

were hydrodynamically or electrokinetically injected and enriched by ITP into narrow bands before introduced into a homogeneous GE channel for separation and detection.

2.2.2. On-chip separation

To study biology at the protein level, it is necessary to know the total number of proteins that are present in a given system before further evaluation of their dynamic interactions. Among many protein separation approaches, CE is one of the most commonly used methods. Micro-CE (μ CE) that takes advantages of system miniaturization has become a highly focused area since its first introduction by Manz and co-workers [56-59].

There are many different separation modes in CE, such as capillary zone electrophoresis (CZE) [60], micellar electrokinetic chromatography (MEKC) [61], capillary isoelectric focus (CIEF) [62], capillary gel electrophoresis (CGE) [63], and capillary electrochromatography (CEC) [64]. So far, most separation modes for conventional CE have been validated for μ CE.

Duffy and co-workers [65] reported a rapid prototyping method for microfluidic systems in PDMS. Using this method, a PDMS-based μ CE system was fabricated in less than 24 hours. Separations of amino acids, charge ladders of positively and negatively charged proteins, and DNA fragments in aqueous solutions were realized with resolutions comparable to those obtained using fused silica capillaries. Hofmann and co-workers [66] reported the first miniaturization of CIEF on a glass microchip. After comparison of three common mobilization methods, electroosmotic flow (EOF)-driven mobilization was found to be most suitable for CIEF miniaturization. Separation of a mixture of Cy5-labeled peptides revealed a maximum peak capacity of 30-40 within 30 seconds. The research group of Yager reported electrochemical formation of pH gradients in microfluidic channel for CIEF in both static [67] and flowing [68] conditions. In a subsequent work [69], they applied this method to perform transverse isoelectric focusing (IEF) in microfluidic channels for continuous concentration and fractionation of proteins. More examples for CIEF miniaturization [70-73] can be found in recent years. Throckmorton et al. [74] developed a microfabricated glass chip containing microfluidic channels filled with polymer monolith for reversed-phase electrochromatography. Separation of bioactive peptides and amino acids labeled with a fluorogenic dye was realized in 45 s with a plate number of 600,000, outperforming the capillary-based separations in both speed and efficiency.

In addition to one-dimensional CE, two-dimensional capillary electrophoresis (2D-CE) can also be achieved by coupling two separation modes orthogonally for enhanced separation resolution [75]. Good examples of 2D-CE were given by the research group of Dovichi [75-78]. However, it usually took several hours to complete a two-dimensional (2D) electropherogram by 2D-CE. Besides, the interface of the two capillaries was relatively complex. Thus, system miniaturization using microfluidic technologies was then proposed for 2D-CE.

The research group of Ramsey [79] earlier reported a microfluidic device coupling MEKC with high-speed open channel electrophoresis for automated 2D analysis of peptides. Second-dimension separation was performed by sampling the effluent from the first dimension. 2D Separation of tryptic peptides was then demonstrated with peak capacity of 500-1000 and enhanced separation resolution compared to either dimension alone. In a subsequent work [80], they further demonstrated 2D separation of β -casein tryptic digests by using open-channel

electrochromatography as the first dimension and capillary electrophoresis as the second. By using an optimized glass microchip with asymmetrically tapered turns in the serpentine channels, Ramsey and co-workers [81] enhanced the resolving power for MEKC-CZE 2D separation of tryptic digests. Results indicated a peak capacity of 4200 and an analysis time less than 15 minutes (Figure 3).

Griebel et al. [82] reported a microfluidic 2D separation system employing a different separation mechanism combining IEF with GE. The microchip integrated a first channel for IEF and a second specially designed transfer channel, 300 parallel microchannels, each having a cross section of $50\ \mu\text{m} \times 50\ \mu\text{m}$, and buffer reservoirs. As a result, efficient separation of proteins can be achieved within 1.5 hours. Li et al. [83] developed an integrated protein concentration/separation system, combining non-native IEF with SDS GE on a polycarbonate microfluidic chip. Comprehensive 2D protein separations were achieved in less than 10 min with an overall peak capacity of ~ 1700 . Another example of 2D IEF-GE separation was demonstrated by Emrich and co-workers [84] using a two-layer borosilicate glass microchip (Figure 4), consisting of a single 3.75 cm long channel for the first dimension IEF, which is sampled in parallel by 20 second-dimension channels for native GE. Reproducible 2D separations of both purified proteins and complex protein mixtures were realized with high repeatability.

Herr and co-workers [85] developed an acrylic microfluidic device that sequentially couples IEF with free solution capillary electrophoresis, resulting in efficient 2D separation with a peak capacity of ~ 1300 . A comprehensive 2D analysis of a fluid volume spanning 15% of the total IEF channel length was completed in less than 5 min. By using on-chip microvalves, Wang et al. [86] realized a 2D microseparation device coupling IEF with high ionic strength electrophoretic separation.

2.2.3. Single-cell proteomics

Single cell proteomics allows the investigation of protein expression in individual cells, providing a valuable insight of that expression within a heterogeneous cellular population. So far, different methods have been reported for realizing single cell proteomic studies. For example, the research group of Ewing [87-90] demonstrated mapping of cellular and subcellular contents from individual cells by time-of-flight secondary ion mass spectrometry. Recently, microfluidic systems have drawn attentions of researchers as an attractive platform for single cell proteomics [6, 91], due to their compatible sizes with cells and potential for automation.

The research group of Ramsey [92] demonstrated a microfluidic device, integrating cell handling, cell lysis, electrophoretic separation and fluorescent detection. The loaded cells were hydrodynamically transported from the cell-containing reservoir to a region where they were focused and rapidly lysed using a pulse electric field. Cell lysis was completed within 33 ms. The total separation time was 2.2 s with absolute migration time reproducibility better than 1%. Wu and co-workers [93] reported an integrated microfluidic device, which allowed cell handling, drug delivery, cell lysis, chemical derivatization, separation and detection. By using three-state microvalves, a volume of $\sim 70\ \text{pL}$ is achieved for the lysis and derivatization of the contents of a single Jurkat T cell. Electropherograms of amino acids from individual Jurkat T cells are recorded as a demonstration of this microfluidic platform. A microfluidic device was fabricated by Huang and co-workers [94], in which they can manipulate, lyse, label, separate, and quantify the protein

contents of a single cell using single-molecule fluorescence counting. Using this device, Huang and co-workers successfully quantified β_2 adrenergic receptors expressed in insect cells. They further demonstrated analysis of phycobiliprotein contents in individual cyanobacterial cells and observation of marked differences in the levels of specific complexes in cell populations that were grown under nitrogen-depleted conditions. More examples for on-chip single cell proteomic analysis can be found recently. The research group of Fang [95] used a simple cross-shaped microchannel design and electrical pulse for the manipulation and lysis of individual cells. Glutathione was used as a model intracellular component in single human erythrocyte cells detected after cell lysis. With a similar method, they further demonstrated on-chip cell lysis and simultaneous determination of released reactive oxygen species and reduced glutathione from individual human erythrocyte cells [96]. Ros et al. [97] fabricated micropost structures at the intersection of cross-shaped microchannels for the docking of single cells. After individual cells are manipulated into the docking area by optical tweezers, cells are lysed by an electrical pulse and the released protein contents are subsequently separated by electrophoresis and detected by laser induced fluorescence detection.

2.2.4. Mass spectrometry coupling

Mass spectrometry (MS) based methods have been extensively used for proteomic studies [7, 98], such as peptide mapping, post-translational modification and protein-protein interactions. With the rapid progress of microfabrication technology, microfluidic chips have been coupled to MS analysis to address issues like speed, throughput, and cost efficiency.

Coupling of μ CE with electrospray ionization (ESI)-MS was demonstrated by Li and co-workers [99]. Disposable nano-electrospray emitters were fabricated and directly coupled to the microfluidic device as the sample introduction interface to MS. Identification of membrane proteins that comprise a subset of the *Haemophilus influenzae* proteome was demonstrated. In a subsequent work [100], an autosampler device and a separation channel array were further developed as updates to the previous system. As a result, a throughput up to 30 samples per hour and a maximum sample carryover of less than 3% was realized. By integrating an additional channel immobilized with affinity media on the microfluidic chip, enrichment of trace-level proteins prior to μ CE and MS analysis was successfully demonstrated [101].

Recently, Gustafsson and co-workers [102] developed a high-throughput microfluidic compact disk (CD) for protein preparation and matrix-assisted laser desorption/ionization (MALDI) mass spectrometry. This microfluidic CD was designed with an array of 96 identical microchannels, enabling parallel preparation of samples with volumes ranging from one to several microliters. Detection of proteolytic peptides down to the 50-amol level was demonstrated. Electrowetting-on-dielectric-based technique for digital microfluidics was used by Wheeler and co-worker [103] to perform in-line sample purification for proteomic analysis with MALDI-MS. A PDMS microfluidic device was demonstrated by Dodge and co-workers [104], which combined on-line protein electrophoretic separation, selection and protein digestion for subsequent MALDI MS analysis. Liu et al. [105] reported a microfluidic enzymatic reactor that allowed rapid digestion of 16 fmol proteins for each MS analysis. Identification of cytoplasm of the human liver tissue was successfully demonstrated using this microfluidic reactor in combination with ESI-MS/MS.

2.3. Metabolomics

Metabolome is a highly dynamic system, where the level of each metabolite such as amino acids and hormones, are strictly dependent on the physiological conditions of the cell. Thus, quantitative investigation of metabolome provides important information for systems-level understanding of biology.

Microfluidic chips have been extensively employed in amino acid analysis. Manz and co-workers pioneered the miniaturization of CE [56, 57], where CZE was adopted as the separation mechanism, resulting in efficient separation of FITC-labeled amino acids. Culbertson and co-workers [106] fabricated a 25-cm-long spiral-shaped separation channel on a glass microchip (Figure 5) for MEKC separation of amino acid. As a result, 19 tetramethylrhodamine-labeled amino acids were efficiently separated in 165 s with an average plate number of 280,000. Recently, Skelley et al. [107] developed the Mars Organic Analyzer, a μ CE system for sensitive amino acid biomarker analysis with a detection limit as low as 0.1 nM.

Hutt and co-worker [108] explored chiral separations of fluorescein isothiocyanate-labeled amino acids using μ CE. With a sodium dodecyl sulfate (SDS)/ β -cyclodextrin carbonate electrophoresis buffer (pH 10.0) and a separation voltage of 550 V/cm, baseline resolution was readily achieved for Val, Ala, Glu, and Asp enantiomers and Gly in 4 min. Ro et al. [109] reported an integrated PDMS microchip for chiral separation of DL-amino acids based on diastereomerization. After diastereomerization in a microfabricated precolumn reactor, amino acid enantiomers were separated by MEKC without chiral selectors in the running buffer. Simultaneous separation of five chiral amino acids was successfully performed in a single run in less than 100 s.

In addition to amino acid analysis, microfluidic systems have also been applied to the investigation of other types of metabolites such as hormones. The research group of Kennedy earlier [110] reported a microfluidic device for the determination of insulin secreted from islets of Langerhans by a capillary electrophoresis competitive immunoassay. In a subsequent work [111], they further developed a high-throughput microfluidic chip that allowed continuous monitoring of cellular secretions from multiple independent living samples in parallel. As a result, 1450 electrophoresis-based immunoassays of biological samples were achieved in less than 40 min.

Liu and co-workers [112] reported a PDMS microfluidic system that integrated μ CE with bioluminescence detection for ATP-conjugated metabolic analysis. As a result, assays of galactose in human urine samples were successfully investigated. Urbanski et al. [113] developed a microfluidic device that can perform microfluorometric enzymatic assays in an automated fashion. After loading of samples and enzyme cocktail, this microfluidic system allowed automated aliquotting, mixing of reagents, data acquisition, and data analysis. Serial measurements of glucose, pyruvate, and lactate in triplicate with submicroliter sample volumes were achieved within 5 min.

3. Analysis at cellular level

Bioanalysis at cellular level gives direct information of a single cell or a group of cells, which plays essential role in understanding each cellular element and their correlations within a complex biological system. Due to their compatible sizes with cells, microfluidic systems are ideal platforms for single or multiple cell manipulation and analysis *in vitro*.

3.1. Cell sorting

Cell sorting is a preliminary step for cellular level investigation of biology. Microfluidic chip based cell sorters that take advantages of system miniaturization have been widely studied in recent years. A large variety of active or passive sorting mechanisms have been employed for microfluidic cell sorters, such as electric, magnetic, hydrodynamic and optical mechanisms [114].

Electric sorting mechanism is the most popular method employed by microfluidic cell sorters, which have advantages of flexibility, integration and potential for automation. Dittrich and co-workers [115] recently presented a microfluidic electrokinetic sorter that allows precise separation of fluorescent cells or particles in solution with respect to their different spectroscopic properties, such as brightness and color. Kang et al. [116] reported a fluorescence-activated particle counting and sorting system. Automatic sorting of fluorescent particles was achieved by electrokinetic switching triggered by a pre-set fluorescent threshold. Cell sorting based on electrokinetic switching is a common approach [115-118]. However, electrokinetic forces are relatively weak for the manipulation of large cells or particles. Recently, Yao and co-workers [119] combined gravity and electrokinetic force for flow cytometry and fluorescence-activated cell sorting. This system was applied to estimate the necrotic and apoptotic effects of ultraviolet light on HeLa cells.

Dielectrophoresis (DEP) is another common method [120-130], which has advantages of low-voltage operation and selectivity for driving cells by frequency. The research group of Soh [122] developed a microfluidic DEP-activated cell sorter (DACS) for the isolation of rare cells by labeling cell with particles that differ in polarization response. When cell mixture entered the channel, the labeled cells were selectively deflected into the collecting stream by DEP. As a result, isolation of rare *E. coli* expressing a specific surface marker was achieved with an enrichment of more than 200 folds and a throughput of 10,000 cells per second. With a similar sorting mechanism, they further reported a microfluidic multitarget DACS [123] (Figure 6). By specific labeling with unique synthetic DEP tags, multiple bacterial target cell types were efficiently separated in a single run with ~1000-fold enrichment.

For cells that have native magnetic susceptibility, such as red blood cells and magnetotactic bacteria, microfluidic magnetic activated cell sorter (MACS) can be used for efficient separation [131, 132]. For nonmagnetic cells or particles, selective magnetic labeling can be used to introduce magnetism to the targets for MACS sorting [133-136]. Inglis and co-workers [134] recently reported a microfluidic device that can isolate cells selectively tagged with magnetic nanoparticles. When cells flew over an array of microfabricated magnetic stripes, a series of high magnetic field gradients trapped the magnetically labeled cells and altered their flow direction.

Hydrodynamic sorting is the most traditional method employed by microfluidic sorters. Generally, cell sorting was realized by active switching of flow directions upon detection of fluorescent signals from cells [137, 138]. Recently, Chabert and Viovy [139] reported a high-throughput hydrodynamic cell sorting approach for the high-throughput encapsulation of single cells into picoliter droplets, and spontaneous self-sorting of these droplets. Successful encapsulation and sorting of 70–80% of the injected cell population was demonstrated. Separation of cancerous lymphocytes from a whole blood mixture was further realized with a >10,000-fold enrichment.

Due to its non-physical contact and minimal contamination during manipulation, optical sorting mechanisms such as optical tweezers and optical traps are another attractive type of method for microfluidic sorters [140-146]. Wang and co-workers [145] demonstrated optical force switching for the separation of fluorescent cells on microfluidic chips. With this method, live stably transfected HeLa cells expressing a fused histone-green fluorescent protein was isolated with an enrichment of 63-71 folds, an 85% recovery and a throughput of ~106 cells per second. Recently, other optical approaches such as optical waveguide [147-149] and optical lattice [150] have also been employed for particle sorting and cell manipulation on microfluidic chips.

In addition to the sorting mechanisms mentioned above, many other type of sorting methods have also been reported lately, such as acoustic method [151-153], affinity-based selection [154-158], concurrent extraction [159, 160], mechanical switching [161] and thermoreversible gelation valves [162].

3.2. Patterning and immobilization

Immobilization of cells is usually necessary before analysis can be effectively conducted on microfluidic chips. One commonly used method is cell patterning in microfluidic channels by soft lithographic techniques [163], such as microcontact printing, patterning using microfluidic channels, and laminar flow patterning.

The research group of Whitesides [164] earlier reported cell patterning using multiple laminar fluid flows in microfluidic channels. This novel method provided a way to simultaneously control the surface properties for cell adhesion, the type of cells in vicinity and the media in contact with the cells. In a subsequent work [165], they further developed a three-dimensional microfluidic system for the patterning of proteins and immobilization of mammalian cells. Multiple types of proteins and cells in complex, discontinuous structures on a planar substrate were realized. Recently, Kaji et al. [166] demonstrated patterning of electrically conjugating cardiomyocytes by microcontact printing. Rhee and co-workers [167] described a plasma-based dry etching method for patterning cell culture inside microfluidic channels. Cell-adhesive and non-adhesive areas on glass and polystyrene substrates were patterned using plasma-based dry etching. The patterned substrates were then successfully used for selective attachment and culturing of human umbilical vein endothelial cells, MDA-MB-231 human breast cancer cells, NIH 3T3 mouse fibroblasts, and primary rat cortical neurons.

Other cell immobilization methods have also been reported for microfluidic systems in recent years, such as cell docking [168, 169], acoustic trap [170] and optical trap [171].

3.3. Cell culture and development

Stem cells differ from other types of cells due to their special ability to regenerate and to differentiate into multiple cell types. Chung and co-workers [172] reported a gradient-generating microfluidic platform for optimizing proliferation and differentiation of neural stem cells in culture. By exposing cells to a concentration gradient of growth factors under continuous flow, proliferation and differentiation of neural stem cells into astrocytes could be monitored by time-lapse microscopy and immunocytochemistry. A different method was reported by Park et al. [173], in which an osmosis-driven pump was used to generate a concentration gradient of fetal bovine serum for the culturing of human mesenchymal stem cells. Kim and co-workers [174]

developed a microfluidic device for culturing adherent cells over a logarithmic range of flow rates. Using this device, they demonstrated culture of murine embryonic stem cells in continuous, logarithmically scaled perfusion for 4 days, with flow rates varying $>300\times$ across the array.

Neural development is another hot topic in cell biology. Martinoia and co-workers [175] earlier reported hydraulically driven deposition of adhesion molecules for the patterning of neurons on silicon micromachined substrates. Entrapment of chick embryo spinal cord neurons in microstructures and formation of patterned neural networks were achieved. Recently, Millet et al. [176] demonstrated culturing of postnatal primary hippocampal neurons from rat in PDMS microfluidic channels. Park and co-workers [177] reported the use of a microfluidic device to culture central nervous system (CNS) and peripheral nervous system (PNS) neurons for neuroscience applications. CNS and PNS neurons were cultured within the device for 2-3 weeks before further applications (Figure 7), serving as an *in vitro* model for axonal injury and regeneration in the CNS.

3.4. Localized chemical treatment

After cells are immobilized in microfluidic channels, localized chemical stimulations can be realized using laminar fluid flows. Takayama et al. [178] reported the use of multiple laminar flows in a microfluidic channel to deliver membrane-permeable molecules to selected subcellular microdomains of immobilized cells (Figure 8). This method was then successfully applied to study the subcellular processes of mitochondrial movement and changes in cytoskeleton structure. With a similar method, Lucchetta et al. [179] studied the robust network in *Drosophila melanogaster* that compensates for variation in the Bicoid morphogen gradient. Results indicated that the compensation system counteracted the effects of extremely unnatural environmental conditions in which the anterior and posterior halves of the embryo are developing at different temperatures and thus at different rates (Figure 9). Sawano and co-workers [180] used laminar flows to study the lateral propagation of epidermal growth factor signaling in single live COS cells following localized chemical stimulation. Results suggested that ligand-independent propagation of epidermal growth factor signaling occurs only when the receptor density on the plasma membrane is high.

3.5. Patch clamp and electroporation

Patch clamp technique is heavily employed in electrophysiological studies of single or multiple ion channels on cell membrane. However, the conventional patch clamp method is labor intensive and time consuming. System miniaturization is then proposed for patch clamp to address such issues [181]. So far, on-chip patch clamp has been realized using both bottom cell trapping [182-186] and lateral cell tapping [187-191] methods. Recently, Li and co-workers [185] reported a microfluidic system with disposable PDMS cell interface for simultaneous patch clamp recording. 2 μm apertures were fabricated in PDMS, which was then reversibly sealed to a microfluidic system with microchannels, isolation valves and microfabricated Ag/AgCl electrodes. Gigaseal recordings from RBL-1 cells were successfully achieved with a 24% success rate. Ionescu-Zanetti et al. [188] developed a PDMS microchip that allowed patch clamp based on microfluidic junctions between a main chamber and lateral recording capillaries. Reliable whole-cell recording of activation of the voltage-sensitive potassium channel Kv2.1 in mammalian cells was demonstrated.

Electroporation is a common technique in cell biology for loading exogenous molecules such as fluorescent dye and DNA into target cells. In transition to microfluidic systems, electroporation has become a more versatile technique for single cell analysis. On a microfluidic chip, polyelectrolytic salt bridges were explored by Kim and co-workers [192] for cell electroporation at low dc voltage. Wang et al. [193] fabricated elastomeric valves on a microchip for cell electroporation. Lu et al. [194] demonstrated an electroporation chip for cell lysis prior to subcellular analysis. Bao et al. [195] used electroporation for selective release of intracellular molecules at the single-cell level. Wang et al. [196] studied translocation of an enhanced green fluorescent protein tagged tyrosine kinase to the plasma membrane in B cells using microfluidic electroporative flow cytometry, which was further used by the same research group for investigating single-cell biomechanics [197].

3.6. Cell-cell signaling

Cell-cell signaling has also been investigated using microfluidic chip based methods. Klauke and co-workers [198] recently studied cell-cell signaling between longitudinally linked primary heart cells in a microfluidic system. By exploring the local ionic conditions that enabled the propagation of Ca^{2+} waves between two heart cells, they discovered that the ability of intracellular Ca^{2+} waves to traverse the intercalated discs is dependent on the relative concentrations of diastolic Ca^{2+} in the two adjacent cells. Wei et al. [199] fabricated a microfluidic coculture system for investigating cell-cell interaction in distance. In their work, substances released from the up-stream macrophages upon activation generated a linear concentration gradient in down-stream wells by a microfluidic mixing system. Cellular responses and viability of the down-stream osteoblasts were subsequently evaluated. Another microfluidic coculture system was reported by Song et al. [200] for distant cell-cell interaction studies, in which the effect of human embryonic germ (hEG) cells on SKOV3 cells was investigated. By unidirectional perfusion from hEG to SKOV3 cells, the growth inhibition of SKOV3 cells was observed and the apoptosis signals in SKOV3 culture area decreased along the flow of the medium.

4. Analysis at organism level

The nematode *C. elegans* is an excellent animal model, which allows biologist to study the development of individual cells or a group of cells within a complex biological system. *C. elegans* is a small (about 1 mm long as an adult) free living round worm with bilaterally symmetry. The anatomy of *C. elegans* is invariant from one another. The standard lab strain *C. elegans* is propagated by self-fertilization. Thus, individual worms are considered to be genetically identical. In addition, *C. elegans* has a life cycle as short as a few days. Conventional methods for studying *C. elegans* are usually time-consuming and labor-intensive. Recently, efforts have been made in microfluidic systems to enable automated high-throughput analysis of *C. elegans*.

Immobilization of *C. elegans* is usually a prerequisite step in such studies. So far, different methods have been reported for the immobilization of *C. elegans* on microfluidic chips, including the use of tapered microfluidic channels [201-203], lateral channel suction [204, 205] and microfluidic valves [206, 207]. With proper immobilization, subsequent analysis of *C. elegans* can be achieved. Rohde and co-worker [204] realized high-throughput whole-animal sorting with lateral channel suction. Chung et al. [205] demonstrated an automated microfluidic device for rapid on-chip sample handling, high-resolution microscopy, phenotyping and whole-animal



sorting (Figure 10). Zeng et al. [206] used microfluidic valves to immobilize *C. elegans* for observation and manipulation of sub-cellular features using two-photon microscopy and femtosecond-laser microsurgery. Imaging of neuronal and behavioral activity in immobilized *C. elegans* was realized by Chronis and co-workers [202] on microfluidic chips (Figure 11). With microfluidic valve based immobilization, Guo et al. realized femtosecond laser nanoaxotomy for in vivo nerve regeneration studies of *C. elegans*.

Recently, Heng et al. [208] developed on-chip optofluidic microscopy for high resolution optical imaging of *C. elegans*. Microfluidic shadow imaging system for studying the behaviors of *C. elegans* was developed by Lange and co-workers [209]. Qin and Wheeler [210] fabricated T-shaped and U-shaped microfluidic mazes for exploring learning of *C. elegans*. An automated microfluidic CD cultivation system was developed by Kim and colleagues [211, 212], which was further used in studying signal transduction induced by hypergravity in *C. elegans* [212].

5. Conclusions

Systems-level understanding of biology requires massive information of individual components and their correlations within complex biological systems. Microfluidic chip based method certainly plays as one of essential roles in such demanding investigations, revolutionizing the means for biological researches due to advantages such as high throughput, small sample consumption and reduced analysis time. But it must be noted that there are also disadvantages due to system miniaturization. For example, reduced sample consumption can result in the requirement for highly sensitive detection schemes due to reduced detection volumes. Micropumps are usually employed for sample injection, which are difficult to be integrated on microfluidic chips, thus complicating the analysis system. But without doubt, microfluidic technology holds high promise to facilitate biological researches in generating enormous amount of information for systems-level understanding of biology. In this review, we documented the recent applications of microfluidic chips in biological researches. At the molecular level, microfluidic chip based methods have been extensively employed for the analysis of DNA, proteins and metabolites. Miniaturization of CE has been a main focus in such investigations. The 384-channel radial μ CAE chip is one good example, which achieves a 20-fold increase in sequencing performance over the conventional 96-capillary instrumentation. At the cellular level, a wide range of applications of microfluidic chips have been reported, which include cell sorting, cell patterning and immobilization, cell culture and development, localized chemical treatment, patch clamp, electroporation and cell-cell signaling. Microfluidic applications at the organism level are just emerging, which have been mainly focused on the model animal, *C. elegans*. More applications of microfluidic chips at the organism level can be expected in near future.

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Figure Legends

Figure 1. (A) Layout of the 384-channel μ CAE device on a 200-mm-diameter wafer. Channels are 60 μm wide and 30 μm deep, and the effective separation length is 8.0 cm. (B) Expanded view of a single quartet of channels with their injectors. (C) All channels in a quartet share a common cathode reservoir located closest to the edge of the wafer. The injector design for every channel is shown at right. S1 – S4 indicate individual sample reservoirs. (Reprinted from Emrich et al., *Anal. Chem.* 2002, 74, 5076-5083, with permission of American Chemical Society, © 2002)



Figure 2. (A) Sequence of fluorescent images of the electrophoretic separation (15 V/cm) of a low DNA mass ladder in a microfluidic chip packed with 0.9- μm silica beads. In this case, the microchip has 100- μm -wide and 20- μm -deep microchannels with a cross-injection design. Dashed lines were added to aid visualization of the microchannels. Frames are time-stamped in seconds. DNA fragments 1-5 are assigned as 200, 400, 800, 1200, and 2000 bp, respectively. The 100-bp band was not detected due to the very low concentration. (B) Four consecutive runs of the same ladder obtained using 0.9- μm silica beads in a device that had been operated for 5 h ($E = 19.2$ V/cm, separation length $L = 5$ mm). (Reprinted from Zeng et al., *Anal. Chem.* 2007, 79, 2289-2295, with permission of American Chemical Society, © 2007)

Figure 3. (A) Image of a microchip with a serpentine channel for two-dimensional separations. Injections were made at valve 1 (V1) for the first-dimension MEKC separation and at valve 2 (V2) for the second-dimension CE separation. The sample was detected 1 cm downstream from V2 at point D using laser-induced fluorescence. The reservoirs are labeled sample (S), buffer 1 (B1), sample waste 1 (SW1), buffer 2 (B2), sample waste (SW2), and waste (W). The channels and reservoirs were filled with black ink for contrast. (B) Two-dimensional separation of a bovine serum albumin tryptic digest. The projections of the two-dimensional data onto each axis are shown. Spots marked with a “T” represent unreacted dye. The dotted line illustrates correlation in the separation mechanisms. (Reprinted from Ramsey et al., *Anal. Chem.* 2003, 75, 3758-3764, with permission of American Chemical Society, © 2003)

Figure 4. Design of the microfluidic two-dimensional separation system. (A) The overall design comprises an arced, 3.75 cm long horizontal channel for first-dimension IEF that is punctuated with 20 6.8 cm long vertical channels through which focused proteins are separated in the second dimension by native gel electrophoresis. (B) The separation channels of the two dimensions are joined by much smaller channels forming a microfluidic interface that fluidically decouples the contents of the two separation dimensions, seen more clearly in electron micrographs C and D. The microfluidic interface channels are 25 μm wide, 400 μm long, and 4 μm deep. (Reprinted from Emrich et al., *Anal. Chem.* 2007, 79, 7360-7366, with permission of American Chemical Society, © 2007)

Figure 5. Image of a microchip with a spiral separation channel. The reservoirs on the interior of the spiral were replaced by a single reservoir located at the injection cross (dashed circle). The small circles are the locations of the detection points: from outside to inside 0.25, 11.9, and 22.2 cm. The inset is a magnification of the injection cross and parts of the spiral channel. The buffer,

sample, and sample waste channels are 219 μm wide (half-depth) for all of their length except for the last 500 μm before the injection cross where they narrow to only 40 μm (half-depth). The separation channel was 24.9 cm long and 40 μm wide (half-depth). The black arrows on the inset indicate the vertexes of the polygonally shaped channels. (Reprinted from Culbertson et al., *Anal. Chem.* 2000, 72, 5814-5819, with permission of American Chemical Society, © 2000)

Figure 6. Multiple bacterial cell sorting procedure using the multitarget DACS device. **(A)** The experimental scheme. Step a: target cells (target A and target B) are labeled with DEP tags (tag A and tag B, respectively) via their respective surface markers. Step b: cells are dielectrophoretically sorted and eluted through independent outlets. Step c: collected cells from the outlets are analyzed via flow cytometry to quantify the sorting performance. **(B)** The physics of multitarget separation via multitarget DACS. Two sets of electrodes are positioned at different glancing angles (θ_1 and θ_2) to select for two different targets. Target A cells labeled with tag A are selected at electrode set A ($\theta_1 = 10^\circ$) and elute through outlet A. Target B cells labeled with tag B are sorted at electrode set B ($\theta_2 = 8^\circ$) and elute through outlet B. The unlabeled, nontarget cells are not deflected by either electrode set, and they are eluted through the waste outlet. (Reprinted from Kim et al., *Anal. Chem.* 2008, 80, 8656-8661, with permission of American Chemical Society, © 2008)

Figure 7. Confocal micrograph of Tau (green) and MAP5 (red) immunostained neurons **(a)** and phase micrographs of 6 DIV neurons **(b,c)** cultured in the microfluidic device. Immunostain results indicate that all processes that cross the barrier are axons. Scale bars, 100 μm . (Reprinted from Park et al., *Nat. Protoc.* 2006, 1, 2128-2136, with permission of Macmillan Publishers Ltd, © 2006)

Figure 8. Differential manipulation of regions of a single bovine capillary endothelial cell using multiple laminar flows. **a, b**, Experimental set-up; **b** shows a close-up of the point at which the inlet channels combine into one main channel. **c**, Fluorescence images of a single cell after treatment of its right pole with Mitotracker Green FM and its left pole with Mitotracker Red CM-H₂XRos. The entire cell is treated with the DNA-binding dye Hoechst 33342. **d**, Image of the same cell as in **c** but taken 2.5 h later, showing intermixing of the red and green subpopulations of mitochondria. **e**, Treatment of a portion of a single cell with latrunculin A. The blue region (Dextran-70,000 Cascade Blue stain) reveals the flow of medium containing latrunculin A. Right, enlarged view of the middle cell and its mitochondria (green). **f**, Phalloidin–Alexa 594-labelled image of the same cell immediately after 10 min of treatment with latrunculin A. The cell in the middle, which was only partly in the stream containing latrunculin, shows disruption of actin microfilaments that is limited to this region (scale bars, 25 μm). (Reprinted from Takayama et al., *Nature* 2001, 411, 1016-1016, with permission of Macmillan Publishers Ltd, © 2001)

Figure 9. Even-skipped expression in embryos exposed to a T-step of 20 °C/27 °C. **a-c**, Embryos with a cool anterior half and warm posterior half. **d-f**, Embryos with a warm anterior half and cool posterior half. Even-skipped stripes were consistently expressed in the warm half first (**a** and **b**, **d** and **e**), but resolved in the correct positions (**c**, **f**). (Reprinted from Lucchetta et al., *Nature* 2005, 434, 1134-1138, with permission of Macmillan Publishers Ltd, © 2005)

Figure 10. Schematics of the system and the microchip. **(a)** A mixed population of worms is injected into the device, worms are imaged, phenotyped and then sorted. **(b)** System block diagram showing the on-chip and off-chip components. **(c)** Photograph of the microdevice. **(d)** Optical micrograph of the microchip's active region (boxed region in c). The channels were filled with dye to show specific features: blue, temperature control channel; green, valves; and red, sample-loading channel. Scale bars, 5 mm **(c)** and 100 μm **(d)**. (Reprinted from Chung et al., *Nat. Methods* 2008, 5, 637-643, with permission of Macmillan Publishers Ltd, © 2008)

Figure 11. **(a)** The microfluidic four-flow device for delivering odor. The microfluidic chip consists of two side channels loaded with fluorescent dye (channels 1 and 4), a stimulus channel (channel 2) and a control, or buffer, channel (channel 3). Scale bar, 2 mm. **(b)** Higher-magnification photographs of the chip with a trapped worm. The dotted lines mark the interfaces between the fluids. Scale bars, 150 μm (top) and 30 μm (bottom). As shown, the nose is exposed to the buffer stream. **(c)** The stimulus and buffer streams are directed by the side flows (gray). In the 'off' state (left), the right dye channel (channel 4) is open and pushes the stimulus stream to exit from the left outlet. In the 'on' state (right), the left dye channel (channel 1) is open, and the worm is exposed to stimulus. (Reprinted from Chronis et al., *Nat. Methods* 2007, 4, 727-731, with permission of Macmillan Publishers Ltd, © 2007)