Chapter 3
Single-Cell Culture and Analysis on Microfluidics

Weiwei Li and Jin-Ming Lin

Abstract Heterogeneity of cell populations is a major obstacle for understanding complex biological processes. In order to have a more comprehensive quantitative comprehending of cellular processes, it is necessary to quantify the distribution of behavior in a population of individual cells. Analysis of single-cell behaviors requires efficient single-cell capture, controllable single-cell culture performance as well as reliable analysis techniques. The microfluidic system provides advanced technology for single-cell culture and observation. This chapter gives a brief account of single-cell capture by microfluidic methods, long-term single-cell culture on both two-dimensional models and three-dimensional microfluidic systems, as well as single-cell growth and differentiation in a microfluidic environment. Furthermore, the advanced methods used for characterizing on-chip single-cell culture were also discussed.

Keywords Single cell · Capture · Culture · Growth and differentiation · Analysis

3.1 Introduction

Single-cell analysis holds much promise to better understand cell behaviors and cell metabolism [1, 2]. Individual isogenic cells are not identical even in the same culture environment [3, 4] known as heterogeneity. Heterogeneity among cell populations is a major obstacle to understand complex biological processes. The averaged results from the sample of large cell populations are insufficient when considering individual cell behaviors and usually obscure the variable response of individual cells. These problems are highlighted in the research of normal and malignant stem and progenitor cell populations, because of the lack of cell purification methods [5] and the inherent stochastic nature of cells' self-renewing and differentiation [6, 7]. Cell-to-cell interactions grown in a monolayer cell culture

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microenvironment are considered as one of the vital factors which influence large distributions in behavior. In this case, both contact and diffusible elements seem to be working. Therefore, single-cell analysis is increasingly being employed to a number of different biomedical fields, such as genetic analysis [8], cancer progression [9, 10], developmental biology [11], and fundamental biological studies on rare stem cells, or cancer cells [12].

The accompanying need with single-cell analysis is cell culture method to interrogate phenotypes through a culture of the rare amount of cells or single cells [13–17]. Single-cell culture is based on multiple generations from single-cell starting points. However, it is technically difficult to establish the single-cell culture platform. Technical difficulties are mainly reflected in (1) precise manipulation of cells and minimal loss of samples; (2) contact with non-biological surfaces and minimum shear stress or other damage stresses; (3) sustainable growth of target cell number and total cell number (need enough culture space, replaceable medium, etc.). In order to solve these difficulties, the development of cell culture platform requires the inherent advantages of microfluidics. Microfluidic platforms have recently been widely applied to realize cell-based assays, for example, manipulation of single cells [18], automated media perfusion [19, 20], providing cellular microenvironment and external stimuli to study cellular responses [21–24], and establishment of long-term cell culture systems.

In general, single-cell analysis requires (1) a large number of captured individual cells in order to obtain statistical significance of single-cell properties, (2) long-term clonal cell culture originated from a start of a single cell, (3) continuous observation of multiple generations of cells on their growth, differentiation, phenotype, metabolism, and other aspects. Single-cell culture is a crucial step in single-cell level analysis followed by single-cell isolation. In this chapter, we mainly present the advances, limitations, and outlooks in microfluidics based on the exploration of single-cell culture, categorized as two-dimensional (2D) and three-dimensional (3D) devices for the cultivation of a variety of cells, and analysis at single-cell level. We first introduce single-cell capture including conventional methods and microfluidic methods, and then we shift to single-cell culture based on microfluidics. In the last section, we focus on the advanced analysis techniques for single-cell culture.

3.2 Single-Cell Capture

Single-cell capture is the first step for single-cell analysis. Conventional methods for single-cell capture include micromanipulation, laser capture microdissection (LCM), and fluorescence activated cell sorting (FACS) (Fig. 3.1). Micromanipulation usually achieves through manual identification and selection with a microscope, which has the advantages of being low cost and easy-to-implement and the disadvantages of being laborious and low throughput [25]. LCM utilizes laser to cut and collect individual cells from tissues also under a microscope

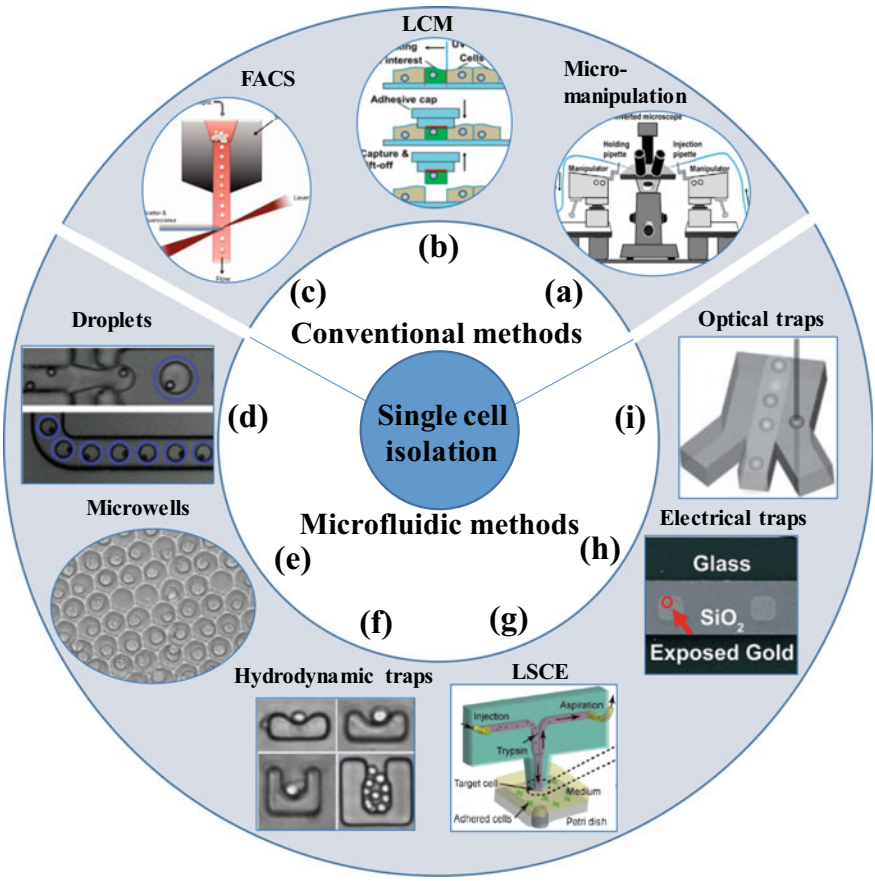

Fig. 3.1 Conventional and microfluidic methods for single-cell capture. Conventional methods includes: **a** Micromanipulation, figure was adapted from Ref. [33]; **b** Laser capture microdissection (LCM), figure was adapted from Ref. [33]; **c** Fluorescence-activated cell sorting (FACS), figure was adapted from Ref. [37]. Microfluidic methods includes: **d** Droplets, figure was adapted from Ref. [59]; **e** Microwell, figure was adapted from Ref. [63]; **f** Hydrodynamic trap, figure was adapted from Ref. [70]; **g** Live single-cell extractor (LSCE), figure was adapted from Ref. [75]; **h** Electrical traps, figure was adapted from Ref. [79]; **i** Optical traps, figure was adapted from ref. [87]

[26, 27]. LCM usually requires fixed tissue or cells and high laboratory skills. FACS is a high-throughput single cell sorting system, which could collect single cell into tubes or microwells for further experiments; while FACS is generally lack of visual inspection of the cells. Conventional micromanipulation, FACS, and LCM, could be referred to previous reviews [28–31], and here are only briefly described.

Modern approaches are based on microfluidic systems for single-cell capture and analysis, including droplet, microwell, hydrodynamic traps, live single-cell

extractor (LSCE), electrical traps, optical traps (Fig. 3.1). Microfluidic devices have more advantages than conventional approaches in single-cell capture. Microfluidic systems offer a closed platform for integrating downstream experiments, which reduce the risk of sample contamination. Miniaturized compartments for single cell reduce the experimental cost and enrich biomolecules with low concentration [32]. Moreover, most microfluidic systems could be automated to save labor, which means easy to achieve high throughput. In this part, we focus on the microfluidic design of single-cell capture for further downstream culture or analysis.

3.2.1 Conventional Methods

3.2.1.1 Micromanipulation

Micromanipulation relies on the manual selection technique typically through a micropipe combined with a microscope [33]. As shown in Fig. 3.1a, target single-cell identification is via microscope visual inspection. The micropipe moves in close proximity and picks a specific suspended cell in a petri dish or well plate. This process is usually performed manually. Micromanipulation can obtain specific cells accurately, but cannot achieve high throughput. Except single-cell capture, the micromanipulation is widely applied in the bacterial analysis [34], reproductive medicine, and forensics.

3.2.1.2 Laser Capture Microdissection

Laser capture microdissection (LCM) is a mature technique to capture single cells or tissue compartments typically from fixed tissue or cells samples [33]. As shown in Fig. 3.1b, the working process of LCM consists of laser cutting procedure and extraction of dissected tissue. Target cell or compartment is observed via a microscope and marked to be cutoff. The focused laser cuts the selected section, and then the operator extracts the dissected tissue. A particular advantage of LCM is that the access to cells in situ is beneficial for the spatial information of single cells [35, 36]. Typical samples are cryo-fixed, fixed in formalin, or embedded in paraffin, which causes cell death. Some modern LCM systems allow the extraction of living cell or tissue for downstream cell culture or analysis, such as Leica LMD7000 with live cell cutting (LCC). However, if the target cells are not precisely cut, they may be contaminated by adjacent cells. LCM allows very limited throughput.

3.2.1.3 Fluorescence-Activated Cell Sorting

Fluorescence-activated cell sorting (FACS) is a high-throughput and automatic method for cell sorting, and large amount of cells can be sorted in a very short time

[37]. FACS uses laser excitation and provides various analytical choices (Fig. 3.1c). Cell characteristics such as relative size and granularity can be obtained as forward scattering (FSC) and side scattering (SSC), respectively. In addition, cells' functional properties can be measured by fluorescence staining. The samples for FACS cover almost every cell type from blood, bone marrow, tumor, plants, protoplasts, yeast, bacteria, and viruses. Thus, FACS has a diverse spectrum of applications, such as quantification of soluble molecules and subpopulations [38], cancer diagnostics, microbial analysis [39], cell cycle analysis, hematopoietic stem cells, DNA content analysis, and apoptosis. FACS has been recognized as a worldwide standard for cell population analysis and sorting. However, the drawback to this approach is that the morphological information is limited to cell size and complexity. In addition, the open environment of FACS system increases the risk of introducing pollution.

3.2.2 Microfluidic Methods

3.2.2.1 Droplets

Droplet-based microfluidics use oil to keep aqueous droplets separated. The droplet like a separate microreactor encapsulates single cells, thus single cells can be collected [40, 41]. Droplet systems for single-cell capture typically generate droplets from a statistically dilute suspension of cells [42]. Single-cell capture via droplets is a feasible and high-throughput method. This method provided a platform for various biological assays on the single-cell level, such as single-cell culture [43, 44], single-cell counting [45], antibody detection [46], drug screening [47], RNA sequencing (RNA-seq) [48, 49], PCR [50, 51], whole genome amplification [52, 53], enzyme screening [54, 55].

Droplet-based single-cell isolation is usually consistent with Poisson distribution, which means that more than half of the droplets are cell-free [56]. Stochastic cell loading is the inherent variability of droplet contents. For single-cell analysis, it is important to improve the droplet encapsulation efficiency of a single cell, while reduce empty droplets and droplets encapsulating multiple cells. Much effort had been made to improve the efficiency of single-cell encapsulation, such as optimizing channel designs [57, 58]. Improving the efficiency of single-cell loading is another method to maximize the number of droplets containing a single cell. Edd et al. proposed a method to controllably load single cells into drops by utilizing inertial sorting to generate two trains of single cells in the loading channel (Fig. 3.1d) [59]. This method caused self-organization of cells, which conquered the intrinsic limitations arose from Poisson distribution, and ensuring that almost every drop contains a single cell. Kemna et al. introduced Dean forces by long helical microchannels and the final cell encapsulation efficiency of $\sim 80\%$ because cells were ordered in the channel before being encapsulated into droplets [60]. Droplet-based microfluidics has becoming a popular technology with extensive

applications, such as biotechnology [61], chemical analysis [62]. Droplet-based high-throughput single-cell biology is promising, but not yet realized.

3.2.2.2 Microwells

Microwell-based single-cell capture generally relies on sized well or modified surface, and cells are separated by the physical boundaries of multi-well. With the microwell array, cells that are attracted to the microwells can be preserved, and other cells outside the microwells would be washed away. Microwells provide a simple method enabling high-throughput single-cell analysis. The prominent advantages of microwells are that the microdevice is simple to operate and easy to achieve the capture of plenty cells. However, the disadvantage is that single-cell capture may occur only in part of the microwell array, and there may be a microwell with multiple cells or without any cells. Adjusting the size and shape of the microwell and optimizing the concentration of the cell suspension are the common solutions to improve the efficiency of single-cell capture.

Microfabrication technology allows the simple preparing of parallel microwells and enables high-throughput single-cell analysis. Liu et al. molded self-assembled polystyrene microspheres in poly (dimethylsiloxane) (PDMS) to produce microwell arrays for single-cell collection (Fig. 3.1e) [63]. For improving the efficiency of single-cell capture, Huang et al. constructed a truncated cone-shaped microwell array, which realized ~90% single-cell occupancy within a few seconds [64]. Surface modification achieved cell collecting without complicated fabrication of cell-sized microwells [65–67]. The combination of surface modification and specific size of microwells could improve the efficiency of cell occupancy. Specific aptamer and protein micropattern could also be modified in the microwell for capturing single cells from a mixture [68].

3.2.2.3 Hydrodynamic Traps

Hydrodynamic trap is a very common method for single-cell capture. The working principle of hydrodynamic trap system is that physical barriers or hydrodynamic tweezers stop the cell and remove it from the flow of cell suspension. In order to trap single cell, physical barriers are designed microscale structures, and hydrodynamic tweezers are specific and complex fluid flow profiles [69]. The advantages of using this method are that it does not require complex experimental steps and operations and could handle a large number of cells in a short time.

U-shaped traps as a popular physical barrier have been widely applied to obtain single cell. One of the typical single-cell U-shaped traps was demonstrated by Di Carlo et al. to collect single cells in large arrays (Fig. 3.1f) [70]. They used this U-shaped traps to determine single-cell enzyme kinetics for three different cell line (HeLa, 293T, Jurkat). Pattern flow channels have been introduced into the U-shaped traps structure, which allowed the fluid to fill the channel and provide a

suitable environment for cell culture [71]. Another type of hydrodynamic trap system is hydrodynamic tweezers. Lutz et al. used four vortices fabricated from cyclic oscillations of the sound waves around a cylinder to trap single cell in the vortex center [72]. In addition, hydrodynamic tweezers also have been used to trap particles [73, 74].

3.2.2.4 Live Single-Cell Extractor

Live single-cell extractor (LSCE) is a newly developing tool for collecting single adhered cell in a tissue culture. LSCE consists of injection part and aspiration part (Fig. 3.1g) [75]. The target cell is digested by the trypsin injected around the cell and then is extracted by aspiration part for further culture or analysis. A prominent advantage of LSCE is to extract live single-cell in situ without damage. Thus, it holds the spatial information of live single cells to their specific positions in the tissue. This method is usually combined with a microscope, which allows for the selecting of the target cell. LSCE takes only a few seconds to extract a single cell each time, without complicated operation. This method will provide a valuable novel tool for studying biology on the single-cell level.

The concept of LSCE was proposed by Mao et al. for revealing the correlation between adhesion strength and viability at single-cell resolution [75]. They used LSCE to extract adhered U87-MG cells (U87) and human hepatoma (HepG2) cells cultured in a culture dish. The microscopy recorded the information of individual cells' morphology and stained metabolites. The relationship among cell adhesion strength, cell morphologies as well as intracellular metabolites were explored, which revealed cell heterogeneity. Deepened on LSCE, Mao et al. clarified the cell-matrix adhesion strength on the single-cell level and revealed the effects of biomaterials on cell-matrix adhesion and heterogeneity of cell-matrix adhesion for adherent cell culture [76]. Moreover, LSCE also clarified the drug effects on the adhesion strength of individual circulating tumor cells (CTCs) on endothelial cells (ECs), which has broad prospects in drug screening for cancer therapy [77].

3.2.2.5 Electrical Traps

The cell membranes generally exhibit negative electronegativity at neutral pH, so the suspended cells are attracted toward the positive electrode. Cells have different charge, sizes, and masses, resulting different electric field force of cells; therefore, different cells can be distinguished. This is the working principle of electrophoresis (EP) [78]. However, the specificity of different cells in electrophoretic migration is not efficient. Electrical traps for single-cell capture generally include electric field-directed adhesion and dielectrophoresis (DEP). The method of electric field-directed adhesion needs cell surface modification. Toriello et al. demonstrate a microfluidic cell capture system comprised of interdigitated gold electrodes covered

in an oxide layer within the PDMS channel (Fig. 3.1h) [79]. The cell surface was modified with thiol functional groups by endogenous RGD receptors. The exposed gold pads bonded the single cell with modified thiol functional group through applying a driving electric potential. However, DEP could electrically capture cells without cell surface modification. DEP uses alternating current and generates a non-uniform electric field. DEP has been widely used for cell trap and further cell analysis. For example, Das et al. coated DEP electrodes on the microscope slide, which could collect different kinds of cells on the slide [80]. Thomas et al. designed concentric ring negative DEP (nDEP) for trapping single cell in the flow system [81]. In addition, DEP combined single-cell Raman spectra (SCRS) to capture single cells for downstream Raman detection [82, 83].

3.2.2.6 Optical Traps

Tightly focused laser beam acts as laser tweezers to trap single cell in aqueous solution. The laser beam could also act as an excitation source to generate a Raman spectrum of samples, known as laser tweezers Raman spectroscopy (LTRS) [84]. Laser tweezers could be integrated into microfluidic devices for transmission, identification, and simultaneous sorting of single cells [85, 86]. Adrian et al. presented the integrated optofluidic Raman-activated cell sorting (RACS) platforms for label-free cell sorting [87]. The specific single cells were identified by Raman spectroscopy and then captured and removed from cell suspensions (Fig. 3.1i). This method achieved automated sampling and high-throughput cell sorting. Another type of optical traps is optoelectronic tweezer, a combination of laser tweezers and DEP, which has been applied to manipulate and select single cells [88, 89].

3.3 Single-Cell Culture on Microfluidics

Many creative microfludic platforms have been proposed for single-cell culture. Except for allowing the collecting of single cells, microfluidic platforms for single-cell culture require sufficient space for cell division and growth, timely nutritional supplementation, and appropriate microenvironment with minimal shear stress or other damage stress. In addition, access to cells of interest should be considered for subsequent study. Here, we classified microfluidic platforms by two dichotomous characteristics: two-dimensional (2D) and three-dimensional (3D). The methods and applications of single-cell culture carried out on these platforms have been described, with multiple types of cells including stem cells, tumor cells, immune cells, and so on.

3.3.1 Two-Dimensional Single-Cell Culture on Microfluidics

2D single-cell culture platforms based on microfluidics divided into two categories: closed and open microfluidics. Closed systems typically integrate hydrodynamic trapping structures and physical barriers into the platforms, which are isolated from the external physical stimulation. In contrast, open microfluidic systems, such as traditional microplate, microwells, and micropatterned surfaces, expose cells to the external physical, which allows direct access to single cells. Closed and open microfluidics with diverse designs have their own merits and demerits.

3.3.1.1 Closed Microfluidic Systems

Closed systems typically rely on hydrodynamic trapping structures and physical barriers. For example, Carlo et al. designed U-shaped hydrodynamic trapping structures on the bottom of the flow channel to trap single cells and cultured the cell in the original position for 24 h (Fig. 3.2) [90]. One of the important advantages of this single-cell culture array is controllable cell–cell interaction by both contact and diffusible elements.

To improve the single-cell capture efficiency, Kobel et al. optimized trap geometries with U-shaped hydrodynamic regions on the edge/wall of a microchannel (Fig. 3.3a) [91]. The single-cell capture efficiency of this device was nearly 100%. And this device allowed a long-term culture of individual non-adherent T-cell lymphoma cell in high throughput without a significant decrease in cell viability. Except U-shaped hydrodynamic trapping structures as efficient trap geometries, Lin et al. [92] fabricated sieve-like traps on adhesive protein micropatterns to capture single cells and cultured the trapped Hela cells on the micropatterns (Fig. 3.3b). The sieve-like trap positioning device was

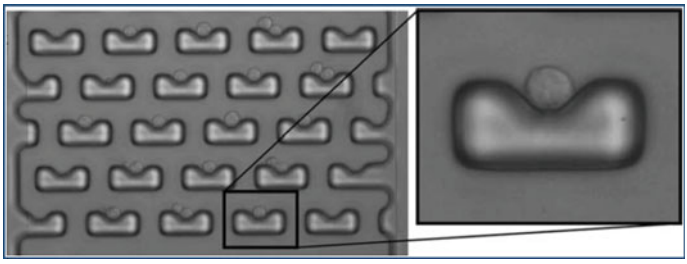

Fig. 3.2 Two-dimensional closed microfluidic systems, U-shaped hydrodynamic trapping structures, for single-cell culture. In most cases, cells rest at the identical potential minimum of the trap, while in some cases two cells are trapped in an identical manner among traps. A magnify-cation shows the details of the trapped cell. Trapping is a gentle process, and no cell deformation is observed for routinely applied pressures. Figure was adapted from Ref. [90]

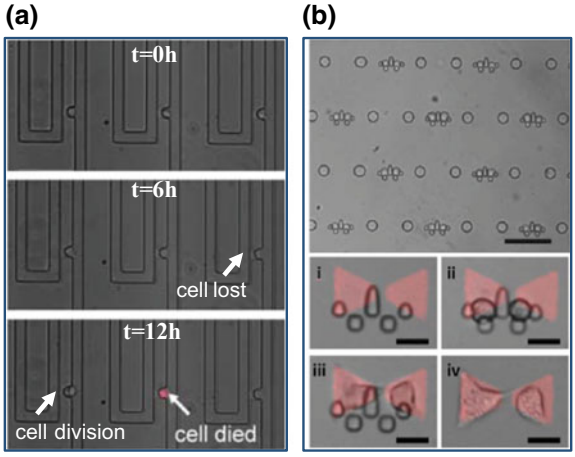

Fig. 3.3 Optimized two-dimensional closed microfluidic systems for single-cell culture. **a** Fates of non-adherent EG7 cells in a microfluidic single-cell trap. Series of typical images from a time-lapse experiment in a 2 mm device at a flow rate of 100 nl/min show a stably trapped cell and one that was lost after 6 h (bright-field image, left panel). Cell death was detected using propidium iodine (PI) added to the medium. Figure was adapted from Ref. [91]. **b** Demonstration of pairwise positioning of cells. (i) Traps were aligned to micropatterns. (ii) Single cells trapped on top of the micropatterns. (iii) Cells were allowed to spread and adhere to the micropattern. (iv) Microchannel peel-off leaving behind cells adhered to the micropattern. Scale bars of i–iv depict 50 mm. Figure was adapted from Ref. [92]

detachable, allowing easy access to individual cells of interest for subsequent manipulations. Moreover, the micropattern with multiple protein pattern clusters could adhere different cell populations, which provided a promising opportunity for studying cell–ECM interactions and cell–cell interactions.

To investigate single cell–cell contact, Frimat et al. reported a highly parallel microfluidic method combined differential fluidic resistance trapping with cellular valving (Fig. 3.4) [93]. They applied differential fluidic resistance to sequential single-cell arraying. Continuous single-cell arrangement of the second cell type relied on the reversal of the fluid. The trapped single-cell pairs could contact with each other through the aperture. This microfluidic system is promising to study homotypic/heterotypic co-culture at the level of a pair of single cells. In addition, the long-term microfluidic culture of mammalian cells was considered difficult for reduced growth rates and deviations from normal phenotypes [94]. Lecault et al. exploited a high-throughput microfluidic system with automated medium exchange to investigate hematopoietic stem cell (HSC) proliferation control at the single-cell level [95]. Closed systems have the advantages of protecting samples from contamination and solvent (e.g., medium) evaporation. However, the disadvantages are the limited accessibility to samples of interest and increased costs from the integrated components (e.g., pumps, tubes).

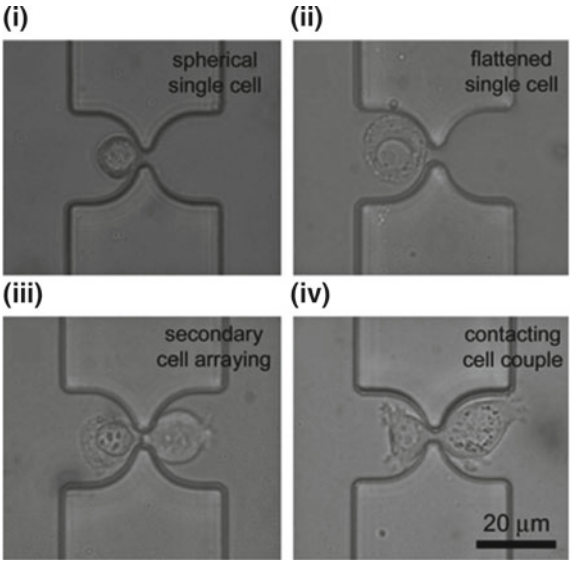

Fig. 3.4 Cellular valving approach for single-cell co-culture. Figure was adapted from Ref. [93]

3.3.1.2 Open Microfluidic Systems

Microwell chips as a kind of common open microfluidic systems have been useful in single-cell culture and analysis [96]. It is easy to achieve large-scale single-cell capture and culture and get the information of single-cell metabolism. A large number of optimization methods for efficient single-cell capture were proposed [97, 98]. Cell suspensions are normally introduced manually into the microwells, and cells are randomly positioned in the wells. Cells outside the well are then washed away. Single non-adherent cells and adherent cell both could be collected by microwell chips, such as single epithelial cells [99], hematopoietic stem and progenitor cells [100], blood cells, and lymphocyte. It is more difficult to fabricate microfluidic devices for the investigation of non-adherent cells, because of restricting non-adherent cells to a known position in the microdevice. Cell seeding efficiency is the most important parameter to evaluate microwell chips. The number of wells containing cells and the number of wells containing single cells, corresponding to well occupancy and single-cell occupancy respectively, in addition, the shape, size, the number of the microwells and material are all parameters should be taken into consideration. Square, hexagonal, and round shapes are common for microwells. Larger wells provide enough space for long-term culture, while smaller wells are propitious to the collection of single cells and instant analysis (hours and days). The number of the microwells (density of wells) need considers two aspects, harboring many cells and communicating with other cells to keep normal functions (without those conditions, cells may subsequently change their normal functions, e.g., diminished viability). Various materials have been used for the fabrication of microwell chips, such as etching/drilling silicon and glass [101], polymer PDMS [102]. The surface of silicon and glass with favorable mechanical properties could

be grinded and polished chemically to be suitable for cell adhesion. Besides the mechanical properties, the optical properties should be taken into consideration. Transparent materials have good satisfactory performance, which facilitates acquiring cell images, such as flat glass surfaces and conventional microscopic slips. Polymer PDMS is another material commonly applied for well-based single-cell chips with the advantages of easy fabrication, facilitated sealing onto other materials, good light transmittance, and biologically inert property. Open microwell chips allowed cell manipulation and handling.

Revzin et al. fabricated microwells composed of PEG hydrogel walls and square glass attachment pads and modified the glass pads with T-cell specific anti-CD5 to capture T-lymphocytes with the single-cell occupancy of ~95% (Fig. 3.5a) [103]. They used laser capture microdissection (LCM) for the retrieval of individual cells from the microwell array, which was prepared for downstream experiments (e.g., genomic or proteomic analysis). Tokimitsu et al. picked the single antigen-specific B-cells from cell-sized microwells by using micromanipulation [104]. Microwell chips should be designed for further cell study with the possibility to retrieve samples of interest. Since most of the existing analysis techniques for cell retrieval are suitable for open microfluidic systems, thus it can be integrated with most microwell chips. Although most microwells are roofless, the wells can be sealed by the addition of a roof (e.g., a glass slide) to capture the cell in the wells from the

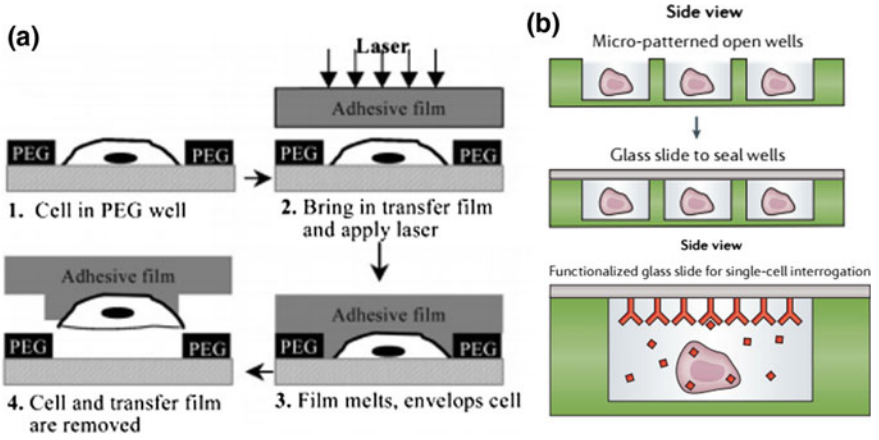

Fig. 3.5 Two-dimensional open microfluidic systems of microwell type for single-cell culture. **a** Retrieval of individual leukocytes from the cell array using LCM system. Step 1: Cells of interest are identified using light microscopy. Step 2: LCM cap containing a transfer film is brought into contact with the cell array after which focused laser beam is pulsed. Step 3: Transfer film melts and fuses with cells lying underneath. When the LCM cap is removed, cells remain preferentially attached to the transfer film. The cap is placed into an Eppendorf tube containing DNA, mRNA, or protein preparation buffers. Figure was adapted from Ref. [103]. **b** Nanowells confine cells by gravity and can subsequently be sealed with a membrane or glass slide to obtain single cells and their components. These cells or components can then be picked out of the wells for further processing or characterized in-well. Figure was adapted from Ref. [105]

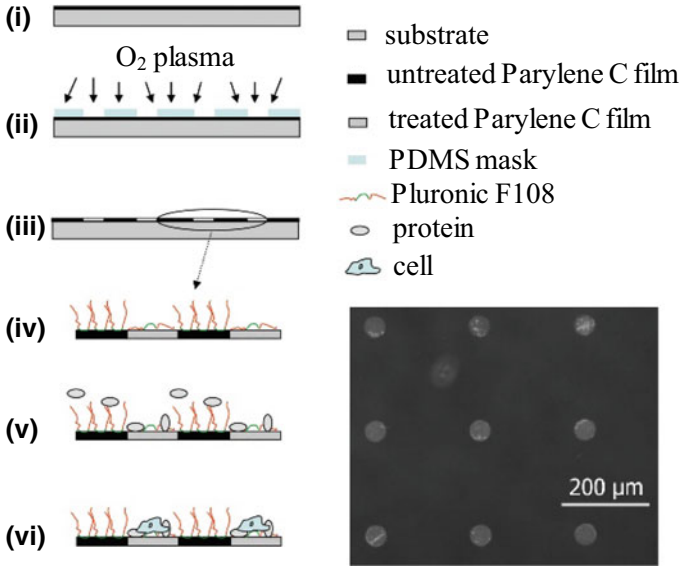

Fig. 3.6 Two-dimensional open microfluidic systems of chemically micropatterned surfaces for single-cell culture. Combination of plasma-assisted surface chemical modification, soft lithography, and protein-induced surface activation to accomplish surface patterning for single-cell culture. Figure was adapted from Ref. [108]

external environment (Fig. 3.5b) [105]. The roof could be modified as a functionalized seal to pick the cells or the components out of the wells for further analysis, for example, the analysis of the secreted cytokines or antibodies [106].

Single-cell culture is required to confine single cells to a specific location on the substrate. Chemically micropatterned surface with cytophilic and cytophobic regions (promoting and suppressing cell growth) is another strategy existing for controlling the position of single cells. Micropatterned surfaces for single-cell culture have been helpful in studying the effects of cell morphology and microenvironment on movement, migration, proliferation, and differentiation of cells [107]. Similar to the most microwell-based assay, the micropatterned surface is also a kind of open microfluidic systems. Various methods have been used to pattern cytophilic and cytophobic chemicals on the substrate for single-cell culture and analysis. Cheng et al. combined plasma-assisted surface chemical modification, soft lithography, and protein-induced surface activation to accomplish surface patterning for single-cell culture (Fig. 3.6) [108]. A polydimethylsiloxane membrane mask was put on the polystyrene film. The patterning was formed by oxygen plasma treatment which could produce hydrophilic areas. Then, the patterned film was incubated with either Pluronic F108 solution or a mixture of Pluronic F108 solution and fibronectin. Protein loading enhanced selective cell attachment on patterned dishes. Long-term (>2 weeks) single-cell culture experiments showed the influence of surface patterning on both cell and nucleus shape and also confirmed

the stability of the produced single-cell molds in serum medium. Ye et al. proposed an alternative approach based on micromolding in capillaries (MIMIC) to achieve chemical surface patterning for single-cell culture [109]: the PDMS micropattern with grid pattern was placed on the cytophilic substrate; cytophobic chemicals filled the microchannels under capillary action; discrete cell adhesion regions surrounded by cytophobic chemicals; removed the PDMS mold and seeded cells in the cytophilic pattern. The micropatterned surface for single-cell culture could be used to investigate the physiological activity of specific cells and employed for further analysis (e.g., drug screening).

3.3.2 Three-Dimensional Single-Cell Culture on Microfluidics

Traditional 2D single-cell culture models generally suspend cells freely in culture medium or randomly allowed cells adhere onto the support surface. Although 2D models have advantages of low cost, reproducibility, accessibility, and easy operation, they are insufficient to recapitulate physiological systems. The poor physiological correlation of 2D models may cause misunderstandings of the cell proliferation, differentiation, cytotoxicity, and metabolism, leading to inaccurate prediction of in vivo behaviors. In addition, most two-dimensional models are faced with some common limitations, such as nutrient depletion, toxin accumulation, influence of the complicated fluid shear, and random cellular migration. Thus, three-dimensional (3D) single-cell culture microfluidic platform mimics the real 3D microenvironment in vivo to reflect cell function and the geometry of tissues. Large amount of microfluidic devices featuring long-term 3D single-cell culture has sprung up. Microgel provides a promising strategy for single-cell 3D culture. Microgel allows long-term single-cell analysis, including heterogeneity of cellular proliferation, differentiation, and drug cytotoxicity. With the advantages of repeatability, flexibility, and highly controllable supporting microenvironment, cells could be independently packaged, cultured, monitored, or manipulated in the microgel. The structures of microgel for single-cell 3D culture are generally divided into three types: hydrogel droplets, microgel column, and microgel arrays.

In order to produce homogeneous hydrogel droplets for encapsulation of single cell, Utech et al. proposed a novel method to form alginate microgels in a highly controlled manner [110]. They added acetic acid into the continuous oil phase to dissociate Ca^{2+}-EDTA into Ca^{2+}, thus releasing Ca^{2+} to react with alginate chains (Fig. 3.7i). They used RGD-functionalized alginate to encapsulate single mesenchymal stem cells, as RGD provided integrin binding sites for cell attachment. The results showed that 25% of the droplets generated carrying single cells, while the majority of drops (70%) remained an empty and very small number of drops in contained more than one cell (Fig. 3.7ii). Improving the single-cell encapsulation efficiency has always been one of the challenges of microgel droplet technology.

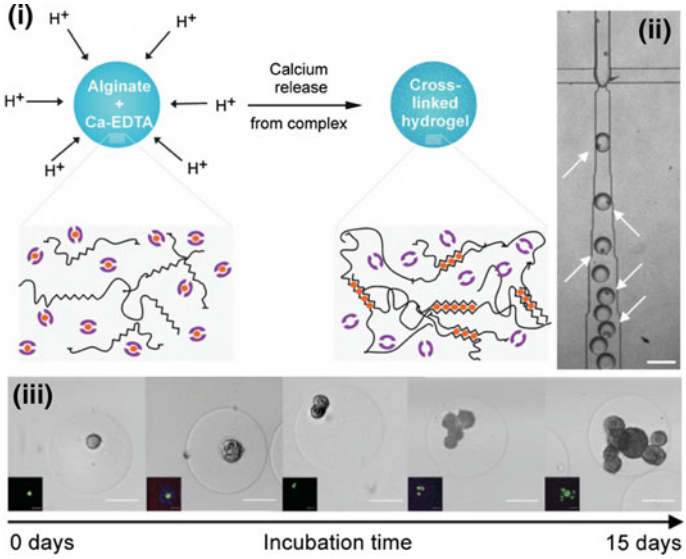

Fig. 3.7 Three-dimensional single-cell culture on microfluidics. Microfluidic generation of homogeneously cross-linked alginate microparticles by on-demand release of calcium ions from a water-soluble calcium–EDTA complex. (i) Schematic illustration of the cross-linking process. Upon addition of acid to the continuous phase, the calcium–EDTA complex dissolves, calcium ions are released, and cross-linking of alginate is induced. (ii) Cells are encapsulated using a 50 μm flow-focusing device (scale bar: 100 μm). Single-cell-containing droplets are indicated by white arrows. (iii) Representative images of cell-containing alginate gels directly after encapsulation and after being cultured for 3, 6, 12, and 15 days, reprehensively. The cells grow and proliferate inside the generated microenvironments while maintaining their spherical morphology. The encapsulated cells are stained using a calcein assay and analyzed via a confocal laser scanning microscope to determine the cell viability (inlets). All scale bars are 25 μm. Figure was adapted from Ref. [110]

The encapsulated cells in the droplets kept high viability for 2 weeks. The cells proliferate inside the microstructure and maintained spherical morphology (Fig. 3.7iii). Similarly, Dolega et al. presented a method for 3D single-cell culture based on a flow-focusing microfluidic system that encapsulates epithelial cells in matrigel beads to analyze clonal acinar development [111]. They cultured single prostatic and breast cells in each individual bead, and the cells proliferated and differentiated into a single acinus per bead. Compared to conventional protocols of bulky 3D culture, this method generated more uniform acini population and recorded acinar development from the very first division to the final stage. Moreover, it provided easy recovery of 3D structures for further analysis, such as the combination with large particle FACS, fundamental genomics.

Hydrogel microcolumn inside microchannels is also one of the microgel types for single-cell culture. Liu et al. developed an photopolymerization approach for controlled encapsulation of single cells in poly(ethylene glycol) diacrylate (PEG-DA) precursor [112]. As shown in Fig. 3.8, cells were suspended in PEG-DA

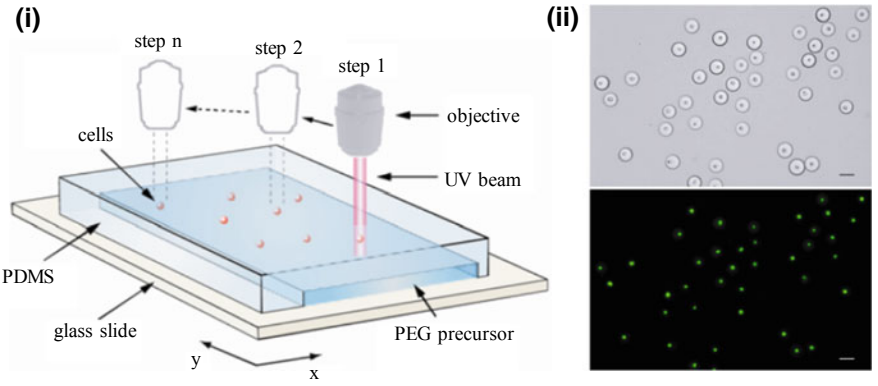

Fig. 3.8 Controlled encapsulation of single cells inside hydrogel microstructures. **i** Schematic setup of the controlled encapsulation of single cells. **ii** Bright field and fluorescence images of the large-scale encapsulation of single cells. Figure was adapted from Ref. [112]

precursor solution in the microchannel. A fluorescence microscope projection provided UV source to generate photopolymerization of PEG-DA precursor. The photopolymerization only occurred where the target cell suspended. Thus, the cell was chosen for encapsulation inside the hydrogel microstructures. The remaining freestanding cells in uncross-linked precursor were removed by TE buffer. Single cells encapsulated inside the microgel structure remained their viability for hours to days, which allowed long-term culture for further single-cell analysis [113].

Microgel arrays provide a feasible and high-throughput 3D mode for long-term single-cell culture. Large-scale microgel arrays carry out high-throughput single-cell analysis, and each unit of the arrays acts as an individual 3D cell culture room. Guan et al. developed a microcollagen gel array (μCGA) for 3D single-cell culture [114]. The process of μCGA fabrication was not complex: soaked the PDMS microwell array in the mixture of collagen solution and cell suspension; pressed the PDMS membrane with cells in-wells with another flat PDMS membrane; removed the cover layer after gelatinization; placed the fabricated μCGA with cells encapsulated into culture medium; added fresh medium to supply nutrients for cell growth and proliferation (Fig. 3.9i). In order to analyze the cell proliferation heterogeneity under 3D culture conditions, they continuously recorded the proliferation of some random single cells for 11 days, which showed growth of five typical cell clones with different proliferation ability during the whole culture process (Fig. 3.9ii). Furthermore, they retrieved the cells of interest in μCGA by integration with a microscale manipulation platform, as open culture conditions based on the microwell array allowed for more convenient retrieve of the target cells.

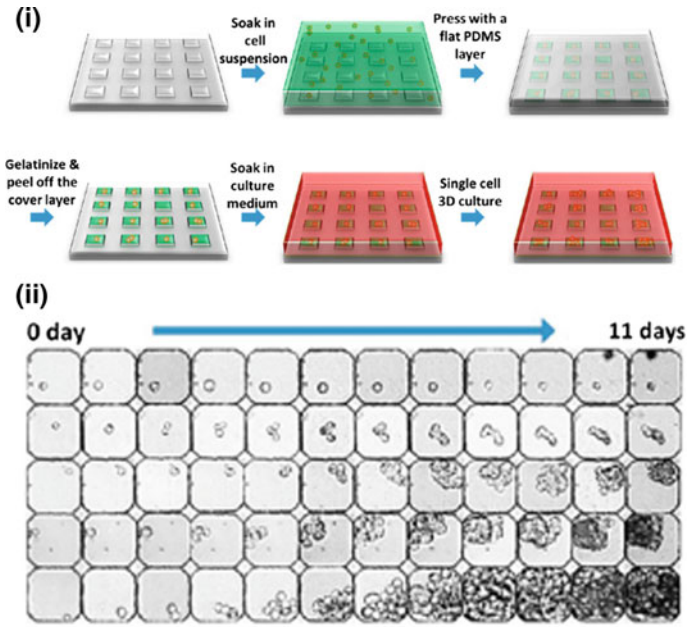

Fig. 3.9 Microcollagen gel array (μCGA) for 3D cell culture. **i** Schematic of μCGA fabrication process. **ii** Images of five typical single cells with significant cellular proliferation ability heterogeneity during the 11 day culture. Figure was adapted from Ref. [114]

3.4 Analysis Techniques for Single-Cell Culture

The final step for single-cell culture is to characterize and screen cell biophysical and biochemical information, such as morphology, behaviors, metabolism, and so on. A variety of technologies has been emerging for obtaining the single-cell information during a series of processes, including single-cell capture, single-cell short-term/long-term culture, single-cell lysis, and drug monitoring. Here, we covered optical, electrochemical, and mass spectrometric techniques for physical, chemical, molecular biology, and gene analysis of single cells.

3.4.1 Optical Characterization Techniques

Optical characterization is very commonly used in single-cell analysis, due to the direct visual observation of cells. Fluorescence methods play a vital role in optical characterization, providing images with high contrast and chemical-specific properties. Through fluorescence imaging, it is easy to reveal the distribution of intracellular molecules and dynamic states of biological processes, and the trace of

biomolecules. However, fluorescence imaging requires the target molecule to generate fluorescence. As to the non-luminescent molecule, fluorescent labeling or chemical modification is needed, which limits the use of fluorescence methods. Thus, label-free optical methods without the need for fluorescent labels or staining have been emerging. In addition, super-resolution microscopy allows the resolution down to 10 nm [115] and permits the observation of the submicroscopic structure of cells. It is necessary to briefly introduce recent advances in optical characterization techniques, including fluorescence techniques and label-free methods.

3.4.1.1 Fluorescence Techniques

Fluorescence resonance energy transfer (FRET) as a specialized fluorescence technique is extensively applied to biochemical reactions. The principle of FRET is that the energy transfer from an excited donor fluorophore to an acceptor molecule leading to the acceptor emitting fluorescence. FRET sensors with different excitation and emission wavelengths can be designed to analyze parameters in parallel. Ng et al. designed multi-color Förster resonance energy transfer (FRET)-based enzymatic substrates in a microfluidics platform to measure multiple specific protease activities from water-in-oil droplets encapsuling single cells [116]. They first mixed suspended cells with multiple modified FRET substrates for cell encapsulation in water-in-oil droplets (Fig. 3.10a). The secreted proteases cleaved multi-color FRET substrates to yield multiple fluorescent signals in cell-encapsulated droplets (Fig. 3.10a). They successfully used four FRET sensors with different excitation and emission wavelengths to measure different metalloproteinases of several breast cancer cell lines on the microdroplet-based platform (Fig. 3.10a). Metalloproteinases (MMPs) have been known as important biomarkers of cancer diagnosis and treatment. For example, MMP9 degrades the basement membrane of the extracellular matrix (ECM), which facilitates cancer cell invasion and metastasis [117]. Metalloproteinases were also regarded as the target in research by Son et al., who designed a micropatterned photodegradable hydrogel array integrated with reconfigurable microfluidics to enable cell-secreted metalloproteinases analysis and specific cell retrieval at the single-cell level. They also monitored the activity of protease molecules secreted from single cells through FRET peptides entrapped inside microfabricated compartments. Moreover, the gel islands could be degraded by UV exposure, which easily allowed to release specific single cells of interest.

Fluorescence in situ hybridization (FISH) relies on fluorescence labeled nucleic acid probes to localize specific sequences of DNA or RNA molecules in single cells or tissues. FISH has many advantages, such as high economy and safety without radioactive isotopes, high stability and specificity, simultaneous detection of multiple sequences through different colors displayed in the same nucleus, yet with the disadvantages of limited throughput. FISH is widely applied to identify heterogeneities in gene expression within single cells or tissues samples. For example, Perez-Toralla et al. presented a protocol for quantitative characterization of ERBB2

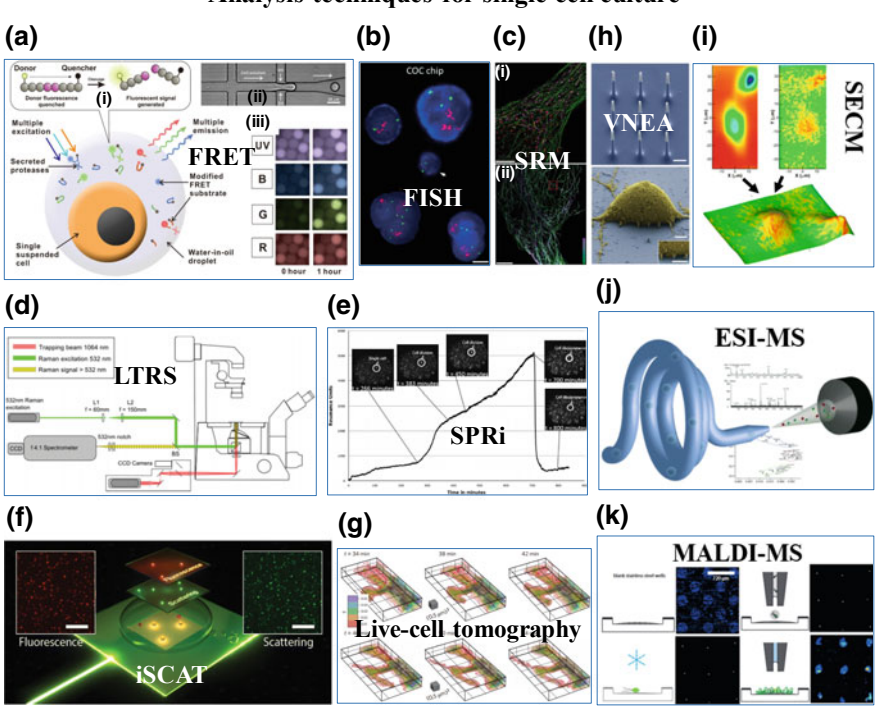

Fig. 3.10 Analysis techniques for single-cell culture. **a** Fluorescence resonance energy transfer (FRET) based enzymatic substrates and in a microfluidics platform to simultaneously measure multiple specific protease activities from water-in-oil droplets encapsuling single cells. Figure was adapted from Ref. [116]. **b** Fluorescence in situ hybridization (FISH) results for HER2 typing of cells from a pleural effusion from a breast cancer patient (sample B) on COC chip. Figure was adapted from Ref. [118]. **c** Super-resolution microscopy (SRM) imaging of fixed single cells. Figure was adapted from Ref. [119]. **d** Laser tweezers Raman spectroscopy (LTRS) setup. Figure was adapted from Ref. [120]. **e** Surface plasmon resonance imaging (SPRi) sensor showing cell division and subsequent cell removal. Figure was adapted from Ref. [121]. **f** Interferometric scattering microscopy (iSCAT). Schematic representation of the planar waveguide chip and detection of fluorescently labeled vesicles in fluorescence and scattering modes. Figure was adapted from Ref. [122]. **g** Live cell tomography. Time-lapsed refractive index change during filopodia formation of a neuronal spine. Figure was adapted from Ref. [123]. **h** SEM images of vertical nanowire electrode array (VNEA) and single cell on the VNEA pad. Figure was adapted from Ref. [125]. **i** Scanning electrochemical microscopy images (SECM) of PC12 cells. Figure was adapted from Ref. [128]. **j** Dean flow-assisted cell ordering system for lipid profiling in single cells using electrospray ionization MS (ESI-MS). Figure was adapted from Ref. [129]. **k** Matrix-assisted laser desorption/ionization MS (MALDI-MS) platform for investigations of single cells spotted into microwells on the stainless steel plate. Figure was adapted from Ref. [131]

gene by FISH based on the microfluidic chip made of cyclic olefin (Fig. 3.10b) [118]. This protocol allowed cell immobilization with minimal dead volume and performed characterization in the liquid phase. The target ERBB2 gene as a

biomarker for the monitoring of HER2+ breast cancer progression was measured quantitatively with a tenfold reduction of sample consumption and decreases the assay time by a factor of two [118].

Fluorescent super-resolution microscopy (SRM) breaks through the original optical far-field diffraction limit in principle. With the help of fluorescent molecules, it could exceed the limit of optical resolution and reach nanometer resolution. This technology is widely applied in biology, chemistry, medicine, and so on. SRM can be used to track target intracellular molecules in single-cell research. Spectacular two- and three-dimensional images of subcellular components have been obtained by SRM techniques. Although SRM could provide images with high resolution, it remains challenging to obtain multiplexed images for a large number of distinct target species. Jungmann et al. used the transient binding of short fluorescently labeled oligonucleotides (DNA-PAINT, a variation of point accumulation for imaging in nanoscale topography) for images with ultra-high resolution that achieves sub-10 nm spatial resolution in vitro on synthetic DNA structures [119]. They experimentally demonstrated spectacular imaging of proteins in fixed cells (Fig. 3.10c).

3.4.1.2 Label-Free Optical Methods

Laser tweezers Raman spectroscopy (LTRS) is promising in the label-free analysis of individual cells, particularly suited to the research of living cells, because it allows cells to be captured and analyzed in an aqueous environment. However, the most reported LTRS methods involved manual trapping of cells, which is time consuming. Casabella et al. proposed a LTRS with an automated microfluidic platform for single-cell Raman spectroscopy (Fig. 3.10d) [120]. They introduced simple microfluidic channel to realize an alternating flow: cells trapped by optical tweezers during low flow intervals and successfully removed once fluid flow increases. During each capture period, Raman spectroscopy was used to measure individual cells. Results showed discrimination in the Raman signals of live epithelial prostate cells and lymphocytes. This method improved the throughput and reduced the manual work of the single-cell Raman measurements.

Surface plasmon resonance imaging (SPRi) is also a popular method used for the label-free measurement of biomolecular interactions. Stojanovic et al. applied SPRi to screen and quantify antibody production from individual hybridoma cells (Fig. 3.10e) [121]. The cells from a hybridoma produced monoclonal antibodies recognizing epithelial cell adhesion molecule (EpCAM) that was preimmobilized on the SPR sensor surface. Through this method, an excreted antibody from individual cells ranged from 0.02 to 1.19 pg per cell per hour.

Interferometric scattering microscopy (iSCAT) is another optical microscopy technique without fluorescent labels. The principle of iSCAT is that light is scattered by an object leading to the change of light intensity based on interference with a reference light field. Agnarsson et al. developed evanescent light-scattering microscopy for label-free interfacial imaging [122]. The core technology of the

evanescent light-scattering microscopy is a waveguide chip consisting of a flat silica core embedded in a symmetric organic cladding with a refractive index matching that of water (Fig. 3.10f). Measurements and theoretical analysis showed that the size of single surface-bound lipid vesicles could be characterized by the light-scattering signals without employing fluorescent lipids as labels.

Live cell tomography is another new label-less super-resolution microscopy technique allowing direct imaging of unstained living biological specimens. Cotte et al. recently presented this microscopic method that 3D imaging of living cells with a resolution less than 100 nm could be obtained by phase contrast of unlabeled single cells [123]. Through this method, they realized longtime neuronal observations for synaptic remodeling in 3D (Fig. 3.10g) and direct study of bacteria (*Escherichia coli*).

3.4.2 Electrochemical Analysis

In recent years, electrochemical analysis has been applied in the field of single cells. Electrochemistry analysis can be integrated on a miniaturized platform, and microelectrodes are suitable for miniaturization of signal acquisition. Microelectrodes can be easily fabricated on various material substrates (e.g., polymers, silicon, or glass). A wide range of electrochemically active molecules could be detected by microelectrodes. Thus, microelectrodes are very useful in the quantitative measurement of neuronal communication and related neuroscience research. One of the hotspots is the fabrication of ultrasmall electrodes for measuring the neurotransmitter's release of single vesicle from living cells. Anderson et al. used carbon fiber microelectrodes to penetrate into individual cells and cell nuclei. The changes of electrode impedance with cell and nuclear penetration were measured [124]. They also monitored transmitter release from single vesicles of individual cells. However, the carbon fiber microelectrodes have to be punched into cells, which requires high experimental operation skills and is very low in throughput. In order to improve the throughput and reduce the cell damage, Robinson et al. fabricated vertical nanowire electrode array (VNEA) for parallel electrical interfacing to multiple mammalian neurons. Depending on VNEA, neuronal activity of rat cortical neurons was intracellularly recorded and stimulated. It was also possible to map multiple individual synaptic connections (Fig. 3.10h) [125]. Another common application of electrochemical methods at the single-cell level is to monitor oxidative stress. Jeffrey E. Dick recently presented a macroscopic setup for the detection of reactive oxygen species (ROS) at single cells level [126]. He monitored the consumption of a single cell's contents upon its collisions with a microelectrode under the presence of surfactants. He obtained large difference with two orders of magnitude between acute lymphoblastic lymphoma T-cells and healthy thymocytes.

Electrochemical analysis is sensitive. The introduction of the enzymatic labeling amplification step further increases the detection limit and gets faster and more

reliable signals. For example, Safaei et al. [127] used velocity valley (VV) chip to capture CTCs tagged with magnetic nanoparticles modified with the anti-EpCAM antibody. And then, they enzymatically labeled alkaline phosphatase on the cells, which catalyzed the reaction of paminophenyl phosphate to an electrochemically active reagent p-aminophenol to be measured. This method shows sensitive performance in analyzing the whole blood samples. The above methods are all based on fixed electrodes, but if the electrode is freely movable, it can be used for the two-dimensional scanning of the electrochemical properties of the underneath samples. Scanning electrochemical microscopy (SECM) is based on this principle. Koch et al. combined SECM with fast-scan cyclic voltammetry (CV) for the simultaneous measurement of impedance and amperometric current of PC12 cells [128]. Amperometric signals were converted into topographical images, because the current density depended on the distance between the tip and the cell (Fig. 3.10i). CV revealed the spatial distribution of minimum oxygen consumption. This method made it possible to achieve chemically and spatially resolved measurements along with imaging topography. However, one unavoidable disadvantage of the electrochemical method is that testing many substances simultaneously is difficult.

3.4.3 Mass Spectrometric Analysis

Mass spectrometry is a powerful tool for single-cell analysis, with the advantages of high sensitivity, simultaneous detection of multiple substances, and the ability to structure the molecules of interest. In particular, mass spectrometry can differentiate hundreds of biological molecules from the sample without labeling, thus making it attractive. However, low abundant cellular targets with any labels are still challenging in single-cell analysis through mass spectrometry. This is why most studies focus on highly abundant metabolites. Moreover, quantification by mass spectrometry generally needs internal references. Various ionization methods have been developed used for desorption/ionization of different kinds of samples in single-cell analysis field, such as electrospray/nano-electrospray ionization (ESI/Nano-ESI), laser ablation/laser desorption ionization (LA/LDI), and secondary ionization mass spectrometry (SIMS). These ionization methods provide multiple choices to ionize and analyze a wide range of substances, such as nucleotide, peptides, proteins, carbohydrates, esters, and small molecular metabolites.

3.4.3.1 Electrospray Ionization MS

Electrospray ionization MS (ESI-MS) is very suitable to investigate the samples with small volumes. In order to be supplied to ESI-MS, the single cell must be lysate. Huang et al. reported a cell ordering platform induced by Dean flow to isolate single cells from cellular suspension. The platform has been integrated with ESI-MS (Fig. 3.10j) [129]. In this method, individual cells were lysate one after

another and then analyzed ESI-MS to identify a single-cell into a subpopulation by lipid profiling. This platform made cells in suspension evenly distributed and significantly improved the efficiency of single-cell mass spectrometry. Lipid is the high abundant target to detect, and some researches more focused on other low abundant targets. For example, Gong et al. developed a probe ESI-MS spectrometry with capillary microsampling to monitor single living leave cells and analyzed the cellular stress of healthy and damaged plant leave cells [130]. The results showed the clear differences in the levels of abscisic acid as compared to other MS analysis methods. However, this method had a disadvantage that the microneedles might hamper normal cellular functions.

3.4.3.2 Matrix-Assisted Laser Desorption/Ionization MS

Matrix-assisted laser desorption/ionization MS (MALDI-MS) utilizes the power of a laser to make the sample embedded in matrix crystals desorption and ionization. This is a soft ionization technique, thus the majority of molecules maintain their original size and weight without fragment. MALDI-MS was employed in single-cell analysis over the last few years. Many efforts have been made to improve the spatial resolution and decrease the interference from the matrix material. For example, Krismer et al. screened different strains of *Chlamydomonas reinhardtii* through MALDI-MS [131]. They embedded microwells to the stainless steel plate for separating individually spotted cells. And then, they fast freezed the samples and deposited the matrix to obtain the mass spectra of single cells (Fig. 3.10k). The MS results showed that the native strain with two different kinds of chlorophyll could be distinguished from the mutant strain lacking one of these chlorophyll subtypes.

3.4.3.3 Secondary Ion Mass Spectroscopy

Secondary ion mass spectroscopy (SIMS) provides a tool to enable and analyze the composition of solid surfaces and thin films. The principle of SIMS is that MS investigates the release of secondary ions from the surface sputtered with an ion beam. Although SIMS has the advantages with the low detection limit and the high spatial resolution, yet samples must be placed in the ultra-high vacuum for analysis, thus inhibiting the study of living organisms. Bobrowska et al. have published a protocol of single cells preparation for the time of flight secondary ion mass spectrometry (TOF-SIMS), including the steps for pretreatment of single-cell samples (fixation, washing, dehydration) and detection by SIMS [132]. TOF-SIMS has frequently been employed for single-cell analysis. For example, it was applied to uncover differences among breast cancer cell lines, and the results showed an 18-carbon chain fatty acid only presenting in the BT-474 cell line [133]. In addition, 3D TOF-SIMS allowed for multiple MS images taken at different heights, thus creating Z-scans for samples [134].

3.5 Conclusions and Outlooks

Single-cell research is a newly established field and develops rapidly. The rapid development of this field is inseparable from the contributions of emerging microfluidic technologies. Although conventional methods for single-cell capture, in particularly FACS, are mature and used routinely, they have one or more following disadvantages: high laboratory skills, low throughput, lack of visual inspection, and the risk of introducing pollution. Emerging microfluidics for single-cell capture overcomes these shortcomings and becomes increasingly mature. However, high-throughput and efficient single-cell separation are still an important goal of microfluidics. In addition, open microfluidic techniques (e.g., microwells) provide convenience for subsequent single-cell manipulation, but also increase the risk of sample contamination; therefore, the work of microfluidic methods for single cell sorting needs continuous development. Microfluidic designers must take full account of the throughput of cell sorting, the efficiency of single-cell separation, the subsequent extraction of single cells, and sample contamination, and visual observation of cells, easy operation, and low cost are other aspects that need to be taken into account.

Single-cell culture is especially important for rare cells. Multiple generations from single-cell starting points are different from the cell populations derived randomly. Single-cell dynamic culture process provides real-time evidences for better understanding of cell behaviors and interrogation of cell phenotypes. Compared to microfluidic methods for single-cell capture, single-cell culture microfluidic platforms need bigger space for single-cell division, timely nutrition supplement for long-term cell growth, and minimum shear stress or other damage stresses. In addition, the single-cell culture platform should consider the easy acquisition of cells of interest. Similar to microfluidic single-cell capture, microfluidics provides 2D and 3D molds for single-cell culture. Although 2D models have the advantages with low cost, reproducibility, and easy operation, 3D molds simulate cell function and the geometry of tissues and organs, which is an irreplaceable advantage of 2D models. We also briefly presented advanced analytical technologies to identify heterogeneities among large cell populations, including optical characterization techniques, electrochemical analysis techniques, and mass spectrometric analysis techniques. Optical characterization techniques based on microscopy and fluorescence have the advantages of high sensitivities and spatial resolutions. Electrochemical and mass spectrometric techniques play unique roles in single-cell analysis in recent years, due to the detection of label-free biomolecules. Single-cell analysis methods are not limited to these technologies, and more novel technologies are emerging to extend our knowledge of cellular processes. However, the reliability of quantitative results is often questioned due to the limited accuracy of many analytical approaches and the lack of references. In addition, combining two or more analysis technologies can provide complementary information,

because of the complexity of cellular processes. We believe that further development of microfluidics and innovations of analytical instruments will cast light on single-cell heterogeneity and potentially promote the development of individual therapy.

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