Review

Inventions and Innovations in Preclinical Platforms for Cancer Research

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Abstract: Three-dimensional (3D) cell culture systems can be regarded as suitable platforms to bridge the huge gap between animal studies and two-dimensional (2D) monolayer cell culture to study chronic diseases such as cancer. In particular, the preclinical platforms for multicellular spheroid formation and culture can be regarded as ideal in vitro tumor models. The complex tumor microenvironment such as hypoxic region and necrotic core can be recapitulated in 3D spheroid configuration. Cells aggregated in spheroid structures can better illustrate the performance of anti-cancer drugs as well. Various methods have been proposed so far to create such 3D spheroid aggregations. Both conventional techniques and microfluidic methods can be used for generation of multicellular spheroids. In this review paper, we first discuss various spheroid formation phases. Then, the conventional spheroid formation techniques such as bioreactor flasks, liquid overlay and hanging droplet technique are explained. Next, a particular topic of the hydrogel in spheroid formation and culture is explored. This topic has received less attention in the literature. Hydrogels entail some advantages to the spheroid formation and culture such as size uniformity, the formation of porous spheroids or hetero-spheroids as well as chemosensitivity and invasion assays and protecting from shear stress. Finally, microfluidic methods for spheroid formation and culture are briefly reviewed.

Keywords: spheroid culture; microfluidic cell culture; spheroids on-chip; tumor microenvironment; in vitro cell culture

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Introduction

A vast number of investigations are being conducted in laboratories and research centers to produce drugs to cure cancer, but few of them can lead to the production of practical and useful drugs. The main reason of that most probably relates to the procedures utilized for experiments and to the *in vitro* platforms for drug screening. As a proof, cancer drug assays in mice, pig, monkeys can be mentioned, which are dominantly being performed in many laboratories [1]. In fact, these tests can be beneficial for a general understanding of what happens during the whole process in a systemic environment, but may not be suitable for drugs that are being generated for human that has different genotype and phenotype of such animals. Those few drugs, which show the effectiveness of cancer treatment in animal bodies, are used to be in human clinical trials. Such clinical trials need complicated protocols and require a large number of cancer patients to take part in the experiment. In the majority of these experiments, the drug fails to perform the expected task efficiently. Accordingly, the whole process and investment become waste and may lead to bankruptcy or at least a significant loss of materials, equipment, time and money.

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Parallel to what we call animal tests, other types of tests for drug investigation also exist which are performed using different kinds of methods and equipment. In these methods, cancer cell lines of human or laboratory animals are used. Although in such platforms the cells belong to human, the deficiency is the lack of physical and chemical parameters that exist in the tumor microenvironment. For instance, at in vivo tumor microenvironment, there is continuous perfusion of oxygen, carbon dioxide, nutrients, and wastes. However, these features are absent in most of the *in vitro* cancer drug screening platforms such as microwell plates or Petri dishes [2]. This continuous perfusion and diffusion cause chemical gradients to be made *in vivo* at tumor sites like hypoxic core which is essential for realistic in vitro assays.

Tumor microenvironment characteristics consist of several features [3], Figure 1. First, tumor microenvironment is hypoxic. Hypoxia occurs as the tumor grows because no capillary has been generated in tumor yet [4]. The second feature is angiogenesis. As a result, blood vessels are generated through cancer tumor to deliver oxygen and nutrients to the cells being proliferated in the tumor [5]. This phenomenon develops oxygen gradients in the tumor to generate hypoxic and necrotic regions in it. As the third trait, tumors are composed of different kinds of cells, including tumor cells, cancer stem cells, fibroblasts, white blood cells (e.g., lymphocytes, macrophages, and neutrophils), fat cells (adipocytes), pericytes and endothelial cells (induced by angiogenesis). So it is evident that for a realistic tumor microenvironment, we need to make tumor cultures that are composed of different types of cells (cell co-culture) as mentioned above. This issue is easy to handle via microfluidic cell culture chips fabricated by many groups all around the world in the last decade. Another feature of tumor cancer cells is their tendency of metastasis. Metastasis is a migration of cancer cells from tumor environment to other places in the body using blood circulation. The act of crossing the endothelial barrier and entering blood flow is called intravasation. After entering blood flow, the migratory cancer cell may find a susceptible region to cross the endothelial barrier and hence diffuse to another organ; this action is extravasation [6].

The three-dimensionality of the tumor cell culture environment also has significant effects on tumor cell responses to cancer drugs due to cell-cell interactions which take place only in a three-dimensional configuration of cells. This fact indicates that monolayer, two-dimensional cell cultures (mostly used cultures) are unable to mimic the in vivo behavior of cancer cells accurately [7].

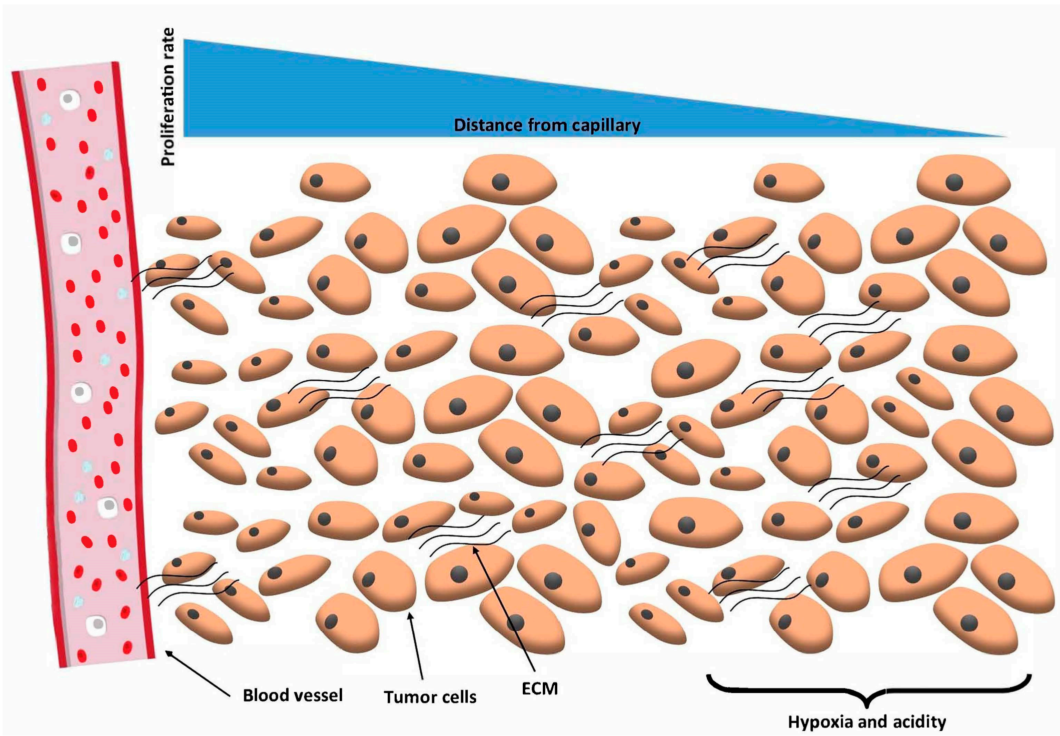

Figure 1. Blood vessels, extracellular matrix (ECM) and the tumor cell in the in-vivo tumor microenvironment.

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The three-dimensional cell culture formation methods have been vastly discussed in the literature, but practically, multiwell plates along with bioreactors and hanging droplet plates have been commercialized and used by many scientists to form spheroids. Although these approaches have several advantages, it has been justified that microfluidic devices are capable of forming 3D cell cultures (like spheroids and hydrogel-based cancer cell encapsulation) and drug tests in high throughput, more efficient and better-mimicked microenvironments [8]. For instance, the static microenvironment existing in a well in a microtiter plate causes fast depletion of oxygen and nutrients while increasing waste concentration in the well. This can influence the spheroid formation and the future results of the drug tests that need be performed on the tumor [9]. The similarities between *in vivo* tumor microenvironment and the tumor spheroids extend further. For instance, the cell proliferation activity in 3D spheroids of malignant pleural mesothelioma is more similar to biopsied cells than 2D monolayer cultures [10]. Several studies have illustrated that gene expressions are altered in 2D-monolayer cancer cell cultures while results obtained from spheroids have captured the *in-vivo* tumor tissue expressions [11] partly as a result of higher production of the cell adhesion molecules such as E-cadherin. Growth kinetics is also a crucial factor in tumor spheroids which resembles that for *in-vivo* tumors [12].

The spheroid culture of cells is not limited to cancer cells. Cell spheroids have been used as 3D cell cultures for mesenchymal stem cells (MSCs) [13], liver tissue [14], cardiac muscle [15], human embryonic kidney cells [16] and so forth. Embryonic stem cells, neural stem cells, pancreatic cells, and hepatocytes also need to be cultured in 3D configurations to induce differentiation and express their own metabolism and proliferation rate similar to the *in-vivo* conditions. Sometimes these cell spheroids are given different names such as neurospheres or embryoid/organoid body according to their cell type [17]. Spheroid formation process with these cells is similar to those made of cancerous cells. These cell spheroids have all the features mentioned above except that some quantities differ among them including spheroid formation time, oxygen uptake and diffusion and hypoxia limit. For instance, oxygen diffusion limitations develop necrotic core in both cancerous and hepatic spheroids when the spheroid grows more than a specified diameter which is 150-200 μm for hepatic cells and 500 μm for cancerous cells [18].

Here, first various spheroid formation phases will be introduced and the effect of hydrogel in spheroid formation and culture will be evaluated. After a brief review of the conventional spheroid formation techniques, the pros and cons of these methods will be presented. Finally, microfluidic methods for spheroid formation and culture will be briefly studied.

2. Spheroid formation phases

In general, we can divide the spheroid formation process into three phases [19]. Forming cellular aggregates and making compact spheroids within the first days is called the first phase. Spheroid diameter decreases during the first phase because cells are attaching to each other and forming stable aggregates [20], Figure 2A. The duration time of the first phase depends on the cell type as well as the method used. For example, Torisawa *et al.* [21] reported that HepG2 cells took three days to form spheroids, while MCF-7 cells only took two days on the same microchip. Chan and colleagues [13] also observed different time durations required for HepG2, MSC, PMEF, and Caco-2 cell lines to form spheroids in a single microfluidic device. Using hanging droplet (HD) method, Kelm *et al.* [22] claimed four days for HepG2 and five days for MCF-7 which were much longer than 24 hr reported by those who used microfluidic spheroid formation chips (μSFCs) from the same cell lines [23]. These data suggest that spheroid formation time depends strongly on the cell type and is attainable to be reduced using dynamic flow μSFCs instead of conventional methods with static flow conditions.

It has been reported that not all cell lines can form spheroids or at least have a lower tendency [24]. Increasing the fetal bovine serum (FBS) [25] or reconstituted basement membrane (rBM) [26] concentration in the culture media can enhance cell aggregation. Hence, it is possible to decrease spheroid formation duration time by elevating the level of FBS or rBM in the culture media. Frey and co-workers [25] investigated the effect of FBS concentration on the spheroid formation. The authors reported that 0% concentration of FBS led to no spheroid formation while the higher concentrations gave rise to larger spheroids.

In the second phase, spheroids face high proliferation rates and biomass production [27]. For human colon carcinoma cells (HT-29) it is declared to continue for seven days from the third day [27], four days from the second day for human colon carcinoma cells (HCT116) [19] and lasted up to the fifth day for co-culture of hepatocytes and hepatic stellate cells [28] on µSFCs. In the third phase, reported by Ziółkowska *et al.* [27], the spheroid growth and cellular proliferation slowed down after ten days of culture and spheroid size tended to a constant diameter (Figure 2B-1). A similar trend was reported by Lee and co-workers [28] where this phase occurred from the fifth day onwards for hepatocyte spheroids in accordance with the decrease in spheroid size. Chen *et al.* [19] also recorded this phase to begin at the sixth day for human colon carcinoma cells (HCT116).

After the occurrence of the three phases, the spheroid cells behave as they exist in *in-vivo* environments. Their proliferation and death obtain a stable condition such that the diameter size does not grow further while maintaining the viability (Figure 2B-3) [19, 27] which can be interpreted as hemostasis.

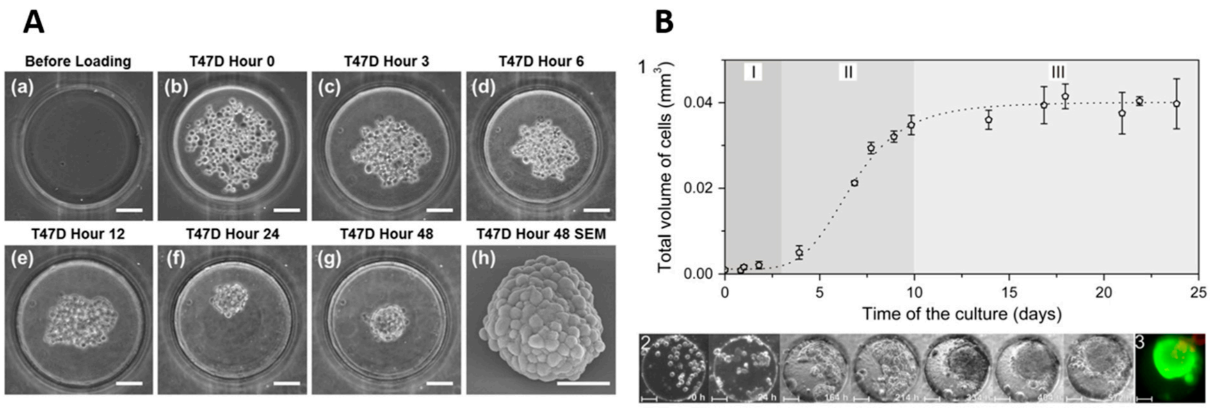

Figure 2. A- shows the first phase in which T47D breast cancer cells aggregate to become a spheroid in 48 hr (a-h). A scanning electron microscopy (SEM) of the tumor spheroid portrays its compactness and roundedness (h). Reproduced with permission from [29] under a Creative Commons Attribution 4.0 International License from Scientific Reports ; B- HT-29 human carcinoma cell spheroid growth on a chip. (1) The curve shows spheroid total volume with respect to time while distinguishing spheroid living phases with the colors. (2) A microwell containing cells for spheroid formation. (3) Viability assay of the spheroid after 25 days of culture. Reproduced with permission from [27] Copyright © 2012 Elsevier B.V.

3. Conventional methods for spheroid formation

There exist several methods for cell spheroid formation other than the microfluidic approach including magnetic levitation [30], 3D-bioprinting [31], hydrophobic surfaces [32], matrix-on-top [33], matrix-embedded [34], polymeric aqueous two-phase system [35], floating liquid marbles [36], multiwell plates [37], bioreactor flasks [38], liquid overlay [39] and HD techniques [32]. Some of these techniques such as HD and multiwell plates are laborious while some others like 3D-bioprinting and magnetic levitation are costly and still lack the standard protocols. A key parameter for cell spheroid formation is the required time indeed. The bioreactor flasks and the liquid overlay method are very time-consuming in comparison with others. The other methods such as those utilizing a hydrogel matrix and the polymeric aqueous two-phase system are not so common again because the required materials are costly or out of access.

The most important thing is the culture microenvironment of the cell spheroid, not only the method used for spheroid generation. A question arises here that are the cell spheroids generated by these methods cultured in an *in vitro* microenvironment which recapitulates the *in vivo* conditions for cells? Maybe it would be easier to form the cell spheroid and culture it in the same platform

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afterward. A platform which gives the necessary conditions for mimicking the in vivo microenvironment for cells would be desired. To find the answer to the question, we go through the following section in which we describe conventional methods routinely used for spheroid formation beside discussing their advantages and drawbacks in comparison with microfluidic techniques. Among non-microfluidic methods above, the bioreactor flasks, liquid overlay method and the HD method are chosen to be discussed because of their conventionality, ease of use and existence of standard protocols.

3.1. Bioreactor flasks

One of the most high-throughput but time-consuming approaches for spheroid formation and culture is the use of bioreactors. In this approach, cells are suspended in culture media while being circulated due to the spinner motion [40] or wall motion [12]. The dynamic environment in the bioreactors is designed to prevent cell sedimentation and also enhance the stirring of the media and oxygen transfer; meanwhile, cells are exposed to nutrients in the absence of large concentration gradients. However, these devices are not suitable for drug screening since they require a high content of drug and culture media and also cannot mimic the *in vivo* microenvironment [41]. Thus, for this purpose spheroids must be retrieved and put into other culture platforms such as multiwell plates [38] or microfluidic spheroid culture chips (μSCCs).

In the bioreactor, cell aggregates of various diameters are formed after a given time period, depending on the type of the cell line and the bioreactor physical features such as speed of stirring [38]. Spheroids may be formed first by other methods and then placed into a bioreactor for culturing [41]. Santo *et al.* [38] recently developed an adaptable stirred-tank bioreactor culture strategy to perform high throughput spheroid formation (HTSF). Although the spheroids were formed at most on the fourth day, large size dispersion still exists and appears to be an inherent feature of this method. Agitation frequency or spinner velocity, as well as cell density, are significant variables in this method of spheroid formation. As reported by Santo *et al.* [38] and Nyberg *et al.* [42] as agitation frequency increased smaller spheroids were generated. However, the agitation or stirring rate must be kept above a specific value to hinder cell sedimentation during the spheroid formation process. Since it is usual to culture cell spheroids for long times (e.g., 2 weeks) in bioreactor flasks, it is crucial to be sure that the shear stress acting on cells in the bioreactor is not high to affect the study results. Therefore, the spinner design and the circulating frequency should be minded such that the cells have a solid body motion to minimize the shear stress [43].

3.2. Liquid overlay and non-adherent surface method

In this method, a cell suspension is placed in a dish with the non-adherent bottom surface. This surface is frequently wrapped with agar or agarose to prevent cell-substrate attachment [39]. PEG (polyethylene/glycol) [44] and polystyrene plastic [45] materials are also used as a non-adherent surface for spheroid formation.

Human cells take one to two days to aggregate. After that, not all cells can generate cell-cell bindings, meaning that a large number of individual cells exists in addition to the cell aggregates. Thus, the excess cells should be extracted from the dish by sedimentation separation or other techniques. Not all aggregated cell clusters are spheroids since some of them have irregular shapes. After spheroid formation, they are pipetted out from the dish and placed in microwell plates or bioreactors for long-term culture and drug efficacy tests because the primitive dishes are not suitable for these purposes [39].

Ziółkowska *et al.* observed that the shear stress on cells was higher in a petri dish when pipetting the culture media in comparison with the microfluidic culture chip [27]. Kuo *et al.* reported a size standard deviation of 104% for on dish liquid overlay and 13% for on-chip spheroid diameters [46]. This illustrates that the spheroid size is much more uniform in the microfluidic approach in comparison with liquid overlay techniques

3.3. Hanging droplet (HD) method

One of the best conventional methods for spheroid formation is the HD technique, Figure 3. In these platforms, highly regular spheroids in a short period of time can be generated in microliter droplets [22]. Kelm *et al.* [22] reported that the coefficient of variation (CV) in regard to spheroid diameter of HepG2 spheroids made by this method was 10% to 15%, even 5% for MCF-7 spheroids. Comparing these results with corresponding values of 40% to 60% for spheroid formation on non-adherent surfaces in the liquid overlay method signifies the capability of this method in uniform size spheroid formation. In case of cells that exhibit the low tendency of aggregation such as pancreatic cancer cells, using methylcellulose can improve the uniformity and compactness of spheroids in HDs [47].

The required time in HD plates for spheroid formation is far less than those for spinner flasks. For instance, Kelm and colleagues [22] reported 4 days to form HepG2 spheroids while it took 4 to 6 weeks in spinner flask bioreactors [48]. However, microfluidic platforms appear to facilitate spheroid formation within a shorter duration of time. Kim and co-workers [49] have shown that spheroid formation took longer in HDs of MCF-7 breast cancer cells than in their μSFC. Their results demonstrate that at the second day of culture, several cell aggregates existed in each HDs while compact spheroids could be observed in the microwell traps of the μSFC.

Tung *et al.* [50] designed a novel HD platform to ease the procedure being traditionally used for HD spheroid formation [51]. The platform was compatible with liquid handling robots as well as conventional plate readers available for 384 & 96-well plates to facilitate high throughput drug screening. Although these advancements were crucial in spheroid formation, the inherent characteristics such as static environment, transient oxygen and nutrient concentrations and osmolality changes due to evaporation confine its ability to mimic *in-vivo* microenvironments. Liquid evaporation within the wells and droplets leads to an increase in osmolality that can negatively affect cell viability [50]. Specific amounts of culture media should be exchanged manually with the delicate droplets every day to compensate for the evaporated liquid.

Recently, the deficiency of lacking dynamic microenvironment in HD platforms has been solved by novel microfluidic designs [52]. In a valuable work by Yazdi *et al.* [52], both pulsatile and steady-state flows were promoted through the device by pneumatic actuation to mimic the *in-vivo* microenvironment for culturing human cardiac iPS-derived spheroids. These platforms enabled closed-looped circulation of medium however still needed adding fresh culture medium to compensate for the evaporated liquid [25].

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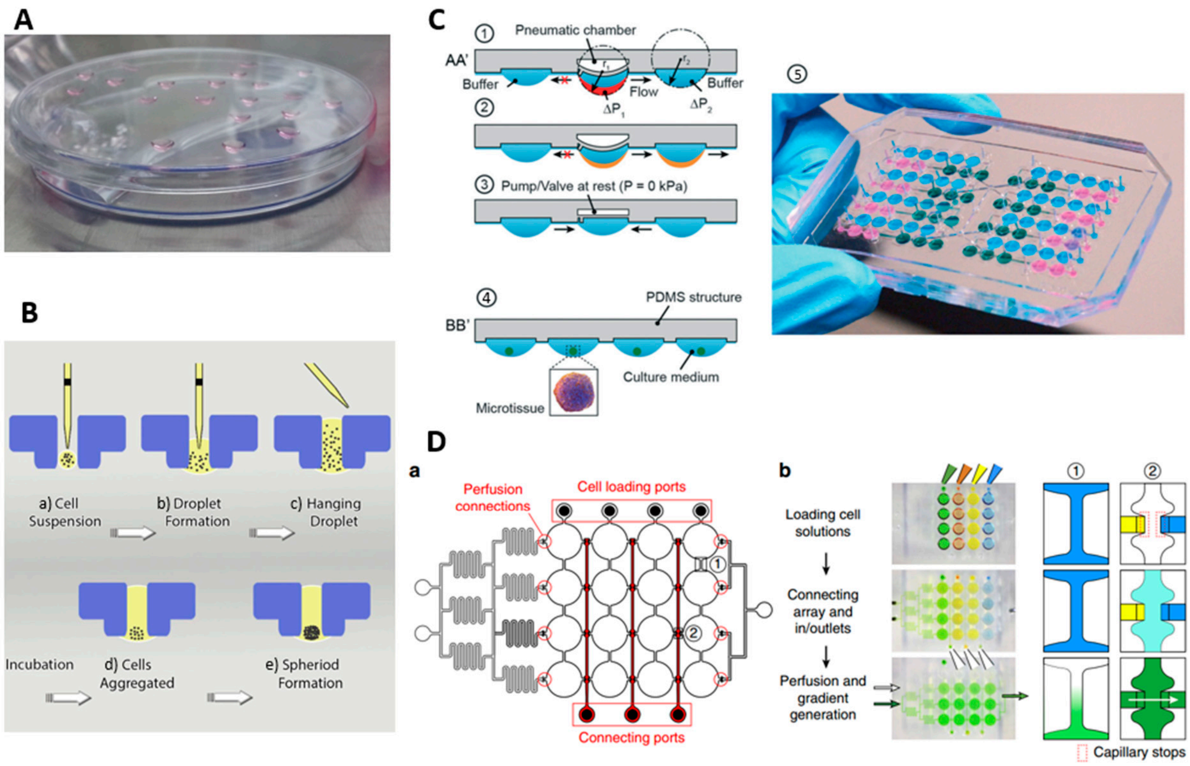

Figure 3. HD methods: A- conventional HD method implemented in petri dish in which droplets are hanging from the lid; The Image was taken at Sharif Stem Cell Laboratory. B- HD spheroid culture in a HD plate: a) introduction of the cell suspension within the holes, b) formation of the droplet by the capilliary forces, c) creation of an HD, d) cell aggregation, e) spheroid formation after one day. Redrawn with permission from [50] Copyright © 2010, Royal Society of Chemistry. C-a HD-based μSFC. The figure depicts the pneumatic chamber being pressurized (1) to promote the flow from the central HD to the right HD (2). The left valve which prevented backflow, is now open to while the pneumatic chamber is unpressurized (3). Part (4) shows the spheroids in the HDs. (5) An image of the HD based μSFC. Reproduced with permission from [52] Copyright © 2015, Royal Society of Chemistry; D-a HD based μSFC integrated with a concentration gradient generator (CGG) whose cell loading ports are distinct from its drug inlet (a). (b) The image depicts the cell loading channels (using four colors) and the concentration gradient generated on the chip (using green). Reproduced with permission from [25] Copyright © 2014, Springer Nature.

4. Hydrogels in spheroid culture

In contrast to 2D monolayer as well as 3D hydrogel based cultures, the existence of the natural extracellular matrix (ECM) between the cells in a spheroid decreases the permeability and the diffusion rate of drugs and other species of the culture media. As the cell secretions construct the natural ECM between cells inside a tissue [53], no synthetic or exogenous hydrogels are required to form tumor spheroids. This effectively reduces the equipment and efforts to have a suitable 3D tumor microenvironment in contrast to hydrogel-based 3D cultures. The hydrogel-based methods require gelification, additional materials, e.g., $CaCl_2$ (in case of alginate) [54] and equipment such as hydrogel handling dishes and heating facilities to adjust temperature for crosslinking. However, using hydrogels entails some advantages to the spheroid formation and culture such as size uniformity [55], the formation of porous spheroids [56] or hetero-spheroids [57] as well as chemosensitivity [58] and invasion assays [59] and protecting from shear stress [13].

Porous spheroids were formed with the goal of increasing nutrient and oxygen exchange [21] between cells and culture medium by Kojima *et al.* [14]. To have porous spheroids from hepatoma cell line HepG2, 20 μm diameter alginate droplets were generated and added to the cell suspension. After creating the spheroids using the cell-droplet mixture, the spheroids were made porous by alginate lyse treatment to remove the alginate from the spheroid's structure. It was shown that 1 μm

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polystyrene particles could enter the central parts while this diffusion was confined only to the few outer layers of conventional spheroids. Yamada and colleagues [56] generated spheroids with various mixtures of HepG2 cells and 10 μm collagen microdroplets in 1024 agarose microwells. They observed that the ratio between the collagen microdroplets and cells influences the hepatic function characteristics noticeably.

Ota *et al.* [57] used collagen hydrogels for strengthening the bonding between hepatocyte and endothelial cells in the spheroids by a coating of 200 nm collagen gel on cells. Collagen gel was also used for covering hepatocyte spheroids with endothelial cells by coating the hepatocyte spheroids initially with the collagen gel [60]. As cell-cell adhesions and attachments between non-identical cells develop slower and weaker [57], collagen gel acts as an anchorage for endothelial cells to stick to the hepatocyte spheroid preference. In an interesting work, Sabhachandani and co-workers [61] used alginate as a hydrogel to encapsulate breast cancer cells (MCF-7) and fibroblast cells to form co-cultured spheroids in a microfluidic device. Alginate hydrogel permits facile de-crosslinking with the aid of calcium chelator, so that, the spheroids can be retrieved for future culture and assays [62].

Placing tumor spheroids in a hydrogel and then crosslinking the gel hinders the dissociation of spheroids [13], since, the hydrogel plays the role of the *in-vivo* surrounding tissue. However, it can damage cells on outer layers of spheroid due to the shear stress of the hydrogel itself [13]. However, hydrogel protects cells from the shear stress caused by the culture medium flow [63]. Sometimes, cells are dispensed in hydrogel droplets and anchored in a chip for spheroid formation and assays [64].

5. Microfluidic methods for spheroid culture

Microfluidics is the science and technology of handling a small volume of fluids in the channels with sub-millimeter length scale [65, 66]. As a science and technology, microfluidics can be used for various fluid mechanics applications, including slip flow in superhydrophobic microchannels [67, 68] and drag reduction [69-71]. In parallel, microfluidic systems hold great promise for cell biology [72], assisted reproductive technology (ART) [73], drug delivery systems [74], anti-cancer drug screening [75] and disease modeling [76]. Recently, microfluidic platforms for spheroid formation and culture have been thoroughly reviewed by our group [8]. We categorized the μSFCs into two main groups, which differ in spheroid formation procedure: emulsion-based spheroid formation and; microwell or U-shaped microstructure-based spheroid formation [8].

Many studies have used flow-focusing droplet generators due to the resulted droplet and spheroid size uniformity, in addition to their high-throughput continuous operation [77]. Single- [61, 78, 79], double- [13, 80] and triple- [77] emulsion droplet generation techniques have been used in μSFCs. Axisymmetric [80] or non-axisymmetric [61, 77, 81] configuration flow-focusing devices exist. This method facilitates the fast production of microdroplets and thus high-throughput spheroid formation (HTSF).

Cell-dispensed hydrogel (Gel) in oil (i.e., Gel/O) and cell suspension (CS) in oil (O) (i.e., CS/O) [82] droplet generation [61, 83] are among the single-emulsion methods which are widely used. Cell suspension in oil in culture medium (CM) (i.e. CS/O/CM) [13] and CS/Gel/CM [80] are double-emulsion techniques. Droplet uniformity can be enhanced with CS/Gel/CM double-emulsion technique which entraps the cells firmly within the droplet. It is facilitated by encapsulating the cell-containing core droplet within an alginate hydrogel shell [80, 84] that acts as an impermeable barrier with respect to the cells.

Microwells [20, 85-88] and U-shaped microstructures [16, 62, 89-92] have been designed for spheroid formation and culture in microfluidic platforms. These structures facilitate short-term [18, 93], controllable and uniform diameter [17, 94] and compact spheroid generation [27, 91]. U-shaped microstructures either are actuated temporarily using pneumatics [90-92, 95] or are fixed within the device [89, 92]. A large number of these U-shaped microstructures were embedded (e.g., 360 [91], 28 [62], 512 [96]) in each microchamber of the μSFC to trap the cells [62, 89, 91, 92, 95] or the cell dispensed hydrogel droplets [62] introduced into the chip. Spheroid diameter is confined to the microstructure size, and the relative position of the microstructures is essential for efficient cell

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trapping. We have recently evaluated the oxygen and glucose distributions inside spheroids in such bioreactor [97] and compare the results with those inside toroidal multicellular aggregates [98].

Microwells have been widely utilized in μSFCs due to their simplicity and ease of operation [99-101]. Uniform cell seeding in microwells and uniformly sized spheroids are achieved by filling the device entirely with the cell suspension before cells begin to enter and trap in the microwells (Figure 4A). Few minutes are needed that cells deposit on the bottom of the microwells and the microchannel (Figure 4B). The cells that did not trap in the microwells are pushed out of the chip before the cells make aggregations and clog microchannels [19, 87, 102] (Figure 4C). Next, the cells begin aggregation and form spheroids (Figure 4D) and are culture for drug screening (Figure 4E).

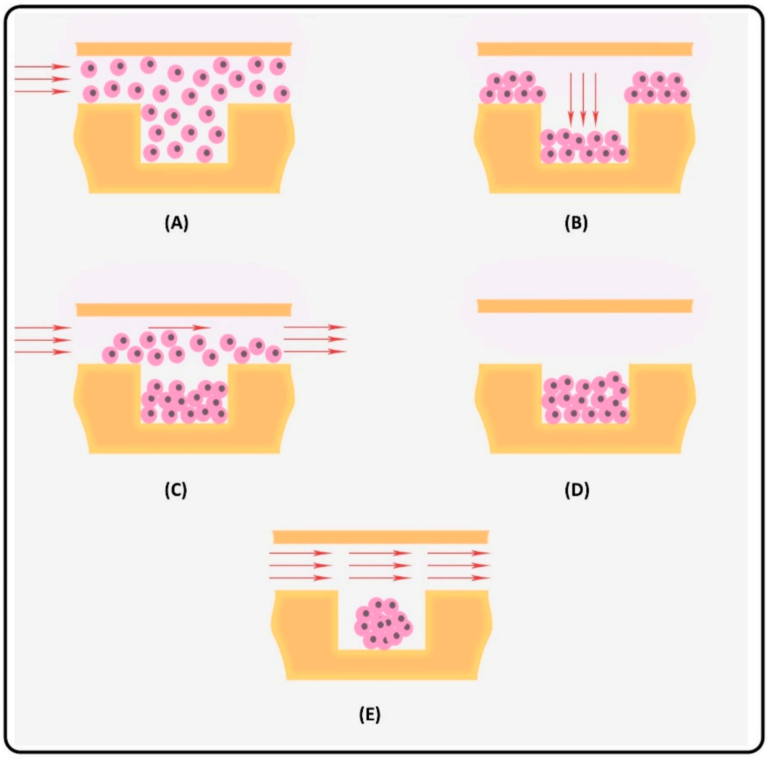

Figure 4. Spheroid formation process in a microwell-based μSFC: (A) Introduction of a cell suspension to the chip inlet. The cell suspension fills all the microchannels and microwells rapidly due to the capillary effect; (B) Cells start depositing on the bottom of the microchannels and microwells; (C) Pure culture medium flows through the chip to rinse the excess cells without disturbing the cells lying on the microwell bottom; (D) Cell secretions and signaling lead to establishment of cell-cell interactions on the non-adherent microwell bottom; (E) Driving spheroid formation under a perfusing flow of culture medium. Reproduced with permission from [8] Copyright © 2018 Elsevier B.V.

Other works have used acoustic tweezers [103], pyramid microwells [21], porous membranes [104], and microrotational flow [18] in μSFCs for more efficient spheroid formation. We have recently shown that electrospinning technique can be efficiently used to fabricate porous membrane [105], and incorporation of such membrane inside a microchip can give rise to the formation of three different cellular aggregates, namely, single cells, monolayer and spheroid-like tissue [106].

Spheroids retrieval is required for flow cytometry analysis, stem cell differentiation-assays, etc., however, these flow rates might create high shear stress on the spheroids while pushing them upward [107]. For the real-time on-chip monitoring of the spheroids, several techniques have been developed including the electrode-based biosensors for oxygen [108], glucose and lactate concentration [109] and also pH and electrical impedance [110] measurements. These monitoring techniques alleviate the need for spheroid retrieval from the chip which effectively reduces the time and cost.

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In designing the μSFCs, the concentration of oxygen and glucose in the culture medium and the cellular uptake rates should be considered. The complicated geometries of the μSFCs and the limited diffusion of glucose and oxygen to spheroids create unpredictable concentration profiles within the cultured spheroids. Thus, mathematical and numerical analyses combined with experimental investigations are needed to predict the condition of hypoxia in the spheroids [107, 111-116].

The microstructure- or microwell-based μSFCs have limited applications in high-throughput screening. Various drug concentrations and combinations into a μSFC have rarely been carried out simultaneously because a suitable microchannel network did not exist. By coupling the μSFC with a concentration gradient generator chip and arranging the microwells in a configuration compatible with commercial microplate readers, we can become a step closer to the automated monitoring and high-throughput screening within μSFCs.

The μSCC are designed for spheroid culture and their spheroid comes from an external source. They have been designed with various purposes including shear stress analysis [117], drug screening [118], multi organ-on-a-chip [119] and analysis of the spheroid fusion process [120]. Digital microfluidic platforms also are used for spheroid formation and culture [121]. In these devices, the cell suspension of droplets is directed towards hydrophilic of hanging droplet sites for culturing [122, 123]. In this method, continuous flow of the culture medium is limited and sequencing delivery of the nutrients is performed [124]. In addition, biofouling and liquid evaporation are the drawbacks of these platforms [125]. The detailed design considerations of μSFCs and μSCCs, such as microstructure design, shear stress, spheroid diameter and retrieval mechanism, have been recently reviewed [8].

Table 1: The table is considered to represent the key variable elements in µSFCs and µSCCs. Those marked with * sign are µSCCs.

Reference	Year	Cell type	Channel dimensions	Hydrogel type	Spheroid formation time	Spheroid Or droplet diameter (µm)	Cells in each spheroid	Cell density (cells/ml)	Media flow rate	Spheroid or droplet or 3D culture formation method	Spheroid size standard deviation	Throughput
McMillan et al. [126]	2016	human glioma cell line (UVW)	-	Alginate	Less than one day	-	-	3×10^6	The medium was refreshed every 2 days	Single emulsion CS/O	-	**48**
McMillan et al.[81]	2016	human glioblastoma cell line (UVW)	-	-	24 hr	300-575	500-1500	5×10^6	Daily Refreshment	Single emulsion CS/O	-	**2000**
Wang et al. [83]	2014	human cervical carcinoma, human hepatocellular liver carcinoma and human umbilical vein endothelial cell	-	Alginate and Matrigel	4 days	-	-	10^7	-	Double Emulsion CS/O and Gel/O	-	-
Sabhachandani et al. [61]	2016	breast cancer cell lines (MCF-7) and fibroblast cell lines (HS-5)	-	alginate	3 to 4 hr	170 (optimum)	-	10^7 (mono) 7.5×10^6 (co)	20 µL h (equivalent to 230 µm s−1)	Single emulsion O/Gel	-	**1000**
Chan et al. [13]	2013	mesenchymal stem cells,	-	alginate	150 min	36 to 84	-	2,5,10 and 20 million cells/mL	-	Double emulsion CS/O/CM	-	-

		HepG2, PMEF and Caco-2										
Yu et al. [62]	2010	LCC6/Her-2 breast tumor cells	-	alginate	4 days for spheroid and	250	100	10^7	0.25µl/min	Single emulsion CS/O and Gel/O	-	**28**
Reference	**Year**	**Cell type**	**Channel dimensions**	**Hydrogel type**	**Spheroid formation time**	**Spheroid Or droplet diameter (µm)**	**Cells in each spheroid**	**Cell density (cells/ml)**	**Media flow rate**	**Spheroid or droplet or 3D culture formation method**	**Spheroid size standard deviation**	**Throughput**
Yu et al. [84]	2015	MCF-7	-	alginate	-	183	-	10^7	-	Double Emulsion CS/Gel/O	4%	-
Alessandri et al. [80]	2013	CT26 mouse colon carcinoma cell line, and HeLa cells and murine sarcoma S180 cells	-	Collagen, alginate	-	100-150	-	-	-	Double Emulsion CS/IS/Gel	-	1000 droplet/sec
Yamada et al. [56]	2015	NIH-3T3 cells and HepG2 cells	diameter = 200 µm, depth = 300 µm	Collagen I	1 day	-	-	2×10^5	-	Flat bottom microwells	-	-
Liu et al. [95]	2015	human glioma (U251) cells	-	-	-	120-200 after 10 days	200-400	5×10^6	at a very slow perfusion rate (5 µL/min)	U-shaped microstructures	-	360

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Wu et al. [92]	2008	MCF-7 breast tumor cells	-	-	7 to 11 hr	50	10	10^6	0.05~10 µl min−1 (0.02 to 4mm/sec)	U-shaped microstructures	-	7500 per cm^2
Shin et al. [63]	2013	MCF-7 breast tumor cells	-	matrigel and a hydrogel scaffold (made of gelatin)	3 days	50	Less than 20	10^6	30 µL/h, equivalent to 278 µm/s	Cell suspension in 50 µm in diameter and 30 µm in height wells	-	-
Reference	**Year**	**Cell type**	**Channel dimensions**	**Hydrogel type**	**Spheroid formation time**	**Spheroid Or droplet diameter (µm)**	**Cells in each spheroid**	**Cell density (cells/ml)**	**Media flow rate**	**Spheroid or droplet or 3D culture formation method**	**Spheroid size standard deviation**	**Throughput**
Albanese et al. [127]	2013	MDA-MB-435 cells	-	-	3 days	260-280	750-1500	-	50 and 450 ml/hr produced a 75–675 mm/s fluid velocity	Hanging droplet plates	-	-
Kwapiszewska et al. [85]	2014	Two human cell lines (HT-29 colon carcinoma and Hep-G2 liver carcinoma)	-	-	48 hr	Almost 50	-	1-5×10^6	4.5 µl/min for 15 minutes daily	in hemispherical bottom micro wells	Up to 30%	216
Aung et al. [23]	2016	human umbilical vein endothelial cells (HUVECs) and	-	gelatin methacrylate (GelMA)	20 hr	200	-	-	10 to 40 µl/hr	Using Petri dish and cultured on an orbital shaker	-	-

		MCF-7 breast tumor cells								(VWR, Model No. DS-500E) at 45 rpm in a humidified incubator		
Ruppen et al. [20]	2015	lung adenocarcinoma + malignant pleural mesothelioma cell line + pericytes	Well diameter: 500 µm Well height: 600 µm	-	48 hr	325 and 210	1250 312	-	Changed once a day	Cell sedimentation in round and flat-bottom wells in the chip	35 to 45 µm	8 in each unit
Jin et al. [90]	2010	non-small lung cancer cells, H1650	-	-	24 hr	197	-	-	-	U-shaped microstructures	11.7 micron	4
Torisawa et al. [21]	2007	MCF-7, HepG2	-	-	2 days for MCF-7 and 3days for HepG2	-	370 for HepG2 with 3×10^6	$1,3,10\times10^6$	-	Pyramidal structures which have a hole at their vertex	-	16
Reference	**Year**	**Cell type**	**Channel dimensions**	**Hydrogel type**	**Spheroid formation time**	**Spheroid Or droplet diameter (µm)**	**Cells in each spheroid**	**Cell density (cells/ml)**	**Media flow rate**	**Spheroid or droplet or 3D culture formation method**	**Spheroid size standard deviation**	**Throughput**
Kim et al. [118]	2015	Human colorectal tumor	-	-	-	180	250	-	13 µl/min. hydrostatic	Hanging droplet of Human	-	8

Reference	Year	Cell type	Channel dimensions	Hydrogel type	Spheroid formation time	Spheroid Or droplet diameter (µm)	Cells in each spheroid	Cell density (cells/ml)	Media flow rate	Spheroid or droplet or 3D culture	Spheroid size standard deviation	Throughput
		and Primary rat liver								colorectal tumor		
Ziółkowska et al. [27]	2013	HT-29 human carcinoma cells	Well: 200, 150 Channel: 50, 1000	-	48 to 72 hr	-	100	1.5×10^6	4.5 µL/min	Flat bottom microwells	not exceeding 20% in cell numbers	45
Lee et al. [28]	2013	Hepatocytes and hepatic stellate cells (HSCs)	Well: 500,400	-	-	200 to 375	-	2×10^6	5.53 mm/h or approximately 1.5 µm/sec	Concave bottom microwells	-	50
Choong Kim et al. [77]	2011	mouse embryonic carcinoma (EC) cells	-	-	3 day	158	178	5×10^5	0.2 ml/h for cell seeding	Flat bottom Microwell trapping	4.5 %	60
Ota et al. [18]	2010	Human hepatocellular liver carcinoma cells.	-	-	120 sec	130–430 µm	1000 for 180 micron spheroid	6.9×10^6	0.4 ±0.05 ml/min.	microrotation	13.2% in the range 150–200 µm and 17.2% in 130–430 µm	1
Choong Kim et al. [49]	2012	MCF-7	-	-	3 days	188	200	-	0.2 ml/h for cell seeding	Flat bottom Microwell trapping	6.06 µm	80

										formation method		
Ota et al. [93]	2011	Hep-G2	-	-	120 sec	134 ± 25, 180 ± 30, and 237 ± 40 µm	-	2-5-13×10^6	1.2 ml/min	microrotation	18.7%, 16.6%, and 16.9%	15
Ota et al. [57]	2011	Hep-G2 and endothelial cells	-	collagen	120 sec	97-226	-	145, 290, 480, and 675× 10^4/ml	1.2 ml/min	microrotation	17%, 18.7%, 16.6%, and 16.9%	15
Patra et al. [87]	2016	human hepatocellular carcinoma cells (HepG2)	Chanel: 250 Well: 200×200×250 and 300×300×250	-	24 hr	130 and 212	-	-	100 µl/min for cell seeding and changed every 12 hr by adding 1 ml of fresh culture media	Flat bottom well	6% for small and 3% for large spheroids	5000
Kangsun Lee et al. [16]	2012	human embryonic kidney 293 cells (HEK 293)	-	-	Less than one day	Less than 300 µm for retrieval	-	1-2-4×10^6	-	sedimentation	5.5%, 7.2%, and 8.9% for 1, 2, and 4×10^6	50
Kuo et al. [46]	2012	human epithelial ovarian cancer cells (SKOV3)	-	-	48 hr	75	-	1.5×10^4	Hydrostatic flow for trapping and media change for culture	Trapping behind a porous membrane	Min of 7.6%	-
Patra et al. [86]	2013	murine ES cell, HepG2, African	Channel: 150, 1400, 25000	-	24 hr for COS-7, 1	COS-7 and HepG2	-	HepG2 and COS-7 cell	1 µl/min for cell seeding	Flat bottom well	standard deviations	5000

Reference	Year	Cell type	Channel dimensions	Hydrogel type	Spheroid formation time	Spheroid Or droplet diameter (µm)	Cells in each spheroid	Cell density (cells/ml)	Media flow rate	Spheroid or droplet or 3D culture formation method	Spheroid size standard deviation	Throughput
		green monkey kidney epithelial fibroblast (COS-7)	Well: 200×200, 250		day for HepG2, 16 h for ES	spheroids are 80 and 200 µm		10^7 and 10^5 respectively	and 20 µl/min for culture refreshment every 48 hr		of 4 and 10 µm, respectively	
Chen et al. [29]	2015	T47D, MCF-7 and SUM159 (breast cancer)	Channel: 100 Well: 250, 400 and 450, 400	-	1 day	-	-	5×10^6	300 µl per minute for cell seeding	Flat bottom well	10%	1024 within an area of 2 by 2 cm
Yongli Chen et al. [19]	2015	HCT116, human breast cancer cell line (T47D) and HepG2	Channel: 100, 3000, 9500 Well: 500, 200	-	24 h	-	-	10^6	-	Flat bottom well	-	120
Choi et al. [102]	2016	Hepatocytes	Channel: 100, 4000	-	-	-	-	1×10^6	4.2 µm/sec (0.12 µl/min)	Concave bottom microwells	-	50
Robillard et al. [128]	2016	ovarian cancer cell line OV90	Channel: 500, 2000 Well: 450×450×500	-	-	170	-	5×10^5cells/ml	The medium was changed Each day	Flat bottom microwells	-	120

Anada et al. [129]	2010	Human osteosarcoma MG63, HepG2	Well: 1000, 500	-	1 day	150 to 320 after 5days of culture	-	$1.25\times10^5$to 8×10^6	-	Pneumatic concave wells	5-8%	1535
Fukuda and Nakazawa [17]	2011	Hepatocytes of Wistar rat	Open Channel: 100, 100 Well: 300, 400	-	2 day	150	-	2.5×10^6	-	Flat bottom microwells	-	1575
Reference	**Year**	**Cell type**	**Channel dimensions**	**Hydrogel type**	**Spheroid formation time**	**Spheroid Or droplet diameter (µm)**	**Cells in each spheroid**	**Cell density (cells/ml)**	**Media flow rate**	**Spheroid or droplet or 3D culture formation method**	**Spheroid size standard deviation**	**Throughput**
Xu et al. [94]	2012	P19 cells	-	-	1 day	100 to 450	-	2-20 $\times10^4$ cells mL-1	2 mm/sec to rinse excess cells, 6 or 0.5 mm/sec for spheroid retrieval	Concave bottom microwells	-	880
Zhang et al. [96]	2009	BALB/3T3 (murine embryonic fibroblast) cell line.	-	-	-	90	85±6.3	10^7	1 µl/min for 10 min every 6 hr	U-shaped microstructures	-	512 totally (8 in each chamber)
Chien-Yu Fu et al. [89]	2014	HepG2 and Balb/c 3T3 fibroblast cells	-	-	1 day	-	-	8.4×10^6	1.5 µl/min for long-term perfusion	U-shaped microstructures	-	56

Tung et al. [50]	2011	COS7, ES-D3, and human epithelial carcinoma cell	-	-	1 day	-	300, 1500, and 7500	-	-	Novel Hanging droplet method (3d-biomatrix, perfecta 3d)	-	384
Santo et al. [38]	2016	MCF7, H1650, H157, HT29, Human Dermal Fibroblasts (hDFs)	-	-	-	100 to 800	-	0.2×10^6 & 0.5×10^6	-	Stirred tank	Up to about 40%	-
Torisawa et al. [130]	2009	COS7;HepG2; ATCC; MDA-MB-231	-	-	-	-	-	10^5	Hydrostatic-driven flow, medium daily exchanged	Patterning on semi-porous membranes	-	-
Hsiao et al. [104]	2009	prostate cancer cells osteoblasts and endothelial cells	-	-	1 day	86	-	-	Hydrostatic-driven flow, medium daily exchanged	Patterning on semi-porous membranes	12 μm	28
Chen et al. [103]	2016	HEK 293, SH-FY5Y, HepG2, and HeLa cells	-	-	1 day	30 to 100	-	$2\text{-}17\times10^6$	medium daily exchanged in petri dish	Acoustic tweezers	-	150

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6. Conclusion

The three-dimensionality of the tumor cell culture environment has significant effects on tumor cell responses to cancer drugs due to cell-cell and cell-matrix interactions occurring only in a 3D configuration of cells. The 3D cell culture formation methods have been vastly discussed in the literature. However, among these methods multiwell plates, bioreactors and hanging droplet plates have been commercialized for spheroid formation. Such conventional methods such as hanging droplets, liquid overlay and non-adherent surfaces and spinner flask methods for tumor spheroid formation lack the ability to precisely control the number of cells in each spheroid. Therefore, it leads to spheroids with various diameters. This is cumbersome to separate and group. Moreover, undesired necrotic cores and acidic environments develop. In addition, drug tests are not usually conclusive on the cells cultured on such platforms. Using these conventional methods also takes a lot of time for spheroid formation and is difficult to achieve cell-cell interactions because cells are not situated close enough to each other to obtain rapid cell aggregates and spheroids. Furthermore, the shear stress presenting in roller bottles, suspension culture and pipetting as well as chemical materials, particularly coating materials (polyethylene glycol (PEG), agarose, agar, etc.), might cause irreversible defects on cells which usually cannot be quelled. On the other hand, microfluidic devices can form uniform 3D cell cultures such as spheroids and hydrogel-based cancer cell encapsulation, and drug screening can be used more efficiently and in a high throughput manner.

Conflict of Interest: The authors declare that they have no conflict of interest

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