

Microfabrication methods for 3D spheroids formation and their application in biomedical engineering

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Abstract—Three-dimensional cell culture systems offer greater understanding of the complex human body structure than monolayer cell cultures. Spheroids, which are the most useful and controllable types of three-dimensional cell formations, are discussed in this review. Conventional spheroid fabrication methods have limitations for the mass production of uniformly sized spheroids, which hinders their further application. As an alternative, microfabrication methods have been proposed to overcome the drawbacks of existing methods. Microfabrication technologies include micropatterning, 3D bioprinting, and microfluidics. Microwell arrays and surface-modified micropatterns can be fabricated through micropatterning methods, and these scaffolds result in the mass production of spheroids with size uniformity. 3D bioprinting technology enables uniformly sized spheroid production at desired locations, and microfluidics allows production of uniform size-controlled spheroids in a large quantity. Recently, efforts have been made to apply 3D spheroid culture systems to regenerative medicine, the study of the tumor microenvironment, drug screening, and organoid fabrication. The 3D spheroid system is an attractive substitute for overcoming the limitations of the conventional 2D culture platform, which cannot precisely imitate *in vivo* physiological environments. Microfabrication methods for spheroids enhance the effectiveness of spheroid formation, allowing for mass production, size control, and spheroid localization. Microfabrication methods have remarkable potential for spheroid utilization in the biomedical field.

Keywords: 3D Cell Culture, Spheroid, Microfabrication, Regenerative Medicine, Microenvironment

INTRODUCTION

Complex 3D structures of the human body can be better understood through three-dimensional (3D) cell culture systems compared to traditional monolayer cell culture systems. Mimicking these complex 3D structures, including simulating the cell-cell and cell-extracellular matrix (ECM) interactions outside living tissue, have multiple merits. These interactions enable the transmission of biochemical and mechanical signals between the cells constituting the 3D structure, inducing them to behave similarly to real tissues. Among the many 3D cell culture methods available, we focus on cell spheroids in this review. A spheroid is an aggregate of cells with a spherical shape, which is the most advantageous and controllable form of 3D cell structure, with enhanced cell-cell and cell-extracellular matrix interactions within it [1]. This intercellular communication network in spheroids that replicates the *in vivo* cell environment can be employed for a variety of applications in the biomedical field, such as drug screening [2-4], cancer research [5,6], organoid formation [7-9], and tissue regeneration [10-12], all of which require mimicking the human body.

For the formation of spheroids, the hanging drop method, culture on nonadherent (or low adherent) surfaces, spinner flasks, or rotating flask bioreactors are conventionally used owing to their

simplicity (Fig. 1). Hanging drop is the most commonly used method, because it is possible to fabricate uniformly sized spheroids without specialized equipment using this method [13]. However, it is time-consuming, labor-intensive, has low throughput, is unstable, and is only capable of short-term culture [5]. Cell culture on non-adherent surfaces is also a widely employed method owing to its low cost, ease of handling, availability for producing a large number of spheroids, and possibility of post-culture recovery [1]. Nevertheless, this method has some critical limitations, such as the generation of spheroids with non-homogeneous sizes, uncontrollable size of spheroids, and difficulty in media exchange [14]. Employing a spinner or rotating flask bioreactor is suitable for the mass production of spheroids and long-term culture under controlled nutritional conditions [6]. However, the method requires specialized equipment, the size uniformity of spheroids cannot be controlled, high shear force may affect the cell function, and the bioreactor has no individual compartments, which may be necessary for further applications [5,14]. The mass production of spheroids with homogeneous volume and shape is essential for organized applications, such as obtaining precise analytical datasets, effective and predictable cell therapy, and sufficient yield of uniform organoids [4,15,16]. Utilization of the microfabrication method has emerged as a prospective solution to overcome the current drawbacks of the conventional spheroid formation methods.

The mass production of uniform-sized spheroids is important for obtaining consistent results in drug screening. In addition, because biological activity within spheroids is significantly affected by their sizes, microfabrication methods have been suggested in

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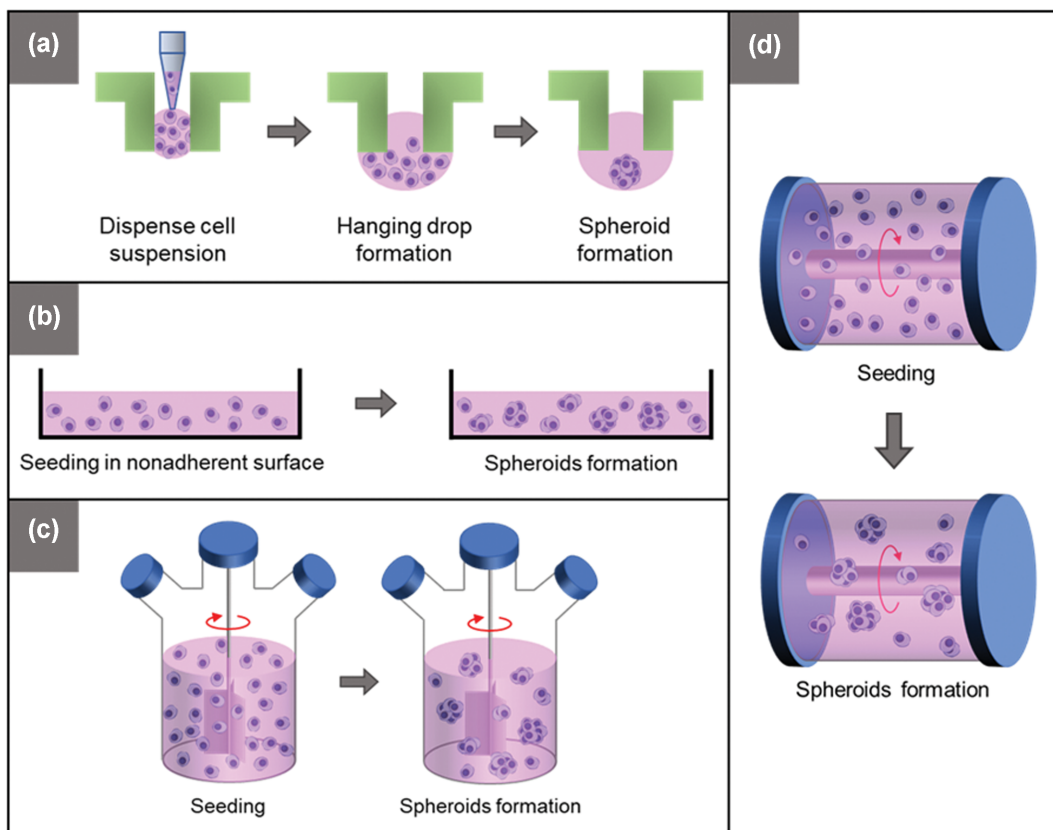


Fig. 1. Conventional spheroid fabrication methods. (a) Hanging drop culture. (b) Culture on nonadherent surface. (c) Culture in spinner flask bioreactor. (d) Culture in rotating flask bioreactor.

many studies for fabricating large number of uniform spheroids [17,18]. Microfabrication mainly consists of methods that use micropatterns, 3D printing, and microfluidics, which involve microscale modifications to culture substrates. Unlike other conventional spheroid fabrication methods, microfabrication enables the large-scale production of uniformly sized spheroids. Moreover, immobilization of spheroids at specific locations is possible for their effective utilization. Owing to these traits, microfabrication technology has received attention not only for the fabrication of uniform spheroids but also for precise analysis of spheroids, formation of spheroid-assembled organoids, and stable production of therapeutic agents secreted from spheroids. We first discuss the microfabrication methods for uniform mass production of spheroids followed by the biomedical applications of spheroids fabricated using these methods. The advantages and disadvantages of several conventional and microfabrication methods are summarized in Table 1.

MICROFABRICATION METHODS FOR SPHEROID FORMATION

Microfabrication methods using micropatterns, 3D printing, and microfluidics for fabrication of spheroids have been widely used in biomedical research [1,7,15,18-22]. This section introduces the microfabrication technologies used for spheroid production. The various microfabrication methods for spheroid fabrication, their applications, and retrieval potentials are summarized in Table 2.

1. Micropattern

Micropatterns are microscopically patterned or textured surfaces that usually exist in the form of large arrays. Micropattern array fabrication methods include soft lithography [11,18,19,21,23,24], photolithography [10,27-29,49], three-dimensional (3D) printing [32], and surface modification [25,30,31,50,51]. Micropattern-based microfabrication methods commonly allow size control, mass production, and localization.

1-1. Soft Lithography

Soft lithography is widely used to create or reproduce three-dimensional structures using molds, elastomeric stamps, and photomasks. Polydimethylsiloxane (PDMS) is commonly used as an elastomeric material owing to its low toxicity, high thermal and chemical stability, sufficient transparency for light penetration, ease of fabrication, and cost-effectiveness [56]. The soft lithography process generally accompanies photopatterning of the photoresist using a photomask and subsequent molding of elastomeric materials such as PDMS or hydrogels (Fig. 2(a)) [1,5,18,19,21,23,24,32].

Microwells fabricated using the soft-lithography method consist of only one type of nonadhesive material. This non-adhesive microwell enables cells to be filled inside the well by gravitational force and to be aggregated by themselves because there are no sites for cells to be attached. In addition, the structural refinement presented by the pattern wall prevents cultured cells from overriding the confined space. Consequently, a large quantity of spheroids of uniform size are stably formed inside the microwells and can easily be har-

Table 1. Advantages and disadvantages of each method

Technical method		Advantages	Disadvantages
Conventional	Hanging drop	<ul style="list-style-type: none"> • Uniformity of spheroid size • Works with small cell population 	<ul style="list-style-type: none"> • Time consuming • Labor-intensive • Low throughput • Low stability • Unsuitable for long-term culture
	Nonadherent surface	<ul style="list-style-type: none"> • Cost-effective • Ease of handling • Possibility of post culture recovery • Mass production of spheroids 	<ul style="list-style-type: none"> • Nonuniform spheroid size • Tedious media exchange • Unsuitable for long-term culture
	Agitation-based approach	<ul style="list-style-type: none"> • Long-term culture • Mass production of spheroids • Able to control nutritional conditions 	<ul style="list-style-type: none"> • Nonuniform spheroid size • May induce damage to the spheroid cells
Microfabrication	Micropattern	<ul style="list-style-type: none"> • Ease of handling • Size controllable • Mass production of spheroids • Ease in localization 	<ul style="list-style-type: none"> • Difficult retrieval of spheroids
	3D bioprinting	<ul style="list-style-type: none"> • Uniformity of spheroid size • Allows for complex and organized structures • Enables precise spheroid production at a desired location 	<ul style="list-style-type: none"> • Mechanical stress during extrusion • Selection of bioink with desired characteristics • Expensive • Time consuming
	Microfluidics	<ul style="list-style-type: none"> • Mass production of spheroids • Uniformity of spheroid size • Uniform cellular composition • Long-term culture • Continuous perfusion • Able to control nutritional conditions 	<ul style="list-style-type: none"> • Low cell recovery • Limitation for post-cell analysis • Complicated setup and device fabrication

vested [18]. However, a microwell with only non-adhesive material cannot avoid cell loss because of its position on the top surface of the compartment, and multiple spheroid fabrication in the well may affect assay chemistry [5,23]. Thus, cylindrical microwells featuring inverted pyramidal openings have been developed to solve these problems [23].

In addition to the fabrication of the microwell array, a surface-modified micropattern can be achieved using a microcontact printing method (Fig. 2(b)) [25]. PDMS, an elastomeric material, is often utilized as a master stamp for surface modification. For example, collagen or other cell-adhesive materials can be coated at specific sites in a patterned manner, while other areas can be coated with poly(ethylene glycol) (PEG) or other non-adhesive materials. This surface modification results in better local adhesion of the cells seeded on it through chemical refinement of the cell adhesion site [25]. Another example includes application of liquid PDMS transferred to a transwell membrane to form a micropattern [26]. Consequently, a micropattern composed of non-adhesive and adhesive surfaces is generated through the PDMS stamping process so that the cells can be selectively attached to the adhesive area and subsequently form spheroids. Although the existence of cell-adhesive sites on the micropattern allows stable culture of spheroids for a long period, the cells and spheroids on one site may invade nearby

areas if no barriers, such as walls, exist between them. In addition, retrieval of fabricated spheroids using methods other than trypsinization is restricted, hindering applications such as spheroid transplantation and organoid generation.

1-2. Photolithography

Photolithography is a simple, rapid, and inexpensive technique for the fabrication of microstructures that only requires a photocrosslinkable prepolymer solution, photomask, and an UV light source. The photocrosslinkable prepolymer solution located on the substrate turns into a solid microstructure when exposed to UV light through a photomask. Monomers that did not crosslink are rinsed to obtain a pattern (Fig. 2(c)) [49]. SU-8 is the most widely used photoresist in this process [30], and acrylated or methacrylated PEG, such as poly(ethylene glycol) diacrylate (PEGDA) or poly(ethylene glycol) dimethacrylate (PEGDMA), are generally used as prepolymer solutions [49,57].

Microwells produced via photolithography have their own advantage of containing two different compartments: cell-adhesive and non-adhesive [27,29,31,50]. A structure solely providing sites for cell attachment results in stable cell growth and spheroid formation. However, the existence of cell-adhesive sites hinders direct spheroid utilization since the retrieval of spheroids from the microwell scaffold is limited. Thermoresponsive polymers such as poly(N-

Table 2. Characteristics of microfabrication methods

Microfabrication methods		Purpose	Spheroids retrieval	Reference
Micropattern	Soft lithography	<ul style="list-style-type: none"> • Drug screening • Tissue regeneration • Organoids • Study of tumor microenvironment • Co-culture studies 	Partially possible (depending on the substrate)	[16,18,23-26]
	Photolithography	<ul style="list-style-type: none"> • Tissue regeneration • Study of tumor microenvironment • Organoids • Cell-based biosensors • Tissue-engineered organs 	Partially possible (depending on the substrate)	[10,27-31]
	3D printed micropattern	<ul style="list-style-type: none"> • Study of tumor microenvironment • Drug screening • Organoids • Tissue engineering 	Partially possible (depending on the substrate)	[32-35]
3D bioprinting	Direct cell extrusion	• Production of replicable, customized, and cost-optimized engineered tissues for therapeutic purpose	-	[34,35]
	Functional bioink printing	<ul style="list-style-type: none"> • Organoids • Drug screening • Development of body-on-a-chip devices 	-	[36]
Microfluidics	Emulsion-based droplet generation	<ul style="list-style-type: none"> • High-throughput drug screening • Study of tumor microenvironment • Personalized treatment • Tissue regeneration • Organoids 	Possible	[7,37-40]
	Array of microstructures for cell trapping	<ul style="list-style-type: none"> • Organoid-on-a-chip • High-throughput drug screening • Tissue simulation • Cell immunosensor 	Possible	[41-45]
	Acoustofluidics	<ul style="list-style-type: none"> • Tissue engineering • Study of tumor microenvironment • Drug screening • Study of disease pathologies 	Possible	[46-48]

isopropylacrylamide) (PNIPAAm) can be used to avoid the damage caused to the spheroids by the routine trypsinization procedure [10,28]. The structural changes based on their critical solution temperature behavior endow polymers with either hydrophobicity or hydrophilicity at certain temperatures. In particular, PNIPAAm undergoes a lower-critical-solution temperature (LCST) transition above and below the LCST. As cells favor adherence on hydrophobic surfaces, they can be stably attached to the polymer substrate above the LCST when it is hydrophobic, and can be released from the polymer substrate after it turns hydrophilic below the LCST. This system enables harmless cell retrieval from the micropatterned scaffold (Fig. 2(d)) [10,28,32,51].

1-3. 3D Printed Micropattern

In recent decades, the 3D printing method has been one of the most extensively studied technologies for fabricating microwell arrays. 3D wall structures comprising microwells can be 3D-printed on a

substrate [35,36]. Various materials can be printed, regardless of their cell adhesiveness. A 3D-printed mold, instead of a soft lithographical elastomeric mold, can be used for reproducible microwell fabrication [32]. Rapid advances are being observed in 3D printing-assisted micropattern fabrication when producing complex three-dimensional microwells. Soft lithography and photolithography methods are not adequate for constructing complex microwell arrays because the micropattern depends on the design of the two-dimensional photomasks. Although studies on the construction of complex 3D micropatterns using soft lithography and photolithography methods have been reported, they require multistep protocols that could influence process sterility and effective spheroid formation [33]. However, the 3D printing method enables a simple one-step fabrication of three-dimensional mold (Fig. 2(e)). Complicated 3D microwell scaffolds have been reported to improve the mass production of regular spheroids by reducing cell

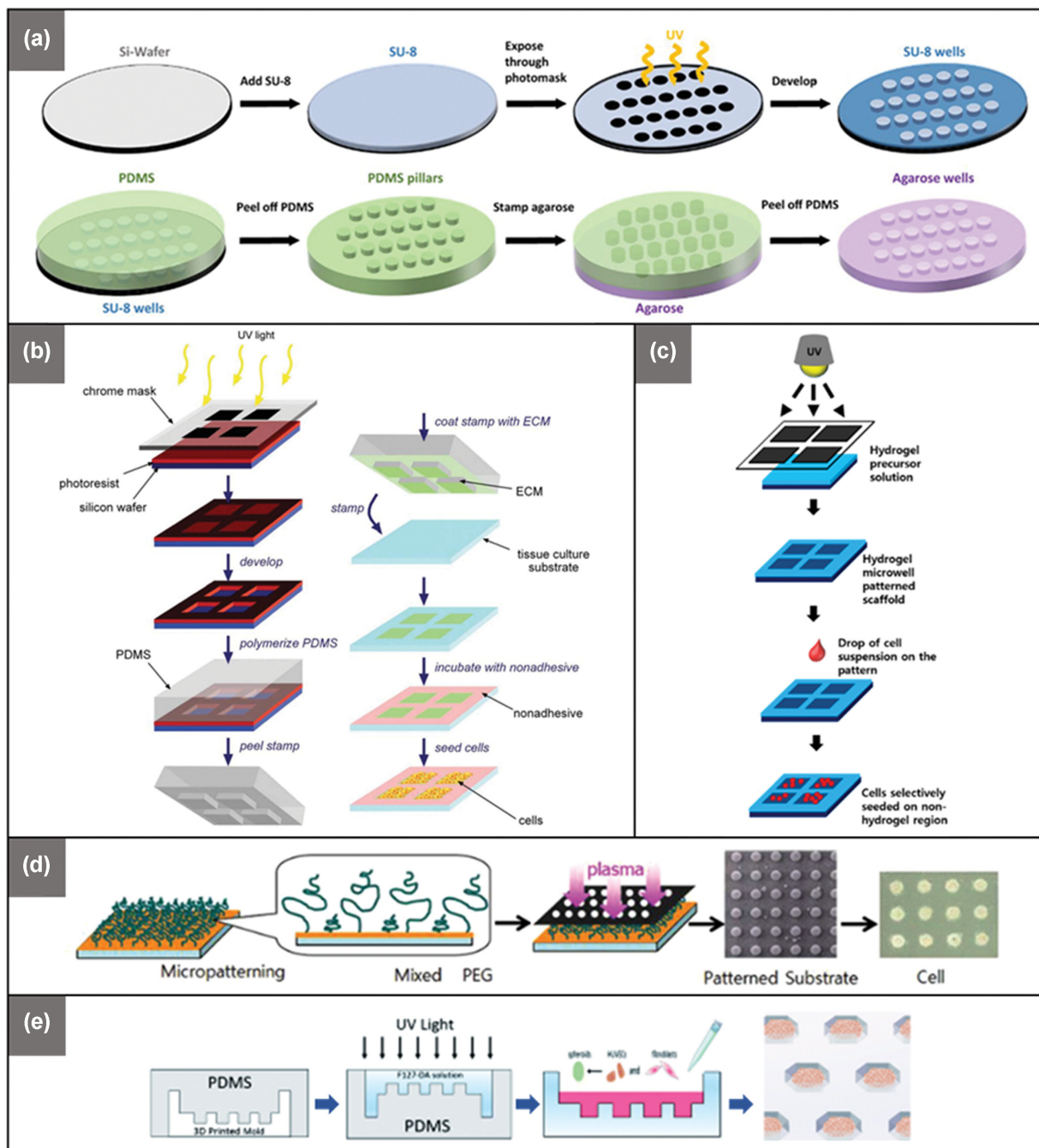


Fig. 2. Micropattern fabrication methods. Schematic representation of (a) microwell scaffold production using soft lithography, (b) surface-modified micropattern production using soft lithography, (c) microwell scaffold production using photolithography, (d) surface-modified micropattern substrate production using photolithography, and (e) complex 3D microwell structure production using 3D printing method. Reprinted with permission from Samy et al. [52], Liu and Chen [53], Hong et al. [54], Lee et al. [50], and Li et al. [55].

loss and improving the usability of the scaffold [33,58].

2. 3D Bioprinting

Although mass production of uniform-sized spheroids is possible with the aid of micropatterns, this method only allows spheroid arrangement in a 2D direction and therefore is not suitable for making 3D constructs with spheroids [36]. As an alternative, 3D bioprinting technology has recently emerged to create 3D models from spheroid cultures. 3D bioprinting methods other than sim-

ple microwell arrays are discussed in this section.

2-1. Direct Cell Extrusion

3D bioprinting enables the precise and versatile printing of complex ingredients, including cell-containing biomaterials, ECM, and cytokines, to construct intricate 3D functional structures *in vitro* similar to living tissues or organs [59]. Bioprinters operate based on additive manufacture and layer-by-layer deposition, forming biological structures in a reproducible and organized manner [15].

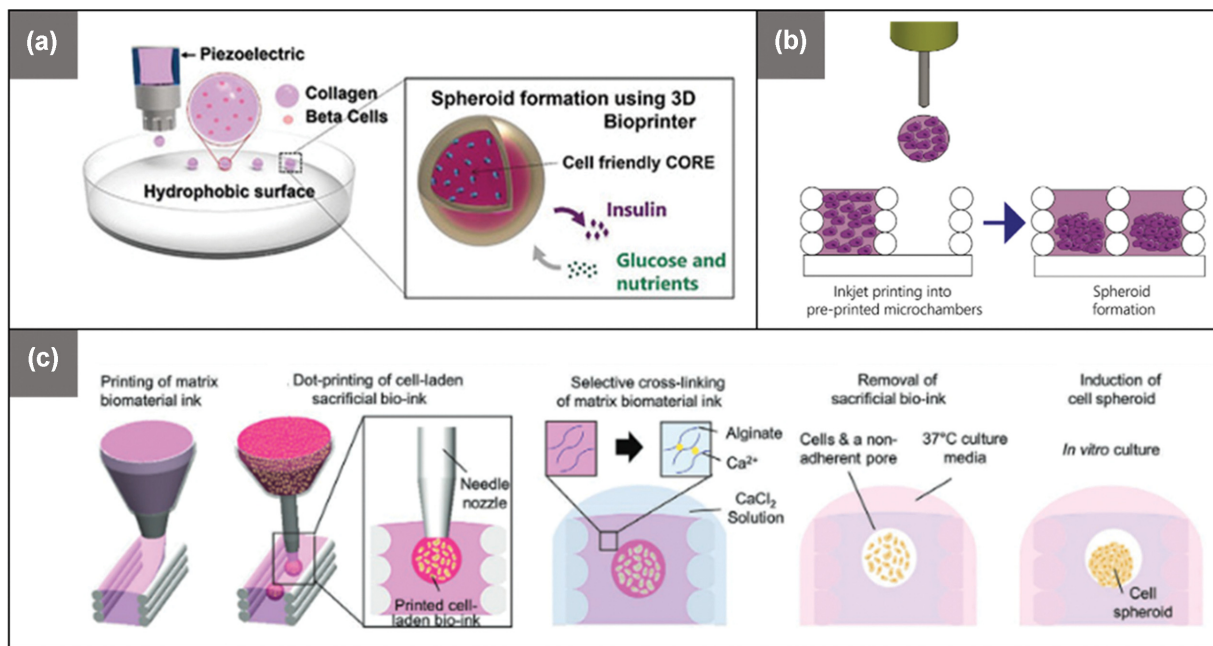


Fig. 3. 3D bioprinting technologies for spheroid generation. Schematic representation of 3D bioprinting methods for mass production of size-controlled spheroids (a) via extrusion of cell-laden bioink into droplets, (b) via direct cell printing on non-adhesive polymeric microchamber array without using bioink, and (c) via extrusion of sacrificial bioink containing suspended cells into supportive matrix biomaterial ink. Reprinted with permission from Clua-Ferré et al. [34], Daly and Kelly [35], and Jeon et al. [36].

The 3D bioprinting method is employed to generate a large quantity of spheroids with controlled size [60]. The extrusion of cell-laden bioinks into droplets results in spheroid formation (Fig. 3(a)). Cell adhesion property of the bioink plays critical role in spheroids formation process. Cell-adhesive bioink provides an ECM-like environment for stable cell attachment and proliferation. Therefore, the droplet is fully occupied with cells and generates a spheroid whose size is similar to that of a droplet [34]. On the other hand, a droplet of cell-repellent bioink promotes aggregation of the cells and intercellular adhesion within it, which produces a spheroid smaller than a droplet [35,61]. The main issue of the bioprinting method is in the selection of bioinks with desired characteristics, such as viscosity, shear thinning, viscoelasticity, biodegradation, crosslinking, and cellular adhesion [62]. A variety of hydrogels possessing optimal characteristics are used as bioinks or supportive surroundings to provide structural support during the process [15,59,63,64]. However, several challenges remain, such as presence of mechanical stress to the cells embedded in the hydrogel during extrusion, external stimuli to the cells from the crosslinking step, and lack of nutrients and oxygen exchange depending on the pore size of the spheroids, which decreases cell viability [63,64].

Various bioprinting technologies have been introduced to overcome the limitations of the early 3D bioprinting methods. For example, direct cell printing onto 3D printed micropatterns without using a hydrogel produced an array of highly uniform-sized spheroids (Fig. 3(b)). Combined with the printed non-adhesive polymeric microchambers, the cell suspension solution containing a controlled cell quantity was directly printed into each microchamber. Consequently, a highly organized array of cellular spheroids with automatically regulated sizes was successfully obtained [35].

2-2. Sacrificial Bioink Printing

A method using a sacrificial bioink instead of a hydrogel was proposed to fabricate precise 3D-patterned spheroids. After sequential printing of the polymer barrier and matrix biomaterial as supportive surroundings, the cell-laden sacrificial bioink was dot printed inside the matrix biomaterial. The matrix biomaterials were selectively crosslinked, and the sacrificial bioink surrounding the cells was dissolved to generate pores enclosed within the matrix biomaterial. Consequently, the printed cells inside the pores were allowed to form spheroids *in situ* through continuous culture, and precise 3D positioning of the spheroids was simultaneously achieved within a single step. Moreover, spheroid size control and accurate localization of spheroids were verified (Fig. 3(c)) [36].

Although 3D bioprinting enables precise spheroid production at a desired location, the practical use of this technology still has limitations because it is expensive, difficult to manipulate, and relatively time-consuming compared to the other methods [59].

3. Microfluidics

Microfluidics refers to the submillimeter-scale control and manipulation of fluids within microstructure devices. It enables the handling of small reagent volumes and simultaneous operation on a single microfluidic device [65]. Because miniaturization, microscale manipulation of fluids, rapid sample processing, high-throughput, and facile handling procedures, microfluidics has become a promising technique in biomedical research [37]. Conventional spheroid formation methods suffer from size distribution problems, are labor intensive, and have low throughput, all of which can be solved with microfluidics [66,67]. In addition, depletion of oxygen and nutrients owing to their static environments is a general limiting factor in spheroid culture. This can be managed through microflu-

idics that provides a dynamic microenvironment owing to the continuous media flow that enables long-term culture of spheroids with enhanced cell viability [41,67].

Three main techniques for generating spheroids using microfluidics are discussed in this section: emulsion-based droplet generation [7,37,38,67], array of microstructures for cell trapping [38,41-43,67], and acoustofluidics [20,46-48].

3-1. Emulsion-based Droplet Generation

Emulsion-based techniques that employ phase separation between two or more liquid phases have been widely used in the fabrication of various droplets. This technique is capable of con-

trolling size in microscale and high-throughput continuous operation [66]. Since the space inside the droplets is limited by volume confinement, when cells are encapsulated, they spontaneously aggregate into uniformly sized spheroids. In the general cell encapsulation method, a water-phase cell suspension solution (CS) is dispersed into the oil phase (O) in the form of droplets. The cell suspension solution passing the continuous phase of oil becomes droplets, where the oil phase flows perpendicular to the flow of the dispersed phase including cells (CS/O) (Fig. 4(a)) [37,38,67]. Hydrogels are also widely used as additive materials in dispersed phases (gel/O) [38,43,71]. Double emulsion techniques, such as cell sus-

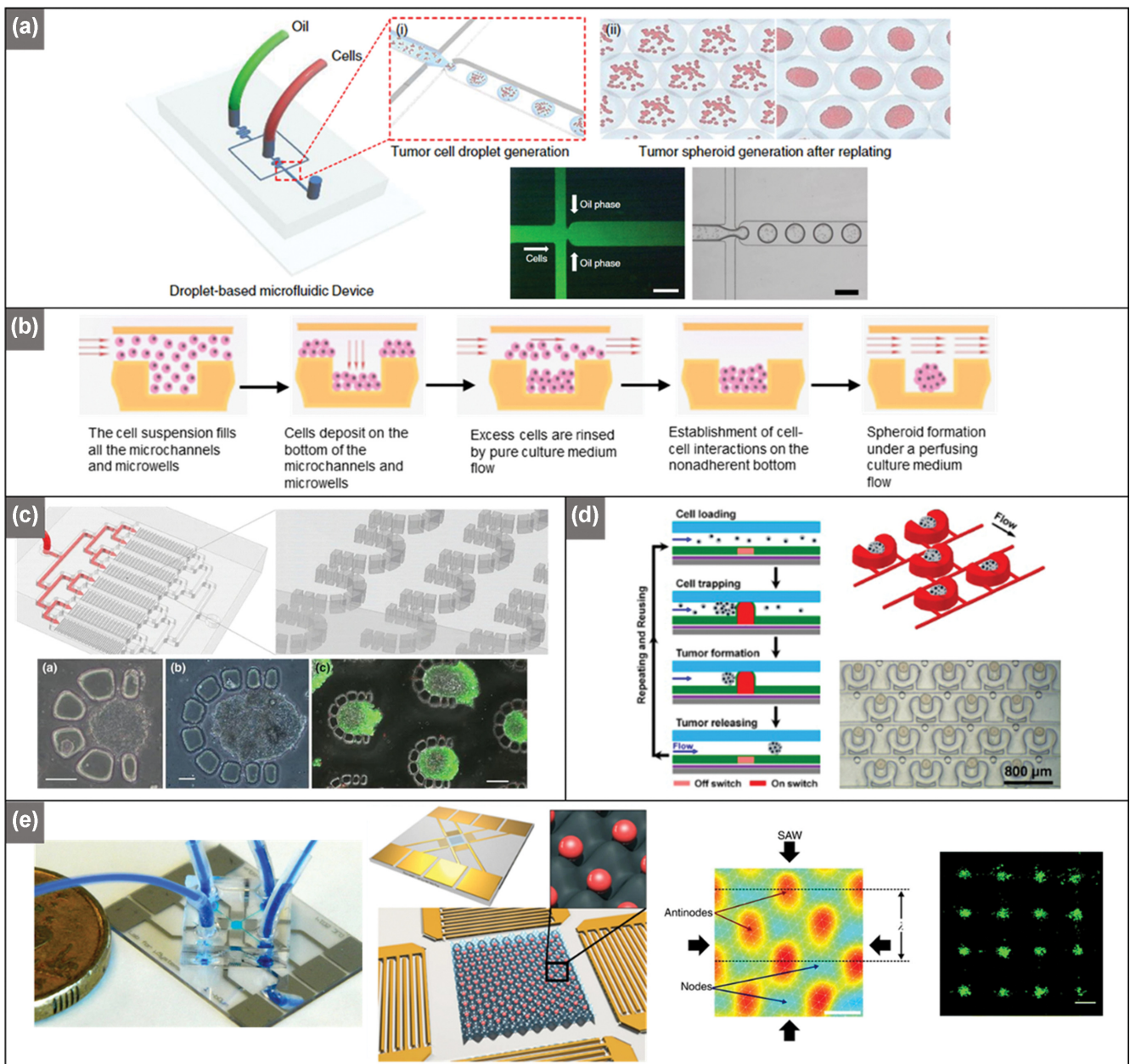


Fig. 4. Spheroid fabrication methods using microfluidics. Schematic representation of the microfluidic methods employing (a) emulsion-based droplet generation, (b) microwell-incorporated microfluidic system, (c) embedded U-shaped microstructure, (d) pneumatic actuation type of U-shaped microstructure system, and (e) acoustofluidics. Reprinted with permission from Lee et al. [37], Moshk-sayan et al. [67], Khoury et al. [68], Liu et al. [42], Armstrong et al. [69], and Collins et al. [70].

pension in oil in culture medium (CM) (CS/O/CM), can be introduced to stably entrap the cells and to enhance the droplet size uniformity by stabilizing the droplet structure [39]. However, emulsion-based methods require precise parameter settings prior to generation of uniform-sized droplets, and handling cell-encapsulated emulsions is difficult [38].

3-2. Array of Microstructures for Cell Trapping

An array of microstructures, such as microwells and U-shaped microchannels, has been introduced to facilitate the mass production of uniform-sized spheroids [67]. These microstructures enable not only localization of the spheroids on miniaturized microfluidic chips but also further long-term culture. Microwell-based microfluidic techniques are frequently used for spheroid generation owing to their simplicity and ease of operation [67]. Cells can be evenly seeded on the microwells by adjusting the cell density, dimensions of the microwells, and flow rate. After spheroid formation in the microwell, spheroid retrieval is possible by employing a suitable flow rate (Fig. 4(b)) [72].

The operation types using the U-shaped microstructure barrier are divided into two groups: the embedded type [43,44] and pneumatic actuation type [45,71]. Embedded U-shaped microstructures entrap cells under precisely controlled conditions that include the flow rate and tilt angle of the microfluidic chip (Fig. 4(c)) [44]. The emulsion-based cell encapsulation technique combined with these microstructures can solve handling difficulties because the beads are localized within stationary microstructures. Stable cell growth in spheroids and further analysis can therefore be achieved easily by employing this system [43]. However, collecting the fabricated spheroids remains a challenge. Pneumatic actuation type of U-shaped microbarriers, which operate through pressure control, has emerged as an alternative, and this structural array effectively traps the cells to form spheroids at restricted locations. After the spheroids have been produced, they can be easily retrieved by deactivating the microbarriers (Fig. 4(d)) [71]. The limitations of these microstructure-array-based microfluidic systems include the requirement for accurate parameter control and post-culture harvesting [72].

3-3. Acoustofluidics

Acoustofluidic techniques in cell culture refer to the combination of surface acoustic waves (SAW) with microfluidic or microfabricated devices [47,48]. In recent decades, SAW-grafting techniques have been widely investigated owing to their ability to provide a biocompatible and flexible environment for cells. This enables contactless and label-free cell manipulation without any shear stress that could influence cell function [20]. Combined with microfluidics, acoustofluidics have been introduced for the rapid and mass production of uniform-sized spheroids [46,48]. Microchannel-embedded SAW devices can help drive formation of cell aggregates on the nodal parts of standing acoustic waves, resulting in spheroid formation within 30 min [47]. It is also possible to produce a large quantity of spheroids without microchannels by placing additional interdigital transducers (IDTs) perpendicular to the existing pair (Fig. 4(e)) [47,69,70]. In this way, the cells move on to the nodes of the generated SAW between the IDT pair at the opposite location, and the cells aggregate at the intersection of the nodes on the generated waves from each IDT pair. The resultant spheroids

can be easily collected by injecting a new inlet inside the microchannel or chamber of the device. This technique requires specific devices for acoustic wave generation, and it is difficult to set parameters for spheroid formation.

CLINICAL APPLICATIONS OF SPHEROID

1. Regenerative Medicine

1-1. Cell Transplantation

Cellular transplantation is one of the most promising strategies for regenerative therapies. In conventional direct cell transplantation techniques, dissociated cells can either be directly injected into target tissues or used as cell sheets. However, the retention time of transplanted cells at the cell injection sites is typically short. The short retention time of directly injected cells is thought to be caused by the lack of a temporary matrix to which the transplanted cells can attach [73]. In addition, mono-layered cell sheets have been shown to be difficult to handle because of their weak mechanical properties, while thick, multilayered cell sheets suffer from limitations of oxygen and nutrient supply [74]. Furthermore, the unfavorable microenvironment of cell graft sites, such as presence of ischemia, hypoxia, or inflammation, can result in cell death and the loss of cellular activity after transplantation [75].

The 3D spheroid culture approach is a remarkable technique to improve the efficiency of cell transplantation. First, the injected spheroids help maintain cell population by enhancing engraftment efficiency after transplantation. Spheroid culture induces cells to increase the expression of surface antigens such as CXCR4, which are responsible for cell adhesion and motility, thus facilitating homing of transplanted cells in new environments [76]. In addition, the aggregated cells show an enhanced paracrine effect compared to the dissociated cells [77]. Higher expression of anti-oxidative and anti-apoptotic proteins [77] and cell-cell and cell-ECM interactions in spheroids also provide resistance to harsh microenvironments [78] including those at the graft sites. Therefore, transplantation of spheroids is a more promising method than transplantation of dissociated cells.

Microfabrication methods facilitate the mass production of uniform spheroids for transplantation. Mass transport deficiency and development of a hypoxic core that result in cell death can be avoided in uniformly sized spheroids, thereby enhancing the survival of cells in long-term cultures. For practical applications, the spheroid diameter should be less than 200 μm to prevent nutrient deficiency and severe hypoxia [79]. Using microfabrication methods, compact and size-controlled spheroids can be generated, thus improving the survival rate of cells [80]. In addition, biomaterials utilized in microfabrication methods, such as hydrogels and fibers, could also provide a supportive matrix to spheroids. They can localize cell spheroids at implantation sites, regulate cell migration, and protect cells from exposure to harsh environments [81].

1-2. Stem Cell-derived Secretome Therapy

One of the major challenges in cell-based replacement therapy is the mutation of transplanted normal cells within tissues, which may contribute to the origin of cancer-initiating cells [82]. Recent studies have focused on secreted factors from stem cells to address this limitation of the direct utilization of cells. The aggregate of fac-

tors secreted from stem cells, including cytokines, chemokines, growth factors, and extracellular proteinases, is defined as the stem cell secretome [83-85]. These molecules are known to regulate cellular activities, such as proliferation, migration, differentiation, angiogenesis, and inflammation [86].

Recent studies have shown that the lack of cell-cell and cell-ECM interaction in traditional 2D cultures makes it difficult for stem cells to behave in the same manner as they would *in vivo*. Stem cells express different secretomes when cultured under 3D conditions, secreting more anti-inflammatory and wound healing-related secretomes in comparison to culture in 2D culture conditions. The 3D spheroid culture model allows interaction between cells, creation of ECM, and releases a gradient of paracrine factors, nutrients, and other metabolic products. In such an environment, the release pattern of immunoregulatory cytokines and angiogenic factors can be more analogous to an actual body-system [87]. The 3D spheroid culture model established using the microfabrication method, which effectively mimics living tissues, improves the effi-

ciency of regenerative therapy [83,88,89]. Cell biology involving cellular function within spheroids is not only affected by the dimension of the culture model, but is also strongly correlated with size of spheroid, [17] and hence the formation of uniform-sized spheroids with microfabrication would be beneficial.

On the other hand, microfabrication methods would be desirable for understanding the composition of a secretome. Despite the therapeutic potential of the secretome, there have been only a few studies on its efficacy as a function of its composition [83]. Consistency of secretome composition is required for reliable analysis and therapeutic applications. The uniformity and mass productivity of spheroids using the microfabrication method would facilitate further understanding and clinical application of the secretome.

2. Study of Tumor Microenvironment

Tumors do not exist in an isolated form *in vivo*. They exist in a heterogeneous population of malignant cells and are involved in complex relationships with various tumor-associated cells. Tumors and their surrounding microenvironments interact continually and

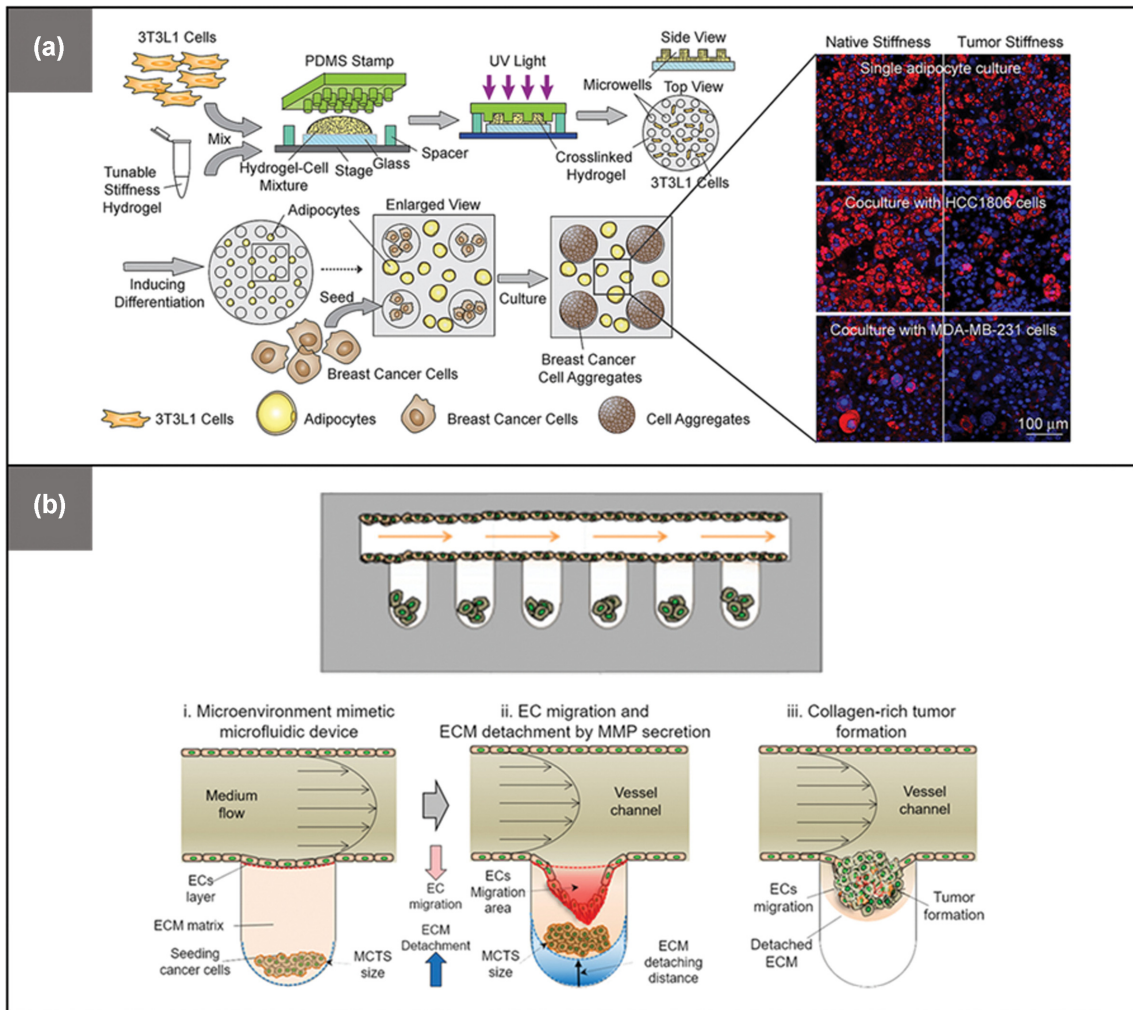


Fig. 5. Application of spheroid microfabrication methods for tumor microenvironment study. (a) Illustration of the fabrication process of adipocyte encapsulated hydrogel-based microwell array and its co-culture with breast tumor spheroids. (b) Schematic representation of the microfluidic device mimicking *in vivo* interactions between the cancer cells and vascular endothelial cells. Reprinted with permission from Yue et al. [93], and Lee et al. [97].

influence one another, either favorably or unfavorably. Therefore, a deeper understanding of the tumor microenvironment is essential to determine the complicated mechanisms underlying tumor progression, metastasis, and immune activation of cancer cells.

Spheroids are likely to offer an attractive approach for studying tumor microenvironments. Research in cancer biology routinely employs 2D monolayer culture systems as a testing platform for studying the tumor microenvironment. Although 2D culture systems have improved our understanding of cell behavior, it is difficult to embody the spatiotemporal complexity of the tumor microenvironment in a 2D culture. 2D cultures cannot maintain the original form, polarization, genetic profile, and heterogeneity of cancer and stromal cells because of their planar cell distribution, which differs from the multifaceted cell morphology in the 3D culture system [90]. Thus, more elaborate models that accurately mimic the microenvironment present in *in vivo* tumors are necessary and these could be accessible with microfabrication methods.

The size of the spheroids is another critical factor. Spheroids closely mimic the heterogeneous structure and internal gradients of signaling factors, pH, nutrients, and oxygenation [91]. Beyond approximately 500 μm , spheroids display typical characteristics of

avascular tumors, including an external proliferating zone, an internal quiescent zone, and a necrotic core due to gradients in nutrient and oxygen concentrations [92]. Cells in the outer zone are highly proliferative because they have greater access to oxygen and nutrients. As we move towards the core of spheroids, the supply of oxygen and nutrients decreases, whereas the amount of carbon dioxide and waste increases [91]. The heterogeneous cell layer structures and nutrient gradients depend on the spheroid size and volume. Microfabrication methods capable of optimizing and controlling spheroid sizes are ideal for imitating such an environment for studying cancer biology.

The tumor stroma is associated with resistance to several cancer therapies. For instance, it has been shown that cancer-associated fibroblasts, which comprise a significant fraction of stromal cells in the tumor microenvironment, are crucial for promoting tumor growth and enabling cancer cells to resist treatment [93]. To further understand stroma-cancer interactions, co-culture systems of microfabricated tumor spheroids and stromal cells have been developed (Fig. 5(a)) [93-95]. Furthermore, vasculature plays an essential role in tumor metastasis and growth [96]. The vascular network is an important component of metastasis because can-

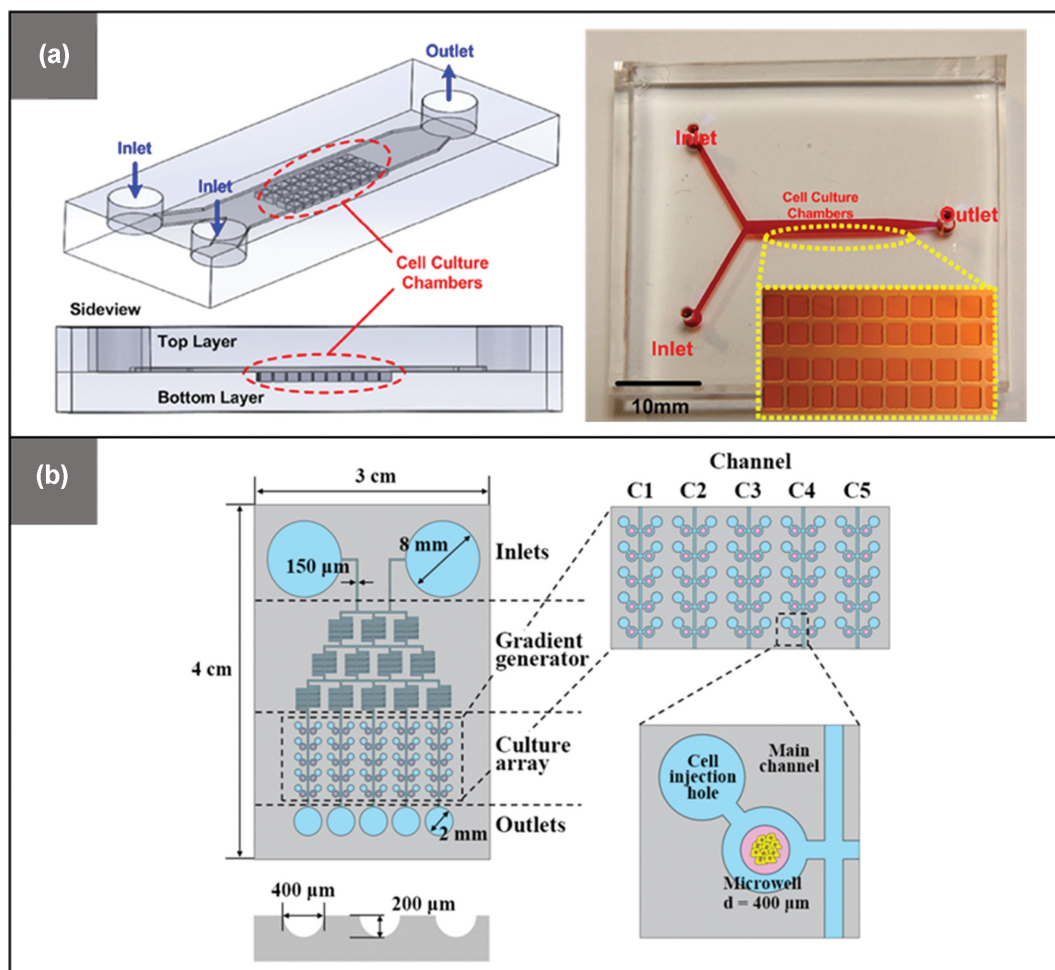


Fig. 6. Application of microfluidic devices for high-throughput drug screening. Schematic representation of the (a) bi-layered and perfused microfluidic system structure, and (b) microfluidic spheroid culture device with a concentration gradient generator. Reprinted with permission from Patra et al. [110], and Lim and Park [111].

cer cells migrate as circulating cancer cells through the blood vessels. The blood circulation system also plays an important role in tumor growth, because malignant cells exchange nutrients and waste through blood vessels. Thus, numerous attempts have recently been made to replicate the interaction between the tumor and blood circulation system through microfabricated tumor spheroids (Fig. 5(b)) [97,98].

3. Drug Screening

Drug development is time-consuming, costly and requires numerous stages of approval for clinical use. High-throughput screening (HTS), which involves the screening of all compound libraries for lead identification, is a crucial phase in drug development. Although animal models, mimicking human-specific biology, have long been used to understand various pathologies, the application of the results obtained in humans is inherently limited owing to the physiological differences between humans and animals [15].

In the case of anticancer drugs, 2D grown tumor cell lines have been used to assess the effectiveness of drugs. However, 2D grown cells on plastic surfaces are unable to simulate the complex micro-environment of *in vivo* tumors, leading to some errors in the HTS results. For instance, numerous anticancer drugs tend to be overestimated on a 2D-culture-based HTS platform, and thus are unable to show the expected efficacy during their clinical application [99, 100]. Hence, 3D spheroid culture models have become essential

tools for drug development [101]. Production of shape- and size-controlled spheroids is mandatory for reproducible and reliable results in drug screening, since the drug screening results depend on the structure and size of the spheroids [91,102,103]. This could be addressed with microfabrication techniques that produce spheroids in a sophisticatedly controlled manner. Moreover, mass production of homogeneous tumor spheroids is directly related to HTS. Gong et al. fabricated microwell-based agarose scaffolds as HTS platforms by stamping with a template containing micro nipples [18]. The authors were able to control spheroid size and demonstrated that drug resistance was strongly dependent on spheroid size.

Recent advancements in microfluidic technology have also contributed tremendously to the development of HTS systems [42,44, 104-108], which enable cell trapping and localization. Providing nutrients and oxygen steadily and removing cellular waste products for a long time to maintain *in vivo* conditions is also advantageous in microfluidic systems [91]. The bi-layered and perfused microfluidic systems are helpful for a continuous nutrition supply, allowing the transport of culture media and cellular waste (Fig. 6(a)) [109,110]. Finally, microfluidic devices with spheroids are beneficial for screening drug combinations (Fig. 6(b)) [111-113]. By mixing more than two drugs and generating a concentration gradient, it is possible to determine the appropriate cancer drug concentration in patients.

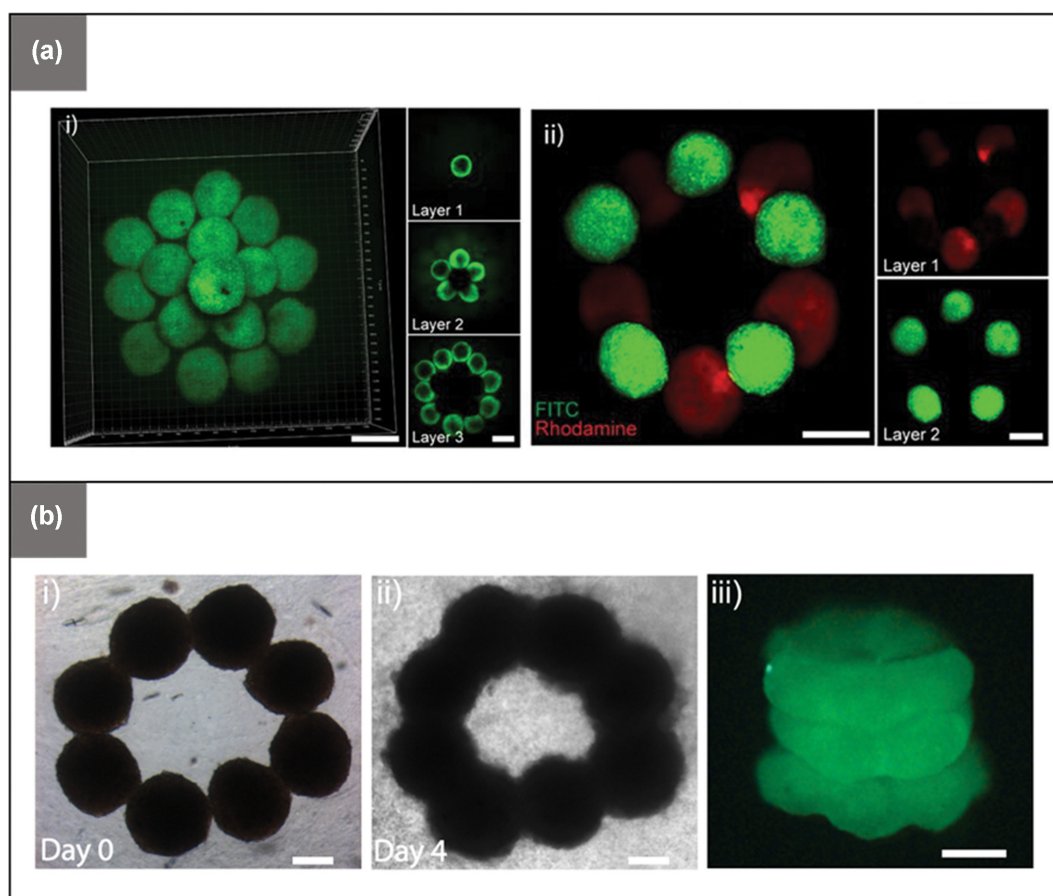


Fig. 7. (a) 3D biprinted mesenchymal stem cell spheroids with diverse geometries. (b) Bright-field images of the cellular pattern that undergoes fusion in both, the vertical and horizontal directions. Reprinted with permission from Daly et al. [63].

4. Organoids

Organoids are 3D cell culture models that self-organize into complex organ-like tissues [15]. Organoids can be established in a variety of organs, including the brain [114-116], liver [117,118], kidneys [119,120] and gut [121-123]. These *in vitro* miniaturized and simplified model systems for organs have attracted immense interest in personalized medicine, drug screening, and regenerative medicine. Although numerous organoid production protocols have recently been introduced, there are still certain production hurdles to overcome. In particular, reproducible production of organoids remains challenging, as small changes in the initial state of cells and their growth can directly affect production [124]. Therefore, further research is required to produce more stable and homogeneous organoids.

Spheroid assembly is an alternative method of organoid production. Microfabrication technologies such as bioprinting enable the programmed assembly of cells into complex 3D structures [125]. The spheroids function as building blocks for constructing and supporting 3D complexes (Fig. 7(a)). Jakab et al. [126] verified the feasibility of using spherical cell aggregates as bioink. Closely placed cell aggregates in 3D gels can self-organize into metastable tissue constructs of the desired shape. Spheroids printed in multiple layers undergo fusion in both the horizontal and vertical directions, forming an organ-like module (Fig. 7(b)). Similarly, microfabricated spheroids can be used as building blocks in 3D bioprinting applications [63,127].

Accelerating the maturation and functionalization of organoid constructs is necessary for highly efficient clinical applications. The spheroid-based model has an advantage in that it can significantly reduce tissue maturation time [128]. Moreover, microfabrication enables the mass production of uniformly sized spheroids, thus allowing the rapid generation of large quantities of cell spheroids necessary for building organ-like complex constructs.

CONCLUSIONS

The 3D spheroid culture system is an appropriate tool to overcome the limitations of the traditional 2D monolayer culture system, which cannot accurately replicate physiological environments such as *in vivo* multicellular microenvironments and biological signal pathways. Unlike conventional spheroid formation methods, microfabrication enables the large-scale mass production of uniform-sized spheroids. In addition, spheroids fabricated via microwells, 3D printing, and microfluidics have the potential for use in regenerative medicine, cancer research, and drug screening because of their excellent localization property.

Although microfabrication has allowed for spheroid production improvement, its potential has not yet been fully realized. Some microfabrication methods still require labor-intensive operations, and it is difficult to retrieve spheroids for further analysis. In addition, the selection of the parameters for the 3D culture model influences the availability of spheroid formation. Thus, a greater focus on exploring and optimizing the detailed techniques for microfabrication would enhance the accessibility of these methods.

In short, the development of microfabrication methods for spheroid formation is essential for its further application and utilization in the biomedical field.

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