

Chapter 2

Recent Development of Cell Analysis on Microfluidics

Ziyi He and Jin-Ming Lin

Abstract Cells are basic structural and functional units of living organisms. Understanding the composition, structure and function of cells, and exploring cellular activities, are quite important for the cognition of phenomena and rules of life. Microfluidics, combined with advanced molecular, imaging and bioinformatics techniques, constitute a robust ‘toolbox’ and revolutionize the way for cell biology researches. In microfluidic systems, small amounts of fluids are manipulated using precisely designed channels with dimensions at micrometer level. Various chemical and biological processes can be transferred and integrated in a small single device, achieving multiple chemical and biological functions. Microfluidic technology displays a number of unique merits over conventional approaches, and has been extensively applied to various fields of cell research. In this chapter, we will review the recent developments and outstanding achievements of microfluidic technology in cell researches. Based on the cell study procedure, the main content is divided into four parts: cell culture, cell manipulation, cell stimulation and cell analysis. This review will also discuss the challenges and directions of microfluidic-based cell analysis, providing important references and ideas for the development of biological and medical researches and applications.

Keywords Microfluidics • Cell analysis • Cell culture • Cell manipulation
Cell stimulation

2.1 Introduction

Cells are basic structural and functional units of living organisms. Understanding the composition, structure and function of cells, and exploring cellular activities, are quite important for the cognition of activity rules and phenomena of life. Cells have small size, low amount of contents and diverse species. And in organisms, cells are

Z. He · J.-M. Lin (✉)

Department of Chemistry, Tsinghua University, Beijing 100084, People’s Republic of China
e-mail: jmlin@mail.tsinghua.edu.cn

located in complex microenvironments and subjected to multiple cues that vary in time and space, including temperature, oxygen concentration, gradients of cytokines and signaling molecules, mechanical forces, and interactions with extracellular matrix (ECM) and other cells [1–3]. The microenvironments are significant for maintaining cellular functions [4, 5]. Therefore, it is highly important and desirable to develop advanced technologies that enable precise cell manipulation, physiologically relevant microenvironment simulation, as well as sensitive, selective, high-throughput and reliable cell analysis.

Since the 1990s, microfluidic technology has experienced explosive developments, and gradually become an important tool for cell research. In microfluidic systems, small (10^{-9} – 10^{-18} L) amounts of fluids are manipulated using precisely designed channels with dimensions of tens to hundreds micrometres [6, 7]. Various chemical and biological processes, such as synthesis, reaction, separation, detection, and cell culture, cell isolation, cell lysis and cell analysis, can be integrated in a single device to achieve multiple chemical and biological functions [8–10]. Microfluidic technology displays a number of unique merits over conventional approaches, which promote the applications in cell researches [11–13]. The dimensions of microfluidic channels are comparable to the sizes of cells, thus facilitating precise cell manipulation. Microchannels reduce sample consumption, avoid sample dilution, and allow rapid mass and heat transfer [14]. These features contribute to highly sensitive cell analysis. And due to the size effect, microfluidic fluids at low flow rate are laminar, which enables precise fluid control [15], and can be applied to partitioned channel modification [16], concentration gradient establishment [17] and regional cell stimulation [18]. A variety of complex microstructures can be designed and fabricated on microfluidic devices, allowing better control of multiple biological cues and more physiologically relevant mimic of cellular microenvironments. The ability of integration is a prominent advantage of microfluidic technology. Microfluidics not only enable the assembly of multiple cell analysis units on a single device, but also can integrate with diverse cell detecting techniques (such as optical and electrical detection, mass spectrometry), thus facilitating comprehensive cell researches [19–21]. For example, combining microvalves, micropumps and microchambers, an automatic single-cell analysis pipeline was established on a microfluidic device, which incorporated cell culture, precise cell stimulation, live-cell microscopy, computerized cell tracking, on-chip staining of key proteins and subsequent retrieval of cells for high-throughput gene expression analysis [22]. Using droplet-based microfluidics [23, 24] or microarrays [25, 26], high throughput cell analysis can be achieved to improve research efficiency and shorten analysis time. Owing to these advantages, recently microfluidic technology has been applied to various fields of cell researches, such as cell culture [27, 28], cell sorting [29], cancer research [30], stem cell research [31], clinic diagnosis [32, 33], drug screening [34, 35] and tissue engineering [36].

In this chapter, we will introduce the recent developments and outstanding achievements of microfluidic technology in cell researches. Based on the cell study procedure, the main content is divided into four parts: cell culture, cell manipulation, cell stimulation and cell analysis. This chapter will also discuss the challenges

and directions of microfluidics in cell research, providing important reference and ideas for the development of biological and medical research and application.

2.2 Cell Culture

Cell culture *in vitro* is the cornerstone of cell biology research. In living organisms, cells are located in complex microenvironments and subjected to multiple cues, including physicochemical properties such as temperature, oxygen concentration, pH, osmotic pressure, stimulation factors such as gradients of cytokines and signaling molecules, mechanical forces, and interactions with ECM and other cells [37]. Compared to conventional cell culture methods using petri dishes or culture plates, microfluidic devices have many unique advantages [38]. Micro-sized channels are comparable to *in vivo* cellular microenvironment, and enable precise regulation of cell number, cell density and spatial location; controllable fluids allow cell culture under flow condition and precise cell stimulation; using microstructures or hydrogels, three dimensional (3D) cell culture and cell co-culture can be achieved, which maintain cell-cell, cell-ECM interactions and are more physiologically relevant; microfluidic technology also facilitates parallel and automated cell culture, improving throughput and reproducibility of cell researches [27, 39].

In microfluidic cell study, how to develop more physiologically relevant cell culture models and make subsequent biological and medical researches more realistic and reliable, is an important research topic. We will review recent progresses of cell culture on microfluidics in this section, according to three parts: 3D cell culture, cell co-culture and tissues/organs-on-chips.

2.2.1 3D Cell Culture

The majority of microfluidic cell-culture systems are 2D cell culture, in which cells are grown as a monolayer on a flat substrate surface (e.g., glass or plastic). Although it is simple to handle, the 2D systems have certain limitations in mimicking the *in vivo* cellular microenvironment, and lack diffusion-limited distribution of soluble factors and cell-cell, cell-ECM interactions [40]. The transition from 2D to 3D cell culture is an important step for better mimicking the *in vivo* microenvironment [41, 42]. Owing to the complex microstructures and well-controlled parameters, microfluidics provide a versatile platform for 3D cell culture, which offer more physiologically relevant cellular morphology and phenotype, and promote metabolic activity and cellular functionality [43, 44].

The most common strategy for microfluidic 3D cell culture is to embed cells in 3D hydrogel scaffolds, such as matrigel, collagen, agarose and synthetic hydrogels [45]. Hydrogels enable cell-ECM interactions and permit diffusive permeability of oxygen, nutrients and metabolites to encapsulated cells [46]. Sung et al. developed

a microfluidic platform to examine the influence of 2D and 3D culture of human mammary fibroblasts (HMFs) on the invasive transition of breast cancer cells (MCF-DCIS) [47]. MCF-DCIS encapsulated in the mixture of collagen and matrigel were co-cultured with HMFs either embed in 3D matrix or grown on 2D surface. Results demonstrated that HMFs cultured in 3D secreted more paracrine signaling molecules and intensified the promotion of the invasive progression through the HGF/c-Met interaction. Combined with high-throughput droplet-based microfluidics, a large number of monodisperse 3D liver model were formed by controlled assembly of hepatocytes and fibroblasts in core-shell hydrogel scaffolds [48]. These droplets were able to be cultured for long periods of time and showed enhanced liver-specific functions. Advances in hydrogel materials promote the development of 3D cell culture with better in vivo relevance. Donald et al. developed an injectable, interconnected microporous gel scaffold assembled from monodisperse microgels generated by microfluidic droplet technique (Fig. 2.1a) [49]. In vitro, cells incorporated during scaffold formation proliferated and formed extensive 3D networks within 48 h. In vivo, the scaffold facilitated cell migration that resulted in rapid cutaneous-tissue regeneration and tissue-structure formation within five days. 3D cell culture can also be realized by gel-free microfluidic systems, such as hanging-drop network [50, 51], micro-well array [52] and dielectrophoretic patterning [53].

2.2.2 Cell Co-culture

Cell-cell interactions are very important for the development and function of multicellular organisms. They allow cells to communicate with each other, respond to signals in microenvironment and regulate basic cellular functions such as survival, apoptosis, migration, proliferation, and differentiation [54, 55]. Cell-cell interactions occur through multiple mechanisms, including direct cell contact, diffusion of soluble factors, electrical signal transmission and transduction of mechanical cues through ECM [56]. Efforts have been made to investigate the interaction mechanisms and their roles in physiological homeostasis and disease states. Microfluidics, as a flexible and reliable technique which is capable of co-culturing multiple cell types in precisely defined positions and delivering biochemical and biophysical stimuli in a spatiotemporal controllable manner, has been extensively developed and widely applied to cell-cell interaction study [57, 58].

Different intercellular interaction mechanisms have been investigated in various microfluidic cell co-culture systems, which can be divided into two categories: contact and non-contact approaches [59]. The non-contact microchips utilize barriers such as hydrogels [60, 61], semi-permeable membranes [62], porous films [63], pressure-controlled valves [64] and narrow channels [65] to separately culture cell in different regions. These microfluidic systems eliminate the influence of direct cell contact and can be used for the study of paracrine signaling and endocrine signaling. Contact approaches, such as microfluidic droplet co-culture [66],

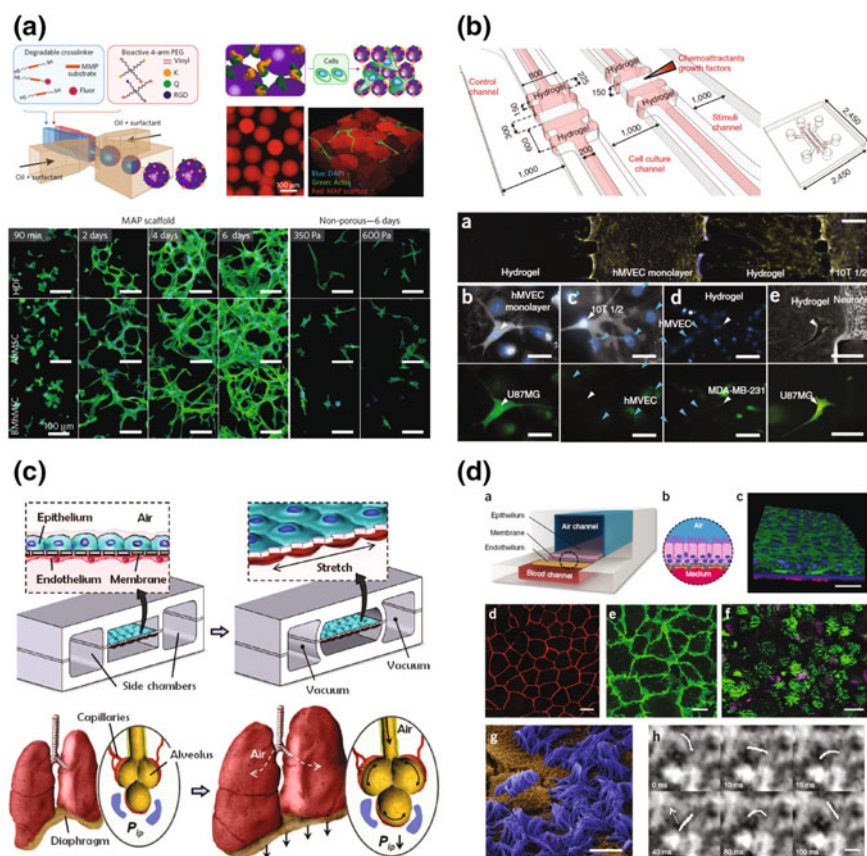


Fig. 2.1 Cell culture on microfluidic chips. **a** 3D cellular network formation on microporous gel scaffolds; **b** Cell migration into scaffolds under co-culture conditions in a microfluidic platform; **c** Human breathing lung-on-a-chip microdevice; **d** The human small airway-on-a-chip. (Reprinted with permission from Ref. [49, 72, 93, 94])

microcontact printing [67] and stencil-based methods [68, 69], culture cells in direct contact and are applicable to all interaction mechanisms, especially gap junction signaling and juxtacrine signaling. Selective cell adhesion on specific functionalized substrates was an alternative method for defined cell co-culture [70].

Cells in microfluidic systems can be co-cultured in 2D, 3D or single cell manner. Combining micropatterned surfaces with microfluidic channels, neurons and astrocytes were cultured in defined locations and communicated with each other through grooves [71]. Pharmacological agents were delivered through microchannels. This device analyzed neuron–astrocyte interactions under both healthy and pathophysiological conditions, and found that calcium dynamics in astrocytes could be modulated by the interactions with neurons. Shin et al. [72] developed a hydrogel-incorporating microfluidic cell culture assay, which facilitated the

interaction of cells in 3D ECM scaffolds. It could be used in various applications, including angiogenesis and cancer metastasis, and provide new insights into how biochemical and biophysical factors regulate interactions between populations of different cell types (Fig. 2.1b). Dura et al. [73] described a microfluidic platform that achieved high-throughput single-cell pairing of lymphocytes with a defined contact time, enabling pairwise-correlated multiparametric profiling of lymphocyte interactions over hundreds of pairs in a single experiment. This platform was applied to characterize early activation dynamics of CD8 T cells and investigated the extent of heterogeneity in T-cell activation and the correlation of multiple readouts.

2.2.3 *Tissues/Organs-on-Chips*

Owing to the progress in 3D cell culture and cell co-culture, as well as the precisely controlled fluid flow and mechanical force, tissue/organ-on-chip microsystems have been extensively developed on microfluidic devices. In tissues/organs-on-chips, living cells are culture in well-organized microchambers, with well-defined physiological factors and mechanical cues, which can not only reconstitute multicellular architectures and microenvironment of living human tissues or organs, but also recapitulate their physiological functions and responses. These systems are more human relevant and cost-effective than animal models, and the optical transparency of microdevices enable the direct real-time imaging and analysis of cellular activities [74, 75]. Tissues/organs-on-chips have great potential to study basic mechanisms of organ physiology and disease such as cancers, and are highly applicable for preclinical drug discovery [76, 77].

Various tissue/organ-on-chip models have been developed, such as blood vessels [78, 79], brain [80], liver [81, 82], lung [83, 84], kidney [85, 86], heart [87, 88], bone [89] and muscle [90]. Microfluidic chip is quite suitable for the development of blood vessel-on-a-chip, because of its fluid perfusion ability which facilitates blood mimic and introduces shear stresses, and the ease of gradient generation for angiogenesis assays [44]. Zheng and co-workers [91] engineered living microvascular networks in type I collagen on a microfluidic scaffold. With long-term (one to two weeks) culture, the microvasculature-on-a-chip emerged appropriate endothelial morphology and barrier functions. This platform could be used to investigate angiogenic remodeling, interactions between endothelial cells and perivascular cells, and interactions between blood components and endothelium with flow. Reconstituting the organ-level functions is the major goal of organs-on-chips study. Ingber's group developed a human 'breathing' lung-on-a-chip which reproduced key structural, functional, and mechanical properties of the human alveolar-capillary interface (Fig. 2.1c) [92, 93]. This bioinspired microdevice demonstrated complex organ-level responses to bacteria and inflammatory cytokines introduced into the alveolar space, and could be used to investigate the role of mechanical breathing motions in lung disease. In follow-up study, this group reconstituted a small airway-on-a-chip containing a differentiated, mucociliary

bronchiolar epithelium and an underlying microvascular endothelium that experienced fluid flow (Fig. 2.1d) [94]. This platform achieved greater robustness and fidelity in modeling of pulmonary diseases and recapitulation of lung inflammatory responses in vitro. As complex heterogeneous diseases, cancer in vitro models have also been intensively studied [95, 96]. Alexandre et al. reported a tumor-on-a-chip system where incorporation of tumor-like spheroids into a microfluidic channel permitted real-time analysis of nanoparticle (NP) accumulation at physiological flow conditions [97]. Taking advantage of the integration feature of microfluidics, ‘human-on-a-chip’ models which interconnect different organ-on-chip compartments through microfluidic circulatory systems have been investigated currently. It can provide more complete physiological biomimicry and become an important direction in further microfluidic study [75, 98].

2.3 Cell Manipulation

Cell manipulation plays an important role in basic cell biology study, drug screening, disease diagnosis and therapy. Because of the unique advantages, microfluidics was an excellent practical technique which provides incomparable possibilities to manipulate cells in an automated, reproducible, fast and efficient way. Various microfluidic techniques have been developed to manipulate cell precisely for diverse biological researches, such as microstructures, integrated valves and pumps, droplet encapsulation, electrokinetic operations, affinity-based surface patterning and free flow manipulation [99, 100]. Different techniques can be integrated to improve performance and functionalities within a single chip. In the next three sections, we mainly focus on the microstructures, electrokinetic operations, and free flow manipulation, and provide some recent examples. Droplet-based microfluidics will be summarized in Chap. 7.

2.3.1 Microstructures

Microstructures, such as microwells, microbarriers and microtraps, can be precisely designed and applied to cell capture, pairing, patterning and subsequent cell culture as well as other biological study [101–104]. This approach is high-throughput, high-efficient and ease of operation, which has been extensively used in both multicellular and single-cell systems. Chung et al. [105] developed a microfluidic embryo-trap array that could rapidly order and vertically orient hundreds of embryos, and this platform was used to quantitatively analyze multiple morphogen gradients in the dorsoventral patterning system (Fig. 2.2a). Sarioglu et al. [106] introduced a Cluster-Chip, which contained a series of triangular pillars to differentiate CTC clusters from single cells in blood. It was used to isolate CTC clusters from unprocessed patient blood samples with high sensitivity, allowing for

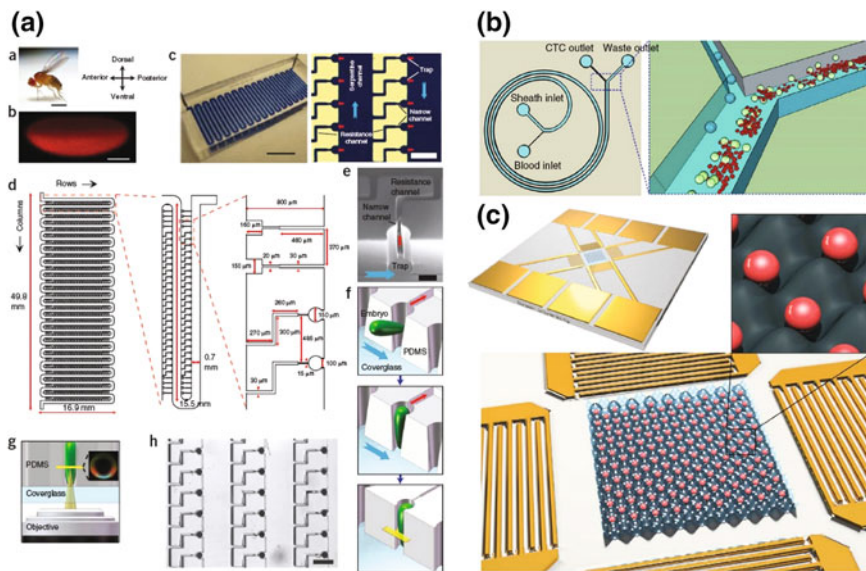


Fig. 2.2 Cell manipulation on microfluidic chips. **a** Microfluidic embryo-trap array for high-throughput arraying of vertically oriented *Drosophila* embryos; **b** Size-based cell isolation in a spiral microfluidic device; **c** Two-dimensional single-cell patterning driven by surface acoustic waves in a microfluidic device. (Reprinted with permission from Ref. [105, 115, 117])

downstream molecular and functional assays. Integrating the microwell array with microfluidic valve and pump systems, Lecault et al. [107] developed a longer-term mammalian cell culture platform which was able to immobilize nonadherent cells during automated medium exchange and recover the cells for subsequent analysis. This platform was then applied to high-throughput investigation of hematopoietic stem cell proliferation at the single-cell level.

2.3.2 Free-Flow Manipulation

The free flow cell manipulation can be divided into passive and active strategies [108]. Passive strategies use rationally designed microfluidic structures to control cell positions, such as pinched flow [109] and deterministic lateral displacement [110]; Active strategies use actuators to manipulate cells based on their electrical, magnetic and mechanical properties, such as dielectrophoresis [111], magnetophoresis [112], acoustophoresis [113] and optical tweezers [114]. These techniques can also be integrated to improve the performance [108]. Warkiani et al. [115] developed a label-free spiral microfluidic device to allow size-based cell isolation by taking advantage of dean migration and inertial focusing in curvilinear microchannels

(Fig. 2.2b). Karabacak et al. [116] presented a CTC-iChip using deterministic lateral displacement, inertial focusing and magnetophoresis to isolate rare circulating tumor cells (CTCs) from blood samples. This device achieved an average of 3.8-log depletion of white blood cells at a rate of 8 ml whole blood/h and a cancer cell yield of $97 \pm 2.7\%$. Collins et al. [117] utilized surface acoustic waves at high frequency to create a 2D acoustic force field with an inter-nodal spacing of the same order as the cell dimensions (Fig. 2.2c). This device was applied to the patterning of multiple spatially separated single cells with one cell per acoustic well.

2.3.3 *Electrokinetic Operations*

Owing to the feasibility of integrating microelectrodes in microfluidic chips, electrokinetic forces stemming from the electric field have been widely applied to microfluidic cell manipulation [118]. Electrokinetic manipulations include several categories: electrophoresis [119], dielectrophoresis [120], electroosmosis [121], electroporation [122], electrofusion [123] and electric cell lysis, and these techniques have all been realized on microfluidic chips.

Dielectrophoresis (DEP) refers to the movement of cells caused by a force, generated due to polarization differences between cells and the medium under a non-uniform electric field. It has been used to move, separate and position cells [124]. Tsutsui et al. developed a quick and active method based on positive DEP traps to pattern embryonic stem cells on PEG hydrogels [53]. Mazutis et al. integrated microdroplet generation and DEP sorting on one microfluidic platform for the high-throughput analysis and sorting of single cells (Fig. 2.3a) [125]. Compartmentalization of single cells in droplets enabled the analysis of proteins secreted by cells, and fluorescence-activated droplet sorting by electrophoretic force enabled target cell enrichment.

Exposing a cell to a strong electric field pulses results in electroporation—the formation of nanoscale aqueous pores in the cell membrane. These permeable structures provide a pathway for diffusive transport of molecules which are physiologically membrane impermeable [126]. Electroporation on microfluidics alleviates heat effect, allows real-time monitoring of cellular response and enables single-cell manipulation [127]. These devices have been applied to cell transfection. Garcia et al. introduced a rapid microfluidic assay to determine the critical electric field threshold required for inducing bacterial electroporation [128]. Qu et al. utilized a droplet electroporation microfluidic platform for nuclear transformation of microalgae, which showed a remarkably higher transformation efficiency than bulk phase electroporation [129]. Kang et al. presented a microfluidic device that coupled long-term cell culture on the device and repeated temporal transfection by localized electroporation (Fig. 2.3b) [130]. This platform enabled on-chip differentiation of neural stem cells and transfection of postmitotic neurons with a green fluorescent protein plasmid.

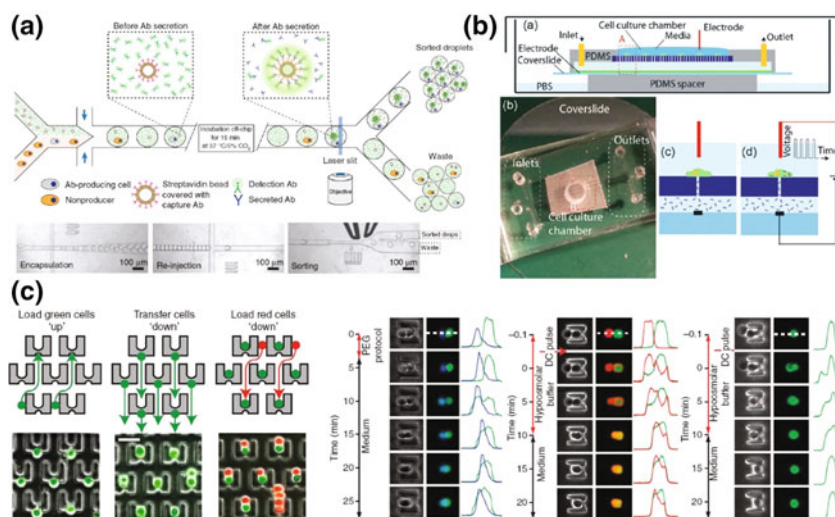


Fig. 2.3 Cell manipulation by electrokinetic operations on microfluidic chips. **a** Dielectrophoresis sorting of microdroplets containing single cells; **b** Microfluidic device for localized electroporation of postmitotic neurons; **c** Microfluidic control of cell pairing and fusion. (Reprinted with permission from Ref. [125, 130, 133])

Cell fusion is an important method to achieve nucleus transfer, hybridoma and epigenetic reprogramming of somatic cells [131]. Due to the ability of precisely controlling cell positions, electrofusion in microfluidic devices shows remarkable advantages [132]. Skelley et al. [133] presented a microfluidic device containing a dense array of weir-based passive hydrodynamic cell traps, which could immobilize and pair thousands of cells at once (Fig. 2.3c). The device is compatible with both chemical and electrical fusion protocols, with better performance of electrical fusion. 50% properly paired and fused cells were achieved over the entire device, fivefold greater than the commercial electrofusion chamber. This platform was successfully applied to the reprogramming in hybrids between mouse embryonic stem cells and mouse embryonic fibroblasts.

2.4 Cell Stimulation

In cellular microenvironment, cells are subject to multiple cues that vary in time and space, including physical conditions such as temperature, oxygen, pH, gradients of cytokines and secreted proteins from neighboring cells, and mechanical forces. Investigating cellular responses to multiple stimulations will facilitate better understanding of biological pathways, cell-fate decisions and tissue functionalities. Microfluidics is a robust technology that enables controlled perturbation of the

cellular environment spatiotemporally in vitro. By precise flow control and well-defined microstructures, it is easy to build concentration gradients and mechanical conditions in microfluidic devices. In this section, we will discuss the recent developments of cell stimulation studies in microfluidics, from three aspects: flow control, gradient generation and mechanical stimuli.

2.4.1 Flow Control

Microfluidic devices facilitate precise flow control, owing to the unique features of flow at micrometer length scale and the feasible integration of valves and pumps. The microfluidic flows are always laminar, allowing for highly predictable fluid dynamics and molecular diffusion kinetics. Laminar flow can route different fluid to specific region in a well-ordered manner, which can alter liquid-phase environment over distances and be applied to controlled cell stimulation in a high spatial and temporal resolution [15]. Lucchetta et al. used microfluidic laminar flow to create temperature differences by flowing two converging aqueous streams around an embryo, each at a controlled temperature (Fig. 2.4a) [18]. This platform was applied to differentially control the rate of development in the anterior and posterior halves of the embryo. Similar microfluidic chips were used to deliver small molecules to selected subcellular microdomains [134], for the study of mitochondrial movement [135] or neurite injury [136].

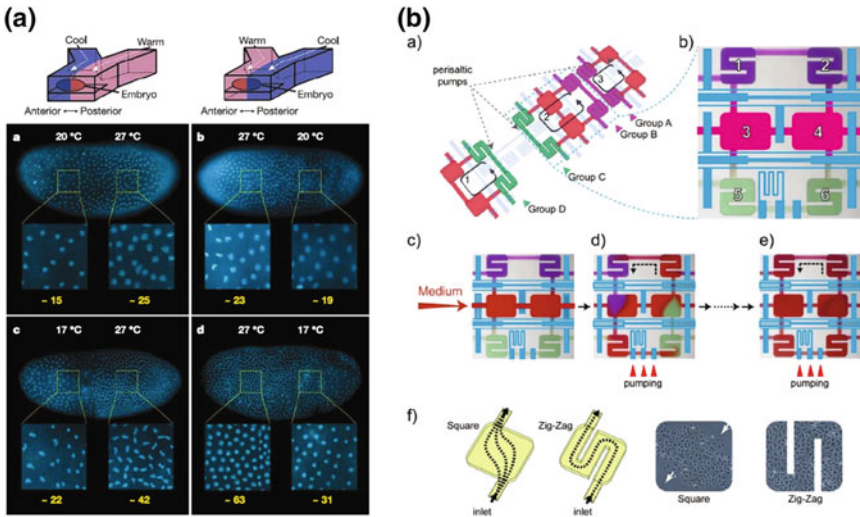


Fig. 2.4 Cell stimulation by flow control. **a** Temperature different stimulation of *Drosophila* embryo by microfluidic laminar flow; **b** Controlled cell stimulation by integrated valves or pumps to study anthrax toxin induced cell lethality. (Reprinted with permission from Ref. [18, 140])

Integrated valves or pumps, which are feasible to be incorporated into elastomeric microfluidic devices, lead to flexible, automated and high-throughput flow control with unparalleled temporal and spatial precision [137–139]. Diverse microfluidic designs with valves and pumps have been developed for various biological researches, including the precisely controlled cell stimulation. For example, Shen et al. reported a microfluidic device that integrated with pneumatic valves and peristaltic pumps to control fluid exchange parallelly and pump conditioned mediums towards the cells that exposed to toxin (Fig. 2.4b) [140]. This platform was used to investigate the roles of Dickkopf-1 in cell susceptibility to anthrax toxin. Taylor et al. developed a microfluidic platform that combined programmable on-chip mixing and perfusion with high-throughput image acquisition and processing [141]. Single-cell network responses under hundreds of combined genetic perturbations and time-varying stimulant sequences were investigated on this platform for dynamic analysis of MAPK signaling.

2.4.2 Gradient Generation

In cellular microenvironment, cells are always exposed to concentration gradients of biochemical signals such as growth factors, hormones and chemokines, which regulate many biological processes including cell differentiation, cell migration, immune responses, angiogenesis and cancer metastasis [142]. Thanks to the ability of accurate and precise flow control, it is convenient to establish concentration gradients in microfluidic systems to mimic the stimulations in microenvironment and study cellular behaviors [143]. Recently, a number of microfluidics-based gradient devices have been developed and used for different cellular studies. These devices can be categorized into two groups: flow-based gradient generators and free-diffusion-based gradient generators [144].

Flow-based gradient generators utilize laminar flows in microfluidic channels and can be divided into two design mechanisms. In Y-junction, T-junction or flow splitter microfluidic devices, streams of fluids composed of different chemical species or concentrations are brought together where the solutes diffuse across the interface as they flow down the microchannel, thus concentration gradients perpendicular to the flow direction can be established [145]. Lin et al. developed a “Y” type microfluidic device to generate concentration gradients of chemokine CCL19 and CXCL12 for T cell chemotaxis investigation [146]. In the other design mechanism, which refers to “Christmas tree”, solutions of different concentrations are introduced from the inlet, and then repeatedly split, mixed in serpentine channel regions and recombined to produce multiple streams of mixed solutions having different proportions of input solutions. These streams can be brought together into a single wide channel to generate a gradient across the channel or introduced into separate channels for parallel cell stimulation with defined concentration gradients [145]. Jeon et al. used “Christmas tree” device to generate linear or complex gradients of interleukin-8 in a single channel to investigate neutrophil chemotaxis

[17]. Similar devices were also utilized to study bacterial chemotaxis [147], to investigate neural stem cell differentiation [148], and to generate gradients of substrate-bound laminin to orient axonal specification of neurons [149]. Our group [150] developed an integrated microfluidic device for high-throughput drug screening with an online mass spectrometry analysis (Fig. 2.5a). “Christmas tree” mixer network was used to generate drug gradient, and cells in the culture chambers were stimulated with different drug concentrations separately. Drug absorption and cytotoxicity were then characterized on this platform.

In free-diffusion-based gradient generators, a gradient across a given area is established as the molecules diffuse from high concentration “source” to low concentration “sink” [151]. Porous materials such as hydrogels and semi-permeable membranes are often used to form concentration gradients between sources and sinks [152]. For example, Haessler et al. developed a microfluidic device that allowed rapid establishment of stable gradients in 3D matrices to show that dendritic cells chemotaxis in 3D could respond to CCR7 ligand gradients (Fig. 2.5b) [153]. Nguyen et al. designed blood vessels-on-a-chip by lining endothelial cells in a cylindrical channel encapsulated within a 3D collagen hydrogel [154]. Emanating from a parallel source channel, gradients of angiogenic factors were established in hydrogel and used to stimulate endothelial cells and recapitulate the angiogenic sprouting in vitro. Apart from porous gels, gradient can also be formed across a

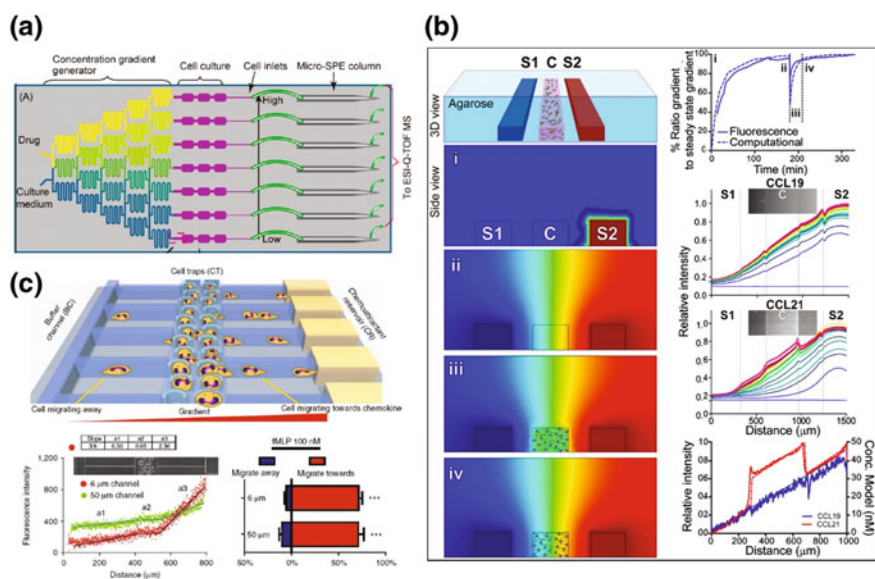


Fig. 2.5 Gradient generation on microfluidic chips for cell stimulation. **a** Drug gradient generated by “Christmas tree” mixer network; **b** Establishment and characterization of matrix bound and soluble protein gradients in a 3D hydrogel microfluidic device; **c** Gradient formed across the interconnecting channels between the source and sink for single-cell migration study. (Reprinted with permission from Ref. [150, 153, 156])

region that connects to the source and sink with microchannels. The interconnecting channels have low height, narrow width and long length, thus creating a high fluidic resistance while minimizing convective flow [152]. Chabaud et al. presented a microfluidic device composed of two fluidic chambers connected by migration microchannels [155]. Perpendicular drug gradients along migration channels were established and applied to investigate the migration and antigen capture processes of dendritic cells. Boneschansker et al. employed two large-scale arrays of microchannels to connect the central main channel with two side channels (Fig. 2.5c) [156]. Cell traps were integrated in the central channel to load precise numbers of leukocytes. Chemokine gradients were developed in the main channel by filling one side channel with chemokines and the other with buffer. This device facilitated the quantification of leukocyte migration patterns at single-cell level.

2.4.3 Mechanical Stimuli

Apart from physical and biochemical signals, cells *in vivo* are also subject to multiple mechanical cues in microenvironment, including shear stress, interstitial flow, substrate strain, confinement, compression and matrix stiffness. These mechanical processes are important for cell growth, migration, differentiation, apoptosis, and dysfunctional mechanotransduction can lead to numerous diseases [157, 158].

Microfluidics offers an excellent strategy for the study of cellular responses to mechanical stimuli. As blood flows through a vessel, it exerts shear stress on endothelial cells. The control of fluidic flow in microfluidics allows to study the impacts of shear stress on cellular morphology, behavior and functions [159, 160]. Sundd et al. integrated a microfluidic device with quantitative dynamic footprinting microscopy to study the mechanisms of neutrophil rolling at high shear stress [161, 162]. They indicated that step-wise peeling of “slings” at the front of rolling cells is responsible for the rolling of neutrophils. Miura et al. fabricated a multilayer microfluidic device to study the microvilli formation in placental transfer process (Fig. 2.6a) [163]. A broad range of fluid shear stress was applied to placental barrier cells, and cellular responses were monitored. Results showed that the fluid shear stress serves as a trigger for microvilli formation in human placental trophoblastic cells, and the molecular mechanisms were also intensively investigated.

Substrate strain is another crucial mechanical force that can manipulate cellular alignment and tissue functions [164]. By incorporating flexible substrates into microfluidic platforms, devices have been developed to study the effect of mechanical stretch on the cells cultured on the deformable substrate. Hsieh et al. developed microfluidic chip which consisted of a concentric circular hydrogel pattern and a flexible PDMS membrane (Fig. 2.6b) [165]. A range of gradient static strains on cells can be generated by compressing the cell-laden hydrogels with the membrane, and cells were elongated with the increase of strain. In previously introduced example, by applying and releasing vacuum in two larger, lateral

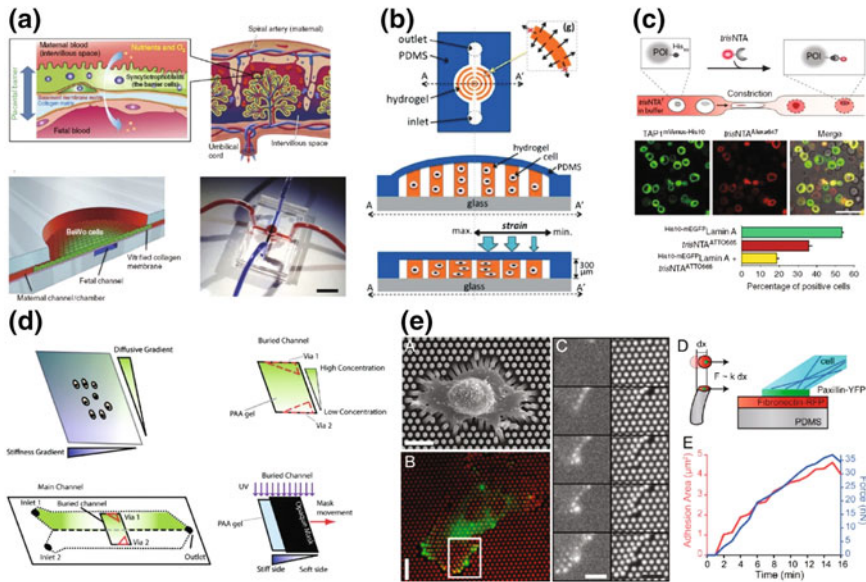


Fig. 2.6 Mechanical cell stimulation on microfluidic chips. **a** Microfluidic platform to study impacts of fluid shear stress on placental barrier cells; **b** Gradient static strain stimulation in a microfluidic chip for 3D cellular alignment; **c** Compression-induced cell deformation for living cell labeling; **d** Generation of stable orthogonal gradients of chemical concentration and substrate stiffness in a microfluidic device; **e** Measurement of mechanical forces generated by cells using micrometre-sized elastomer post arrays. (Reprinted with permission from Ref. [163, 165, 166, 170, 176])

microchambers, PDMS membrane with the adherent tissue layers were stretched and recoiled [93]. This “breathing” human lung-on-a-chip could replicate the dynamic mechanical distortion of the alveolar-capillary interface caused by breathing movements.

Compressive stress on cells was also realized in microfluidic devices. Kollmannsperger et al. utilized the compression-induced cell deformation for rapid and efficient transfer of trisNTA probe into living cells (Fig. 2.6c) [166]. Cells were compressed through micrometre constrictions in a microfluidic device, causing the formation of transient holes in the plasma membrane. Si et al. developed an air-driven microfluidic device to apply a compressive force on *Escherichia coli* cells [167]. With compression, cells no longer retained their rod-like shapes but grew and divided with a flat pancake-like geometry.

The mechanical stiffness of extracellular matrix has proven to be a crucial regulator for cell growth, differentiation, movement and functions [168]. Microfluidics offer versatile platforms for the study of cellular responses to matrix stiffness. Sundararaghavan et al. designed and developed a microfluidic device to investigate the neurites growth under stiffness gradients [169]. An “H”-shaped, source–sink network was utilized to generate a gradient of genipin (a collagen

crosslinker) in type I collagen solution. After self-assembly, hydrogel with a gradient of mechanical stiffness, linearly ranging from 57 to 797 Pa, was formed in the device. Chick dorsal root ganglia was introduced into the gradient and results shown that the neurites extended preferentially down the stiffness gradient. García et al. presented a robust microfluidic device that generated a stable, linear and diffusive chemical gradient over a biocompatible hydrogel with a well-defined stiffness gradient (Fig. 2.6d) [170]. Matrix stiffness was regulated by UV exposure level of the polyacrylamide hydrogel. This device was then used to study the cell scattering in response to perpendicular gradients of hepatocyte growth factor and substrate stiffness.

Except for the mimicking of cellular mechanical environments, microfluidic techniques can also be used to measure mechanical forces generated by cells [171]. Christopher S. Chen group has developed micrometre-sized elastomer post arrays to manipulate and measure mechanical interactions between cells and their underlying substrates. Cells attached to, spread across, and deflected multiple posts. And the deflections of the posts directly reported the subcellular distribution of traction forces. Values of the forces could be measure according to the geometry change. By controlling cell adhesion on these micromechanical sensors, it showed that cell morphology regulated the magnitude of traction force generated by cells. Unspread cells did not contract in response to stimulation by serum or lysophosphatidic acid, whereas spread cells did [172]. Similar micro-fabricated device has also been utilized for mapping mechanical forces during epithelial cell migration [173]. Besides, by alternating the post heights or pillar diameters, the stiffness of substrates could be regulated, and their effects on cell morphology, focal adhesions, cytoskeletal contractility and stem cell differentiation were then investigated [174, 175]. The mechanosensing mechanism for cellular adaptation to substrate stiffness was also intensively studied (Fig. 2.6e) [176].

2.5 Cell Analysis

In native cellular microenvironment, cells are surrounded with multiple biophysical and biochemical cues and also respond to the various stimuli. Thus analyzing cells, including the cell morphology, cellular contents, cell signaling and cell secretion, is crucial for intensive understanding of biological processes and mechanisms. Owing to the unique merits, including flexible design, low sample consumption, high throughput, ease of integration and automation, microfluidic technology has been regarded as a robust and promising tool for cell analysis [11, 31, 177]. Almost every analytical tool available in a conventional biology lab has an equivalent microfabricated counterpart on microfluidic devices. And the microfluidic chips are also feasible to integrate with diverse analytical instruments, such as microscopy, electronic operation, mass spectrometry and nuclear magnetic resonance, to analyze cells for various purposes [21]. In this section, we will introduce the recent developments of cell analysis on microfluidics, both in sample preparation and analytical systems.

2.5.1 Sample Preparation

2.5.1.1 Cell Sorting

Isolating and sorting cells from complex, heterogeneous cell mixtures is a critical preparatory step in many biological and medical assays, enabling the enrichment of cell samples into well-defined populations or the isolation of rare cells such as circulating tumor cells (CTCs) and hematopoietic stem cells (HSCs) from much larger population of background cells [178]. It enhances the efficiency in biological researches and diagnosis, and facilitates the understanding of accurate underlying biochemical information of specific cell types in a mixture. Recent advances in microfluidics promote high-throughput cell sorting, and this has led to various novel diagnostic and therapeutic applications that are difficult to implement using conventional technologies [179]. Microfluidic cell sorting techniques are either based on the inherent physical properties of cells, such as cell size, morphology, electrical properties and cell-fluid interactions, or on account of the differences after affinity labeling [180, 181]. And depending on the utilization of external forces, microfluidic cell sorting can also be classified as passive and active strategies. Passive sorting relies on the channel geometry (pillar and weir structures, microfilters), hydrodynamic forces (pinched flow fractionation, hydrodynamic filtration, inertial separation) and surface modification (affinity-based separation) for functionality. While active sorting applies external forces such as electric, magnetic, acoustic and optical forces for cell separation [182]. Detailed examples are listed in Table 2.1 [183].

Affinity ligands for cell surface markers can be used either to provide a force for separation, such as in cell affinity separation and capture, or as labels in fluorescence activated cell sorting (FACS) and magnetic activated cell sorting (MACS). It improves the selectivity and are quite suitable for cell types that are physically similar to the background cell populations. In cell affinity separation, affinity ligands such as antibodies, aptamers and proteins are modified on chip surface. Cells with specific markers can be selectively captured in channel through the ligand-receptor interactions, while other cell types are passed through the device. The large surface to volume ratio of microfluidic channels significantly increases the possibility of cell-to-surface interactions and leads to a better isolation performance [180, 184]. The geometry of the channel can affect the cell flow and cell capture efficiency. Stott et al. demonstrated a herringbone-chip (HB-chip) that allowed passive mixing of blood cells through the generation of microvortices to significantly increase the number of interactions between target CTCs and the antibody-coated chip surface (Fig. 2.7a) [185]. The HB-chip was used for CTC separations, identifying CTCs in 93% of patients with metastatic disease. The low shear flow properties enabled the isolation of previously unappreciated micro-clusters of CTCs, facilitating the investigation of the association between expression of mesenchymal markers and CTC clusters [186]. Chen et al. developed a microfluidic chip with a microwell array that was encoded with cell-recognizable

Table 2.1 List of various active and passive cell separation methods. (Reprinted with permission from Ref. [183])

| | Method | Mechanism | Separation markers | Flow rate/throughput |
|---------|----------------------------------|---|--|---|
| Active | Flow cytometry (FACS) | Fluorescence | Fluorescence labels | 100 s ⁻¹ |
| | Magnetic | Homogeneous/inhomogeneous magnetic field | Size, magnetic susceptibility | 10,000 s ⁻¹ |
| | Dielectrophoresis | Inhomogeneous electric acid | Size, polarizability | 10,000 s ⁻¹ |
| | Electrophoresis | Homogeneous electric acid | Size, charge density | 0.1–0.2 mm s ⁻¹ |
| | Optical | Optical force | Size, refractive index, polarizability | 1500 min ⁻¹ |
| | Acoustic | Ultrasonic standing waves | Size, density, compressibility | 0.1–0.4 mL min ⁻¹ |
| Passive | Pillar and weir structures | Laminar flow | Size, deformability | 1000 μm s ⁻¹ |
| | Pinched flow fractionation (PFF) | Hydrodynamic force (parabolic velocity profile) | Size | ~4000 min ⁻¹ 20 μL h ⁻¹ |
| | Hydrodynamic filtration | Hydrodynamic force | Size | 20 μL min ⁻¹ >100,000 min ⁻¹ |
| | Inertial | Shear-induced and wall-induced lift | Size, shape | ~10 ⁶ min ⁻¹ |
| | Surface affinity | Specific binding to surface markers | Surface biomarkers | 1–2 mL h ⁻¹ |
| | Biomimetic | Hydrodynamic force/Fahraeus effect | Size, deformability | 10 μL h ⁻¹ |
| | Hydrophobic filtration | Pressure field gradient | Size | 4 × 10 ³ s ⁻¹ |

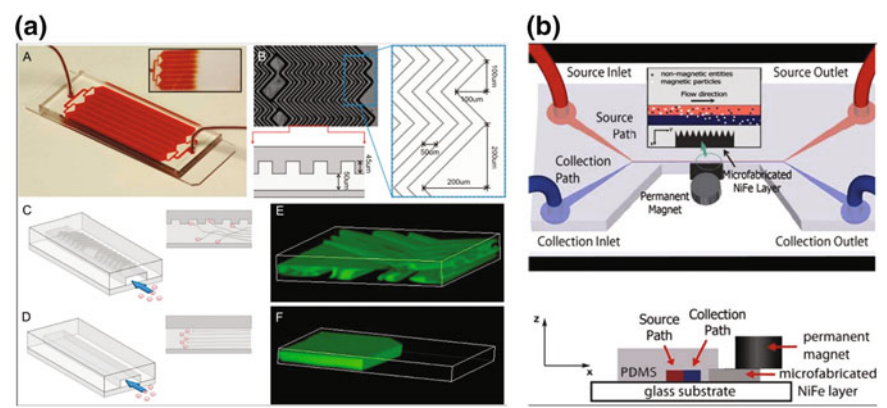


Fig. 2.7 Cell sorting on microfluidic chips. **a** Isolation of CTCs using a microvortex-generating herringbone chip; **b** Lateral magnetophoresis for cell separation. (Reprinted with permission from Ref. [185, 198])

aptamer [187]. Single tumor cells were isolated with 88.2% single-cell occupancy, and various cellular carboxylesterases were studied by time-course measurements of cellular fluorescence kinetics at individual-cell level. Recovery of captured cells can be achieved by using specific enzymes for antibodies or aptamers digestion or photocleavable linkers for ligand immobilization.

In FACS, cells are labeled by antibodies conjugated to fluorophores and selected according to their fluorescence signals. Traditional FACS has been thoroughly developed and widely applied in biological and pharmaceutical researches and industries [188]. More recently, FACS has been implemented in microfluidic devices, achieving high separation purity, high efficiency and low sample consumption. Microfluidic FACS generally operates by ordering cells in flow streams for: (i) serial interrogation by laser light, (ii) real-time classification, and (iii) rapid, command-driven sorting [29]. Sorting process can be driven by electrokinetic mechanisms (including electrophoresis, dielectrophoresis and electroosmotic flow), acoustophoresis, optical manipulations and mechanical forces [189–194]. However, the throughput of microfluidic FACS is not high enough to compete with the commercial, large-scale instruments systems. Thus further efforts should be made to improve the throughput, for example by using a pulsed laser triggered sorting to reduce the switching time [195] or by building a parallel array of microfluidic sorting units for simultaneous operation.

In MACS, cells are first labeled by magnetic beads coated with affinity ligands, and a strong magnetic field is used to isolate the magnetic beads that are attached to the desired cell population. The immunomagnetic-based separation is highly specific, biocompatible and high-throughput. Enrichment of up to 10^{11} cells in less than 30 min has been reported [184]. Many macroscale MACS separations have been adapted in microfluidic systems, which have the advantages of low sample costs, fluid flow condition and parallel operations [29, 196]. Among these approaches, continuous separation by means of magnetophoresis has been well-studied and applied to cell separation. For example, Hoshino et al. developed an immunomagnetic microchip for CTC detection [197]. As the blood sample flowed through the microchannel closely above arrayed magnets, cancer cells labeled with magnetic nanoparticles were captured and separated from blood flow. CTCs with low cancer cell to blood cell ratios (about $1:10^7$ to 10^9) were detected, at a fast screening speed (10 mL/h). Xia et al. developed a lateral magnetophoresis for cell separation (Fig. 2.7b) [198]. This method used a horizontal magnetic field to drag labeled target cells from the sample flow into the buffer flow. Living *E. coli* bacteria bound to magnetic nanoparticles were efficiently removed from flowing solutions containing densities of red blood cells similar to that found in blood. Similar design was also utilized in CTC separation, which isolates about 90% of spiked CTCs in human peripheral blood with a purity of 97%. The overall isolation procedure was completed within 15 min for 200 μ L of blood [199].

2.5.1.2 Cell Lysis

Cell lysis is an essential step for the analysis of cellular contents such as proteins and nucleic acids. Microfluidic cell lysis has several advantages over conventional approaches. The unique geometries and precise dimensions allow for finely tuned mechanical or chemical cell perturbation. Micrometer length scale minimizes lysate dilution. And laminar flow limits the convective transport of lysate. These properties facilitate the increase of analyzing sensitivity [200, 201]. Microfluidic cell lysis approaches can be categorized into four major groups: mechanical lysis, thermal lysis, chemical lysis and electrical lysis [202]. In this section, we will discuss these four cell lysis approaches and provide some recent examples.

Mechanical cell lysis tears or punctures cell membranes by mechanical forces, which include shear stress, collision with sharp features, friction forces and compressive stress. In this way, cell structures are disrupted and intracellular components are released [201]. Yun et al. presented a handheld mechanical cell lysis chip with ultra-sharp nano-blade arrays (Fig. 2.8a) [203]. Cells bumped into the blades were easily ruptured by these ultra-sharp nanostructures. This chip can be directly connected to a commercial syringe, and the protein concentration obtained by this chip is quantitatively comparable to the conventional chemical lysis method. Kim et al. developed a microfluidic CD (Compact Disc) platform for cell lysis [204]. Cells were mixed with granular particles, and the solution was placed into an annular channel on the chip. When the disc was rotated at high speeds around a horizontal axis, cells were broken up by the frictions and collisions between the cells and particles. Mammalian cells, bacteria and yeast cells could all be effectively lysed and the lysis efficiency relative to a conventional lysis protocol was approximately 65%. Combining a magnetically actuated bead-beating system with this CD chip could further improve the lysis efficiency [205]. These platforms were utilized in nucleic acid extraction from clinical samples. Mechanical lysis can relatively minimize the protein damage and avoid detergent interferences. However, it requires additional instrumentation or operation for activation, and the cell debris produced in mechanical lysis may hinder subsequent extraction.

In electrical cell lysis, cells are exposed to strong electric fields. The membranes are destabilized and pores are formed. As the osmotic pressure between the cytosol and the surrounding media becomes unbalanced, cells swell, rupture and eventually lyse. The electric field can be tuned for rapid cell lysis without denaturing target biomolecules. And considering difference between trans cell-membrane potential and trans organelle-membrane potentials, appropriate electric field strength and exposure time can selectively rupture the cell membrane while leave organelles intact [2, 201]. Electrical cell lysis is well suitable for microfluidics applications. Mellors et al. developed a microfluidic device for automated real-time analysis of individual cells using capillary electrophoresis (CE) and electrospray ionization mass spectrometry (ESI-MS) [206]. Cell lysis occurred at a channel intersection using a combination of rapid buffer exchange and an increase in electric field strength (4 kV). The cell lysis rate is 0.2 cells per second. Jokilaakso et al. reported a microfluidic device which positioned individual cells on silicon nanowire biological field effect transistors by

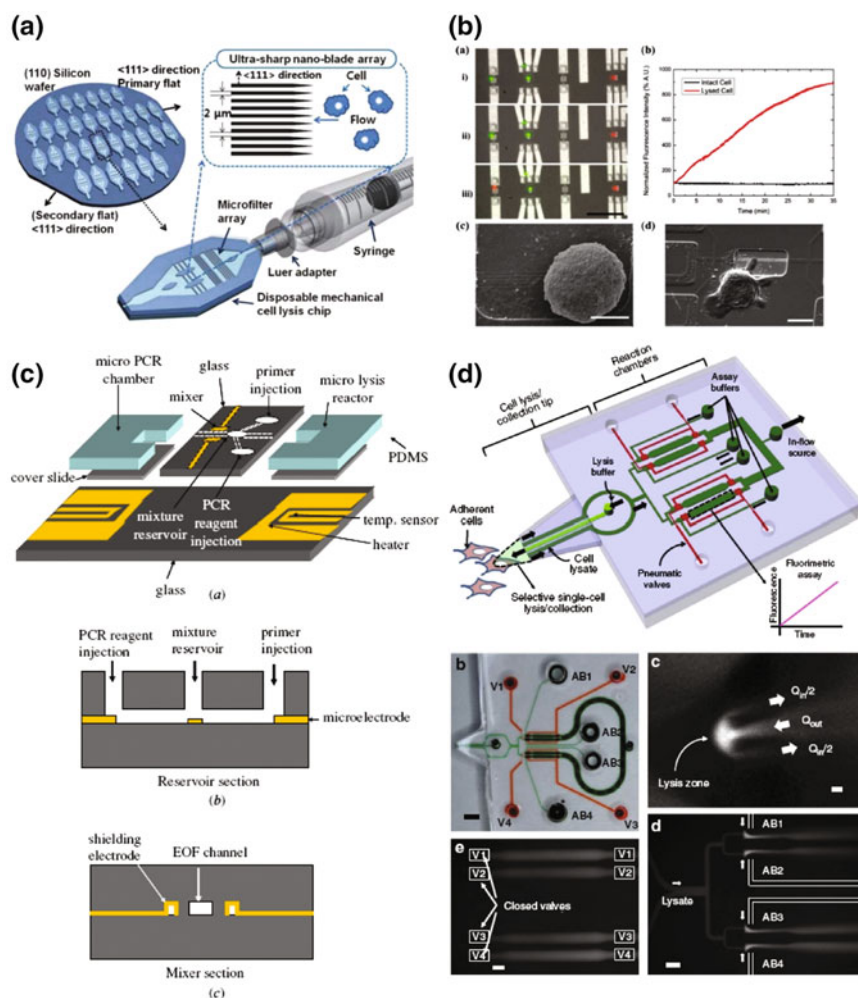


Fig. 2.8 Cell lysis on microfluidic chips. **a** Mechanical lysis; **b** Electrical lysis; **c** Thermal lysis; **d** Chemical lysis. (Reprinted with permission from Ref. [203, 207–209])

manipulating magnetic beads using external magnetic fields (Fig. 2.8b) [207]. Ultra-rapid cell lysis was subsequently performed by applying 600–900 mVpp at 10 MHz for as little as 2 ms across the transistor channel and the bulk substrate. This system was used to study the single cell variation within a population.

Thermal cell lysis utilizes high temperature to denature the proteins within cell membranes, thus irreparably damaging the cells and releasing the cytoplasmic contents. This method is commonly used in nucleic acid preparation, combining with PCR-based assays. Thermal lysis can be performed by ohmic heating, induction heating or heat generated by irradiating nanoparticles. For example,

Lee et al. presented an automated microfluidic chip capable of performing thermal cell lysis, electrokinetic sample/reagent transportation and mixing, and DNA amplification (PCR) (Fig. 2.8c) [208]. Two sets of micro-heaters and micro temperature sensors were integrated in cell lysis reactor and PCR chamber, to regulate and monitor the temperature. Cells could be lysed within 2 min at a constant temperature of 95 °C and PCR amplification of a 273 bp *Streptococcus pneumoniae* were demonstrated. Thermal lysis is a simple method and has been well-established for nucleic acid analysis. However, the thermal damage of proteins restricts its application in immunoassays.

Chemical cell lysis uses lytic agents to break down the cell wall and/or membrane. There are various chemical reagents which are chosen based on the cell types and target molecules. Detergents such as sodium dodecyl sulfate (SDS) and Triton X-100 disrupt cell membranes by solubilizing membrane proteins and lipids and creating pores. Ammonium chloride can only lyse the erythrocytes. For bacterial lysis, enzymatic degradation step such as lysozyme treatment is essential to destroy the cell wall. Chaotropic salts such as guanidinium thiocyanate and guanidinium chloride lyse cell membranes by disrupting protein intermolecular forces. They are most commonly used in nucleic acid preparations [200]. Chemical cell lysis is easily incorporated in microfluidic chips. Sarkar et al. presented a microfluidic probe that chemically lysed single adherent cells from standard tissue culture using commercial lysis buffer and captured the contents to perform single-cell biochemical assays (Fig. 2.8d) [209]. This device was applied to measure kinase and housekeeping protein activities from single human hepatocellular carcinoma cells in adherent culture. Chemical cell lysis is simple to implement and needs only mixing for activation. However, the chemical reagents in lysis buffer may interfere subsequent extraction and detection, for example when target molecules are analyzed by mass spectrometry. Thus additional separation steps will be required to remove the reagents.

2.5.1.3 Sample Separation

Preconcentration and separation of biomolecules (nucleic acids, proteins, metabolites etc.) from complex cell lysate are quite important for sensitive detection and successful downstream processing. Microfluidics offer unique advantages for sample separation compared to conventional bench-top methods [32]. Microscale channels reduce sample and reagent consumption. The ease of integration on microfluidic devices enables the coupling of multiple separation techniques, which improves the separation efficiency. The separation step can also be integrated with lysis and analysis steps in an automatic manner, and this can minimize manual sample handling and preserve sample integrity, thereby improving the accuracy and reproducibility [210].

Many separation techniques can be operated in microfluidic devices, including chromatography, electrokinetic separation, solid-phase extraction (SPE), liquid-liquid extraction and filtration [211]. Each method has its own merits and applicative analytes. These separation techniques can also be integrated in one

device to develop multidimensional separations works, which increase the capacity, throughput and efficiency.

Microfluidic electrokinetic separation, such as microchip capillary electrophoresis (MCE), gel electrophoresis, electrochromatography, isoelectric focusing (IEF) and isotachopheresis (ITP), are more commonly used for biomolecule fractionation and enrichment [212–214]. In microchip electrophoresis, charged molecules are separated based on their electrophoretic mobility under an electric field. Liu et al. developed a portable lab-on-a-chip system comprising a PCR reactor and a 7-cm-long separation channel for capillary electrophoretic analysis (Fig. 2.9a) [215]. This device was utilized in real-time forensic short tandem repeat (STR) analysis. Alleles which differed by 1 bp could be separated, and the CE separation process was completed in 8 min. Lin et al. presented a multiplex protein assay based on tunable aptamer by MCE [216]. Different lengths of aptamers could modulate the electrophoretic mobility of proteins, allowing the proteins to be effectively separated. This method was applied to analyze the PDGF-BB and VEGF₁₆₅ from cell secretions. ITP uses a heterogeneous buffer system consisting of high-mobility ions in the leading electrolyte (LE) and low-mobility ions in the terminating electrolyte (TE); an applied electric potential separates the ionic species based on their electrophoretic mobility, thus focusing the analytes at the LE/TE interface [217]. Schoch et al. demonstrated an on-chip ITP for the simultaneous extraction, isolation, preconcentration and quantitation of small RNAs from cell lysate [218]. It separated small RNAs from precursor miRNAs in less than 3 min, and the minimal cell number for small RNA extraction and detection is 900 (from a 5 mL sample volume).

In microchip chromatography system, stationary phase plays a critical role in molecules separation. There are a variety of stationary phase materials, including chromatography resins, monoliths and nanowires. In addition, surface

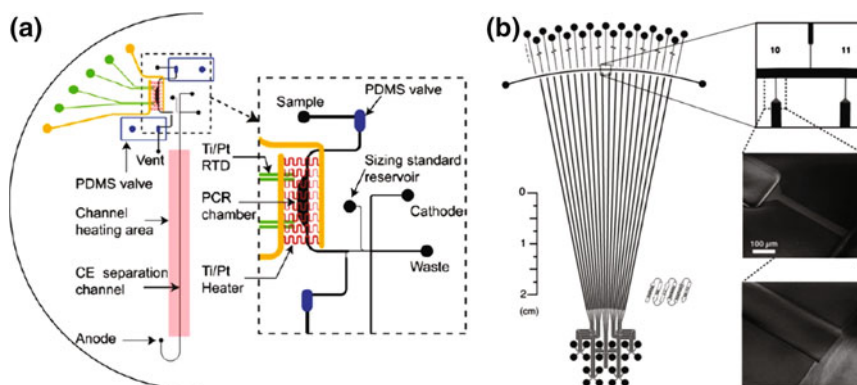


Fig. 2.9 Sample separation on microfluidic chips. **a** A portable lab-on-a-chip system comprising a PCR reactor and a 7-cm-long separation channel for capillary electrophoretic analysis; **b** Two-dimensional differential gel electrophoretic separations (IEF-CE). (Reprinted with permission from Ref. [215, 231])

functionalized microchannels (open channel system) and “pillar structured” microchannels are also studied as stationary phases [219, 220]. Several companies have launched nanoLC systems with integrated liquid chromatography (LC) microchips for chip-based LC-MS analysis. The cHiPLC-nanoflex system from Eksigent Technologies, Trizaic UPLC nanoTile system from Waters and Chip Cube from Agilent are currently commercially available and have been used in various fields of bioanalytical applications including proteomics, glycomics, and clinical and pharmaceutical studies [221]. Chen et al. coupled the cHiPLCnanoflex system to an Orbitrap spectrometer [222]. After multistep sample preparation, peptide mixtures were separated in cHiPLC-nanoflex equipped with a 15 cm \times 75 μ m C18 column. high peptide coverage for 12 γ -aminobutyric acid type A receptor (GABA_A receptor) subunits was obtained from 2 pmol of affinity-purified GABA_A receptors from rat brain neocortex.

Microchip solid-phase extraction is also a widely used method for sample pre-concentration and clean-up. In microchip SPE, sample is retained on a solid phase, allowing the matrix to be rinsed away, and then the retained analytes was eluted for analysis [211]. Various solid phases, including packed-bead columns [223], monolith columns [224] and affinity columns [225] have been incorporated in microfluidic devices. Our group has developed a series of microfluidic systems which integrated drug injection, cell culture chambers and micro-SPE column in one device and coupled to mass spectrometry. The micro-SPE was used for desalination and extraction of cell metabolites. These devices have been applied to study drug metabolism in cell co-culture systems [150, 226–228]. Hagan et al. utilized chitosan-coated silica as a solid phase for RNA purification in a microfluidic device [229]. The effectiveness of the chitosan phase was demonstrated by the successful purification of RNA from cancer cells, with 3.5-fold greater extraction efficiencies than that purified by the simple silica phase.

Multidimensional separation is a significant advantage for microfluidic separation techniques. Various multidimensional microfluidic systems have been developed, such as micellar electrokinetic chromatography (MEKC)-CE, IEF-CE, ITP-CE, IEF-free-flow electrophoresis (FFE) [210, 230]. Emrich et al. developed a microfluidic separation system to perform two-dimensional differential gel electrophoretic separations of complex protein mixtures (Fig. 2.9b) [231]. This two-layer borosilicate glass microdevice consisted of a single 3.75 cm long channel for IEF, which was sampled in parallel by 20 channels effecting a second-dimension separation by native CE. The connection between the orthogonal separation systems was much shallower, narrower channels, which could prevented media leakage between the two dimensions and enabled facile loading of discontinuous gel systems in each dimension. Reproducible separations of both purified proteins and complex protein mixtures produced by *E. coli* were performed with minimal run-to-run variation.

2.5.2 Cell Analysis

Microfluidic devices are feasible to integrate with diverse analytical techniques, including optical detector, electrical analysis, mass spectrometry and nuclear magnetic resonance, to perform both qualitative and quantitative cell analysis for various purposes [21]. Optical detection, such as absorbance, fluorescence, infrared (IR) and surface plasmon resonance (SPR), are the most widely used techniques in microfluidics for cell analysis [232–234]. Owing to the transparency of microfluidic chips, various types of microscopy can be integrated to imaging the cell morphology, structure, movements, as well as specifically labeled cellular contents. Optical detector such as absorbance, laser-induce fluorescence (LIF), chemiluminescence (CL) can also be coupled with microfluidic separation channels, for the detection and quantification of fractionated biomolecules [235]. Electrical analysis, such as amperometry, electrochemical impedance spectroscopy (EIS), or patch-clamp, can be incorporated in microdevice to monitor cell secretion, cell morphology and migration, cell monolayer permeability, cell membrane electrophysiologic activity, and so on [236–239]. Mass spectrometry, as a powerful analytical technique, is commonly coupled with microfluidics for the analysis of cellular contents (DNA, protein, glycan etc.) and metabolites [240–242]. Integrating with these analytical techniques, microfluidic systems enable rapid, sensitive, reproducible and high-throughput cell analysis, which promotes the development of both basic biological researches and clinic diagnosis and therapy [243, 244]. In this section, we will introduce cell analysis using microfluidic devices integrated with diverse analytical techniques, according to the analysis objects. Some recent examples are also reviewed here.

2.5.2.1 Cell Morphology and Movement

In cellular microenvironment, cells are surrounded with multiple physical and biochemical cues. Cell morphology and movement are the most intuitionistic parameters that can reflect cellular responses to the diverse stimuli. By integrating biomimetic cell culture systems with various types of microscopy or electrical techniques, microfluidics offers a robust platform for real-time monitoring of cell morphology alteration and cell movements.

Fluorescence imaging are the most commonly used technique for cell observation. Cells cultured in microchannels are labeled by fluorescent dyes or proteins and visualized under fluorescence microscopy. Giobbe et al. described a multistage microfluidics-based approach for the differentiation of human pluripotent stem cells [245]. Cell morphology changed during factor stimulation and cell differentiation were observed by immunofluorescent staining of F-actin. And with the increase of acetaminophen concentration, morphology of human embryonic stem cell was disrupted, especially at 25 mM with almost complete loss of cell structure and function. Cell movement can also be altered under external stimuli, which is a

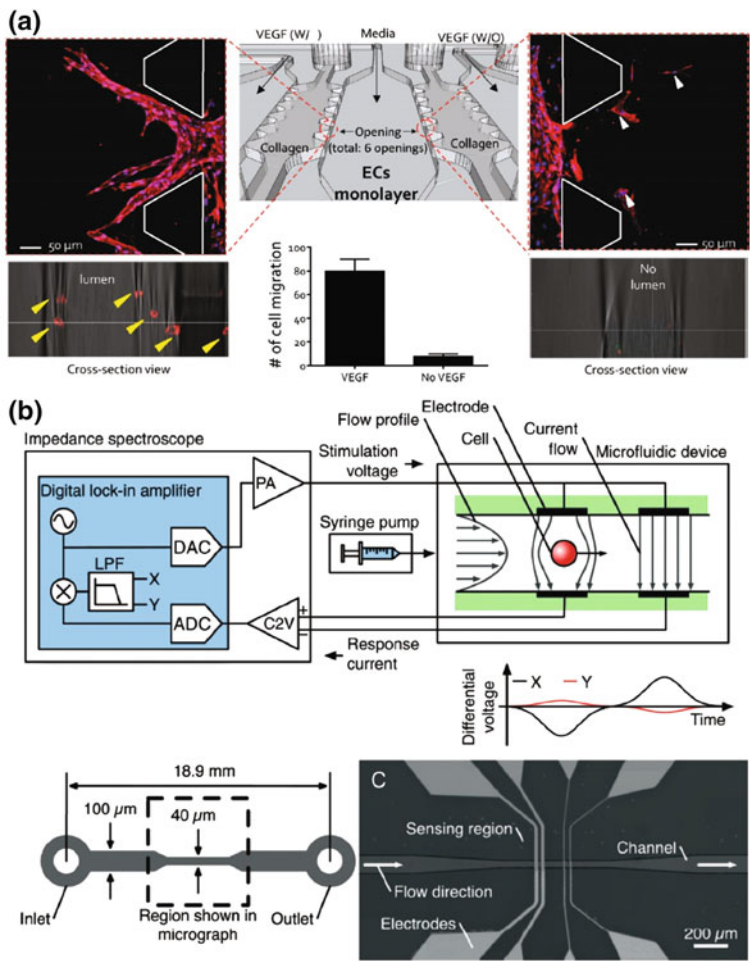


Fig. 2.10 Cell morphology and movement analysis on microfluidic chips. **a** Microfluidic cell angiogenesis assay combining with fluorescence imaging; **b** Microfluidic impedance cytometer for subcellular morphology characterization. (Reprinted with permission from Ref. [249, 250])

pivotal step in angiogenesis, cancer invasion and metastasis [246–248]. Kim et al. proposed a microfluidic platform that could monitor and quantify cellular behaviors, including morphological changes, cell migration and formation of angiogenic sprouts, under the treatment of anti-angiogenic drug (Fig. 2.10a) [249]. Results showed that cells rapidly and actively migrated from the endothelial channel into the 3D hydrogel scaffolds toward the VEGF-supplemented media channel, and this process could be inhibited by anti-angiogenic drug bortezomib.

Electrical techniques can also be incorporated in microfluidic devices for cell morphology analysis. Haandbæk et al. demonstrated a microfluidic single cell impedance cytometer capable of dielectric characterization of single cells at

frequencies up to 500 MHz (Fig. 2.10b) [250]. The increased frequency range allowed for characterization of subcellular morphology, such as vacuoles and cell nuclei, in addition to the properties detectable at lower frequencies. This device could discriminate wild-type yeast cells from those with a mutant, which differed in size and distribution of vacuoles in the intracellular fluid.

2.5.2.2 Genetic Analysis

Genetic analysis is one of the most extensively developed field in microfluidic cell analysis. Since the amount of the nucleic acids extracted from cells is relatively small, nucleic acid amplification processes play a critical role in sensitive detection and quantification [251]. Polymerase chain reaction (PCR) is the most widely used non-isothermal amplification technique, which performs thermal cycling to amplify a few copies of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence. Quantification of RNA can be achieved by performing reverse-transcription PCR (RT-PCR). There are also various isothermal amplification techniques, such as strand displacement amplification (SDA), nucleic acid sequence based amplification (NASBA), rolling circle amplification (RCA), and loop mediated isothermal amplification (LAMP) [252]. Both isothermal and non-isothermal amplification have been successfully performed in microfluidic devices, with advantages including faster reaction times, low sample consumption, precise temperature distribution and the ease of integrating with separation techniques [253, 254]. Zhang et al. developed a targeted RNA sequencing method that coupled microfluidics-based multiplex PCR with deep sequencing [255]. This system could uniformly and simultaneously amplify up to 960 loci in 48 samples on a single microfluidic chip independent of their gene expression levels. Resulting PCR amplicons were barcoded for each sample and then subjected to deep sequencing to obtain high coverage allowing accurate measurement of allelic ratios. Fang et al. demonstrated a portable microchip-based LAMP platform, which performed rapid DNA release, exponential signal amplification and naked-eye result read-out in single or multiplex format [256]. This device was successfully used for point-of-care identification of bacteria.

Microfluidic techniques have been applied to various areas of genetic analysis, including sequencing, gene expression analysis [257], pathogen detection [258], and forensic STR typing [259]. High-throughput single-cell transcriptome sequencing (RNA-Seq) offers an unbiased approach for understanding the extent, basis and function of gene expression variation between seemingly identical cells. Currently, several microfluidic-based single-cell RNA-Seq platforms have been developed and applied to study transcriptional heterogeneity of cancer [260, 261], immune [262] and stem cells [263]. Streetsv et al. [264] presented a microfluidic-based system to perform single-cell whole-transcriptome sequencing (Fig. 2.11a). Single cells were captured and lysed in a microfluidic device, where mRNAs with poly(A) tails were reverse-transcribed into cDNA. Double-stranded cDNA was then collected and sequenced using a next generation sequencing

platform. This technique enabled the identification and quantification of biological variation in a population of mouse embryonic stem cells at the single-cell level. Shalek et al. used a commercially available microfluidic device (C1 single-cell Auto Prep System, Fluidigm) and a transposase-based library preparation strategy to help prepare over 1700 single-cell RNA-seq libraries along time courses of bone-marrow derived dendritic cells responding to different stimuli [265]. Transcriptome-wide changes in single-cell expression variation across a variety of conditions was tracked and how both antiviral and inflammatory response modules in dendritic cells were controlled by positive and negative intercellular paracrine signaling were illustrated.

Chromatin immunoprecipitation coupled with deep sequencing (ChIP-seq) has also been successfully operated on microfluidics for the examining of *in vivo* genome-wide chromatin modifications (Fig. 2.11b) [266]. Magnetic beads coated

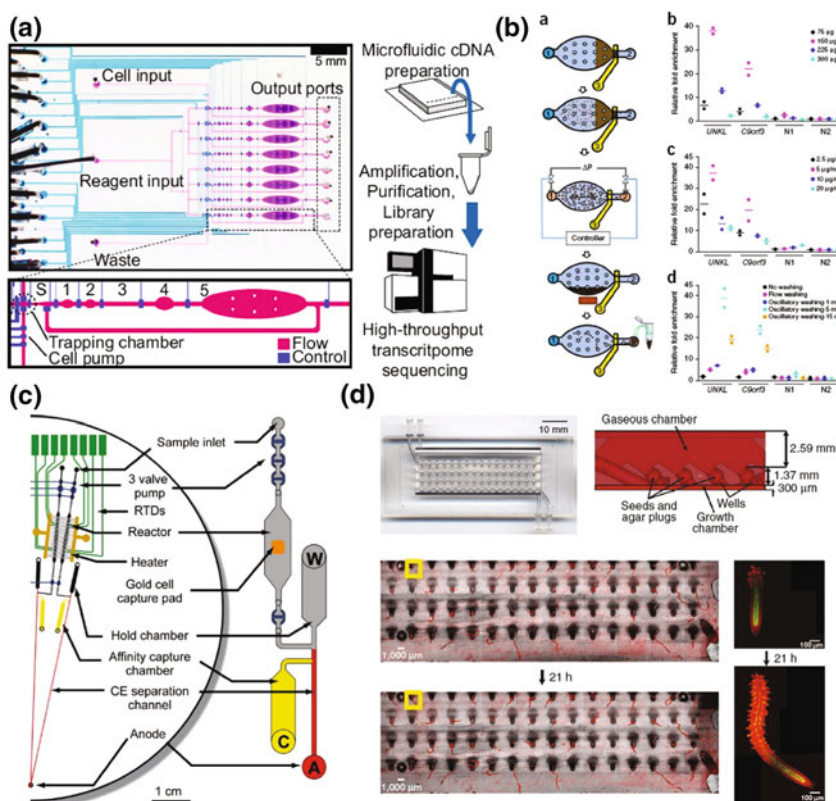


Fig. 2.11 Genetic analysis on microfluidic chips. **a** High-throughput single-cell transcriptome sequencing microfluidic system; **b** Microfluidic ChIP-seq; **c** An integrated microdevice for the analysis of gene expression in single cells; **d** A microfluidic platform for high-throughput live imaging of gene expression. (Reprinted with permission from Ref. [264, 266, 268, 269])

with a ChIP antibody were flowed into the microfluidic chamber and form a packed bed. It facilitated rapid and high-efficiency adsorption of target chromatin fragments, and the nonspecific adsorption was removed by effective oscillatory washing. These target chromatin samples were then purified and sequenced. Using this technology, many new enhancers and super enhancers in hematopoietic stem and progenitor cells were uncovered, suggesting that enhancer activity was highly dynamic during early hematopoiesis.

Even genetically identical cells with seemingly identical cell histories and environmental conditions can have significant differences in gene expression levels, due largely to the alteration of mRNA production by random fluctuations or complex molecular switches. Thus quantitative analysis of gene expression at single-cell level is important for the understanding of basic biological mechanism and disease onset and progression [267]. Toriello et al. developed an integrated microdevice for the analysis of gene expression in single cells (Fig. 2.11c) [268]. This device consisted of integrated nanoliter metering pumps, a 200 nL RT-PCR reactor with a single-cell capture pad, and an affinity capture matrix that was coupled to a microchip capillary electrophoresis separation channel for product purification and analysis. It was used to measure siRNA knockdown of the GAPDH gene in individual Jurkat cells and indicated the presence of 2 distinct populations of cells with moderate or complete silencing. Busch et al. developed a high-throughput microfluidic device in which 64 *Arabidopsis thaliana* seedlings could be grown and their roots were imaged by confocal microscopy over several days without manual intervention (Fig. 2.11d) [269]. This device was used to quantify expression patterns of 12 reporter genes in roots growing in different conditions and identified several cases of transient or heterogeneous expression.

2.5.2.3 Protein Analysis

Proteins are one of basic component of cells, which perform and regulate various cellular functions. Owing to the low abundance and high complexity, the development of sensitive and reliable protein analysis techniques are highly desirable. Microfluidics offer rapid, sensitive, reproducible and high-throughput platforms for protein analysis. Various aspects, including protein species, amounts, activity, as well as protein interaction with other biomolecules, can be analyzed using microfluidic devices, with tremendous advantages over conventional methods [270]. In this section, we will review the main microfluidic-based protein analysis methods, with some recently developed examples.

Cellular staining assays are commonly used methods which are easy to be applied in microfluidic devices for protein analysis. Proteins in cells are specifically labeled by tags or fluorescent antibodies, and their locations and expressions can be imaged using microscopies. Sun et al. reported a microfluidic image cytometry which was capable of quantitative, single-cell proteomic analysis (Fig. 2.12a) [271]. Simultaneous measurement of four critical signaling proteins (EGFR, PTEN, phospho-Akt, and phospho-S6) within the oncogenic signaling pathway in human

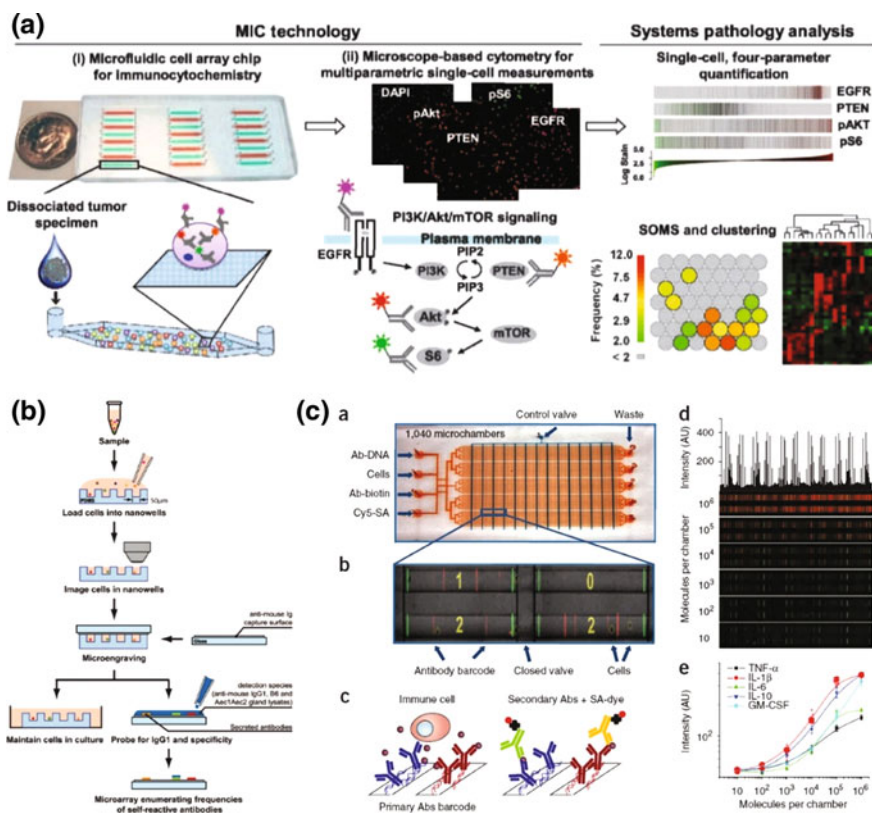


Fig. 2.12 Protein analysis on microfluidic chips. **a** Microfluidic image cytometry for quantitative, single-cell proteomic analysis; **b** Microengraved single-cell proteomics chip; **c** Microfluidic barcode chip for proteomic analysis. (Reprinted with permission from Ref. [271, 272, 275])

brain tumor biopsies was performed by multicolor analysis of fluorescent antibody-labeled cytoplasmic proteins. This platform required much smaller amounts of reagents ($\sim 2 \mu\text{L}/\text{channel}$) and samples (< 3000 cells) than that for flow cytometry, and results were in good correlation with the clinical immunohistochemistry method. In previously mentioned example, native proteins were genetically encoded with oligohistidine sequence (His_{6-10}), which could interacted with a fluorescent multivalent chelator head trisNTA , leading to high-affinity protein labelling in living cells [166]. And the transfer of trisNTA probe into cells was facilitated by microfluidic cell squeezing. Multiplexed protein labeling as well as super-resolution (nanometer precision) tracing of target proteins in live mammalian cells were achieved using this technique.

Another important microfluidic protein analysis technique is surface-based immunoassay. Proteins are specifically captured by affinity ligands modified on microchannel or microbead surface, and sandwich immunoassays are then

performed. This method enables high level of multiplexing and quantitation, and intracellular, membrane, and secreted proteins can all be analyzed from the same single cell [270]. Love's group developed a microengraved single-cell proteomics chip which employed an array of microfabricated subnanoliter wells to isolate and culture single cells (Fig. 2.12b) [272]. This microwell array was capped with an antibody-coated microengraved substrate, and proteins secreted by single cells were captured and then characterized using sandwich ELISAs. These devices were used to analyze the antibody production behavior of B cells collected from both a healthy mouse and a mouse model with autoimmune disorder. Microfluidic barcode chip developed by Heath group was also a versatile and robust tool for proteomic analysis (Fig. 2.12c). Single cells, or defined numbers of cells, were isolated within microchambers, each of which contained a full barcode array. Each barcode stripe was initially patterned with a unique ssDNA oligomer, and the barcode was converted into an antibody array using a cocktail of antibodies labeled with complementary ssDNA oligomers (DNA-encoded antibody library, DEAL) [273], just prior to running an assay. This platform could be used to capture and detect secreted proteins from living cells, or proteins, nucleic acids and metabolites from lysed cells, using ELISA or fluorimmunoassay (FIA) coupled with a standard array scanner [274]. This technique was quantitative, sensitive, highly multiplexed and high-throughput, and has been applied to study single-cell proteomics, cell signaling and cell-cell interaction [275, 276].

Recently, protein immunoblotting assay has been operated on microfluidic devices, overcomes several limitations associated with conventional immunoblotting, including multiple steps requiring manual intervention, low throughput and substantial consumption of reagents [277, 278]. Polyacrylamide (PA) gels in glass microfluidic devices were regionally photo-patterned and served as a platform for rapid and automated protein immunoblotting. All assay stages were programmably controlled by a high-voltage power supply and monitored by an epifluorescence microscope equipped with a charge-coupled device camera. Microfluidic single-cell western blotting (scWestern) has also been developed by the same group (Fig. 2.13a) [279, 280]. PA gels were photo-patterned to form a microwell array, in which single cells were settled and lysed *in situ*. Gel electrophoresis was then performed, and separated proteins were immobilized by photoinitiated blotting and detected by antibody probing. This scWestern method enabled multiplexed analysis of 11 protein targets per single cell with detection thresholds of < 30,000 molecules. It was applied to monitor single-cell differentiation of rat neural stem cells and their responses to mitogen stimulation.

Protein activity, and the interactions between protein and other biomolecules, can also be analyzed on microfluidics. In previously mentioned example, Sarkar et al. presented a microfluidic probe that lysed single adherent cells from standard tissue culture and captured the contents to perform single-cell assays [209]. Kinase and housekeeping protein activities were measured simultaneously or separately by mixing them with assay reagents in nanolitre scale integrated chambers, and imaging over time for fluorescence. This approach was used to elucidate the biological heterogeneity in Akt kinase activity levels among cells under insulin

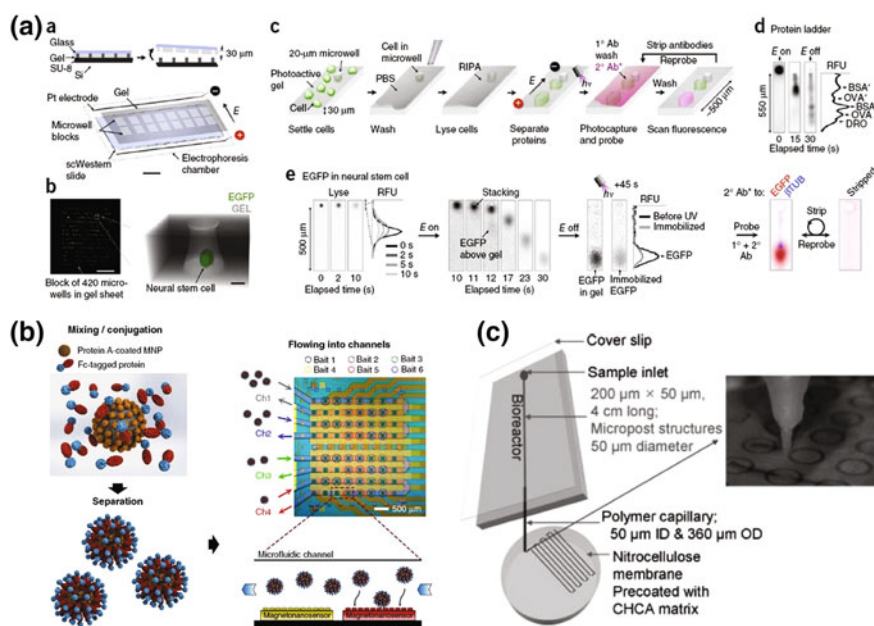


Fig. 2.13 Protein analysis on microfluidic chips. **a** Microfluidic single-cell western blotting (scWestern); **b** A magneto-nanosensor microfluidic platform for protein–protein interaction study; **c** A microfluidic device integrated with MALDI-TOF MS for protein analysis. (Reprinted with permission from Ref. [279, 281, 286])

stimulation. Lee et al. presented a magneto-nanosensor platform integrated with a microfluidic chip that allowed measurement of low-affinity protein–protein interactions (Fig. 2.13b) [281]. Prey proteins were pre-conjugated to magnetic nanoparticles (MNPs), and then introduced into microchannels and specifically interacted with magneto-nanosensors modified with bait proteins. The magneto-nanosensors produced signals proportional to the number of bound complexes, and real-time binding curves were measured and kinetic parameters were calculated. This platform was used to characterize the binding affinities of the PD-1—PD-L1/PD-L2 co-inhibitory receptor system, and discovered an unexpected interaction between the two known PD-1 ligands, PD-L1 and PD-L2.

Coupled with mass spectrometry (MS), microfluidic technique offers a robust platform for proteomic analysis [282, 283]. A commercially available HPLC-chip/MS system has been developed by Agilent Technologies, which uses a multilayer polyimide chip consisting of preparation channels and an integrated electrospray tip [284]. Trypsin-digested proteins are introduced into the HPLC-chip, concentrated on a small volume (40 nL) enrichment column and separated in the analytical nanochannel. Eluting compounds are directly sprayed into the MS instrument with the electrospray tip. This system was combined with strong cation exchange chromatography and applied to the analysis of the nucleolar

proteome, resulting in the identification of more than 200 proteins which corresponded to 2024 unique tryptic peptides. Microfluidic devices can also be integrated with matrix-assisted laser desorption and ionization mass spectrometry (MALDI-MS), in either off-line or on-line manner [285]. Lee et al. constructed an automated proteolytic digestion bioreactor and continuous deposition system in a plastic microfluidic device for off-line interfacing to MALDI-TOF MS (Fig. 2.13c) [286]. Proteins were digested by trypsin immobilized on an array of micropost structures in bioreactor, and the obtained peptides were continuously deposited onto a MALDI plate modified with nitrocellulose solution containing a matrix by a 50 mm capillary tube attached to the end of the bioreactor. Various proteins were analyzed in this platform with good sequence coverage.

2.5.2.4 Metabolite Analysis

The intracellular levels and spatial localizations of metabolites reflect the state of a cell and its relationship to its surrounding environment [287]. Microfluidic device is an ideal platform for cellular metabolite profiling both in physiological environment and under drug treatment, owing to the ability of integrating cell culture, stimulation, metabolite enrichment and detection on a single chip coupled with various analytical instruments [288].

Among diverse analytical techniques, MS is the most powerful and promising tool for cell metabolite analysis, because of its broad detection range, high sensitivity, high mass resolution, rapid operation, and the ability for multiplexed analysis. Microfluidic devices can integrate with different types of MS, including ESI-MS, MALDI-MS and paper-spray ionization MS [289]. Recently, our group has developed a series of chip-MS platforms for cell metabolites analysis. Functional sections including cell medium/drug introducing channels, cell culture chambers, micro-SPE columns were integrated in one microchip, and coupled to ESI-MS via capillaries [150, 226–228, 290–292]. Chen et al. developed a stable isotope labeling assisted microfluidic chip electrospray ionization mass spectrometry (SIL-chip—ESI-MS) platform for qualitative and quantitative analysis of cell metabolism (Fig. 2.14a) [293]. MCF-7 cells were cultivated *in vitro* and exposed in anticancer agent (genistein) for cell-based drug assay. A dual-isotopic labeling was presented for effective qualitative analysis of multiplex metabolites. Three coeluting pairs of isotopomers could be easily recognized and identified. This chip-MS technique can also be extended to study cell-cell communication. Zhuang et al. [294] designed a membrane integrated microfluidic device to achieve the co-culture of PC12 cells and 293 cells to study nephrocyte-neurocyte interaction. The neuro-like differentiated PC12 cells induced by mNGF released neurotransmitter acetylcholine, which stimulated the 293 cells and led to the secretion of hormone epinephrine. Secreted epinephrine could be detected by ESI-MS, providing a chemical insight into the understanding of cellular interaction.

Optical detecting technique can also be integrated with microfluidic device for cell metabolite analysis. Wang et al. presented a flexible high-throughput approach

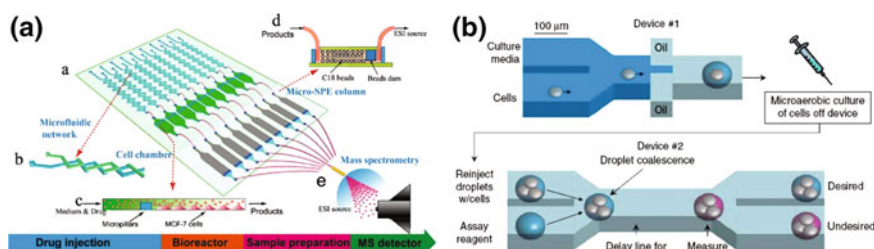


Fig. 2.14 Metabolite analysis on microfluidic chips. **a** Chip-MS platform for drug metabolism analysis; **b** Microfluidic optical detection for cell metabolite analysis. (Reprinted with permission from Ref. [293, 295])

that used microfluidics to compartmentalize individual cells for growth and analysis in monodisperse nanoliter aqueous droplets surrounded by an immiscible fluorinated oil phase (Fig. 2.14b) [295]. Fluorescent assay system was used to measure the concentration of the metabolites (oxidase enzymes), and the assay reaction started when a cell-containing droplet coalesced with an assay droplet. Based on the cellular metabolism behavior, this system was able to identify xylose-overconsuming *Saccharomyces cerevisiae* cells from a population containing one such cell per 10^4 cells and enrich L-lactate-producing *Escherichia coli* clones $5800 \times$ from a population containing one L-lactate producer per 10^4 D-lactate producers.

2.6 Conclusion and Perspective

Microfluidics, combined with advanced molecular, imaging and bioinformatics techniques, constitute a robust 'toolbox' and revolutionize the way for cell biology researches. Multiple processes including cell culture, cell manipulation, cell stimulation and cell analysis can be transferred and integrated in a small single microfluidic device, promoting the development of cell researches. Owing to the unique advantages of microfluidic technology, it has been applied to various biological fields, such as single-cell analysis, cancer research, drug discovery and screening, clinical diagnostics, stem cell research, intra- and inter-cellular signaling, tissue models and microbiology. These applications have been partially introduced in previous sections and will be reviewed in detail in the following chapters in this book.

Despite the rapid development and significant progress of microfluidic technology in recent years, there are still challenges and hurdles that should be addressed for microfluidic cell analysis. One pivotal challenge is the low adoption of novel microfluidic techniques in mainstream biology researches. Most microfluidic-based techniques for cell biology applications recently are only iterative improvements on methods that already exist. And owing to the gaps in

expertise between microfluidic engineers and biologists, it is not easy for biologists to handle complex microfluidic systems. Thus the superiority and particularity of microfluidic techniques over traditional methods are not obvious, and biologists will generally prefer to use conventional macroscale methods than to learn and adopt the new microfluidic techniques. To address this challenge, efforts can be made from different directions. First, collaborations between multidisciplinary researchers (physicists, chemists, engineers, molecular and cell biologists, and clinicians) should be further strengthened, which can make the microfluidic-based techniques more biologically compatible, allow direct interaction and feedback between designers and end-users, and accelerate the applications of newly developed techniques. Second, more attentions should be paid to research areas in which microfluidic technology shows unique advantages and irreplaceable roles, such as diagnostic devices for low-resource settings, rapidly assaying biofluids for research and clinical applications, and development of more physiologically relevant *in vitro* models. Third, simplifying microfluidic devices and operation processes is an effective way to lower the barrier to entry for biologists and expand practical applications. In addition, developing automated microfluidic systems with diverse functions can also improve the adoption of microfluidic technology in mainstream cell researches [7, 15].

In summary, with twenty-year development, microfluidic technology has become one of the most powerful and promising tool for the study of cell biology and cell analysis. It has been extensively utilized in various fields of cell research and is still in rapid progression. We believe that with further advance and maturation, this versatile and robust technology will continue to introduce new paradigm for cell study, and make contributions to the deep cognition and development of both basic biological research and clinic applications.

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