Dan Liu ORCID iD: 0000-0002-5192-695X

A review of manufacturing capabilities of cell spheroid generation technologies and future development

Dan Liu*[,1], Sixun Chen[1], May Win Naing[1,2]

*Corresponding author, Email: liu_dan@bti.a-star.edu.sg, 20 Biopolis Way, #06-01 Centros, Singapore 138668

[1]Bioprocessing Technology Institute, Agency for Science, Technology and Research, Singapore

[2]Singapore Institute of Manufacturing Technology, Agency for Science, Technology and Research, Singapore

Abstract

Spheroid culture provides cells with a 3D environment that can better mimic physiological conditions compared to monolayer culture. Technologies involved in the generation of cell spheroids are continuously being innovated to produce spheroids with enhanced properties. In this paper, we review the manufacturing capabilities of current cell spheroid generation technologies. We propose that spheroid generation technologies should enable tight and robust process controls to produce spheroids of consistent and repeatable quality. Future technology development for the generation of cell spheroids should look into improvement in process control, standardization, scalability

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1002/bit.27620.

and monitoring, in addition to advanced methods of spheroid transfer and characterization.

Keywords: cell spheroid, spheroid generation, spheroid manufacturing, 3D cell culture

Introduction

The tight spatial arrangement of cells *in vivo* enables complex interactions between individual cells as well as between cells and the extracellular matrix (ECM). This arrangement provides the cells with unique cues to guide specific cellular function and growth. To replicate this spatial arrangement *in vitro*, cells can be cultured in 3D culture systems that enable cell-cell contact without foreign scaffolding material known as spheroids. Culturing cells in spheroids has shown to be supportive of cell growth that leads to better cellular functions such as potency, angiogenesis, survival, and differentiation compared to monolayer or scaffold-based culture. Cell spheroids have been increasingly applied in a wide variety of areas such as cancer research (Ferreira, Gaspar et al. 2018), drug screening (Kunz-Schughart, Freyer et al. 2004, Langhans 2018, Verjans, Doijen et al. 2018), and tissue assembly (Okudaira, Yabuta et al. 2017).

Compared to cell cultures performed in traditional monolayer, cell spheroid generation involves many more and different process parameters making it complicated even to persons who might be experienced with handling different types of 3D cell culture. The first step in spheroid culture is the generation of spheroids from a population of cells. There are several technologies that are currently available for spheroid generation. Common

ones include hanging drop, low-adherence substrates, microwells, bioreactors and magnetic levitation. There have also been new developments in the recent years, such as microfluidics, smart material and cell membrane engineering.

As of today, cell spheroids are mostly generated and grown in small batches with the aim of addressing specific applications in the laboratories. Outside of research laboratories, commercial laboratories such as Tebu-Bio and Cytoo have started offering customized 3D cell spheroid production services. It is clear that cell spheroids are still too complicated to handle for many and the current spheroid generation technologies are still limited in their capability to produce quality cell spheroids. The relevant industries would require more advanced spheroid generation technologies that could enable cell spheroid production with easier process control and at larger scales to meet the increasing application needs.

This manuscript aims to provide a review on the manufacturing capabilities of current cell spheroid generation technologies. In this manuscript, “spheroid generation” refers to the formation of cell spheroids irrespective of the production scale, while “spheroid manufacturing” refers to large-scale production of cell spheroids. We will review the existing cell spheroid generation technologies and at the same time comment on their manufacturing capability. We will also share our views on the desired characteristics of manufacturing process, and the technological advancement needed to address the needs of future large-scale spheroid manufacturing.

Current mainstream technologies for cell spheroid generation

Figure 1 shows the schematics of different mainstream spheroid generation technologies.

Insert Figure 1 here

1. Hanging drop (Figure 1a)

In this method, cell solutions are dispensed in typical volumes of 10-20 microliters onto the underside of a surface to create a “hanging drop” that adheres to the surface (Lin, Miao et al. 2016). Under gravity, cells slowly settle to the lowest point of the drop, aggregating and forming spheroids. Co-culture of different cell types and size control of the spheroids are easily achievable by varying the content and volume of the cell solution (Prestigiacomo, Weston et al. 2020).

The hanging drop method is straightforward and cost effective as it requires only basic labware such as pipettes, petri dishes and well plates. However, given the small volume of the “hanging drop”, there is a high risk of cell or spheroid loss during cell solution dispensing, medium change and spheroid transfer (unpublished data). The method thus requires careful and precise liquid handling, which could be challenging if done manually. To overcome this challenge, automated liquid handling stations or commercial hanging drop plates can be used.

Commercial hanging drop plates comprise arrays of open holes with specific configurations. Most of the plates are designed to be top loading and compatible with commercial liquid handling stations to enable high throughput. The major market products include Perfecta3D, GravityPLUS™, Elplasia and MAPlate™ 5RC96.

Overall, hanging drop is an easy to use method and can be used to generate cell spheroids with good control over spheroid size in the laboratories (Damman, Lucini Paioni et al. 2020). It is scalable as users can easily transfer their process from manual pipetting to liquid handling stations. The cost involved in usage of liquid handling stations should be carefully weighed against savings in labor and reduction in human error. With all considered, hanging drop plate when integrated with automatic liquid handling stations, is a technology that enables spheroid manufacturing with high throughput and consistent spheroid quality.

2. Low-adherence substrate (Figure 1b)

In this method, culture substrates are modified either chemically or topologically to become low-adherent. Cells that are dispensed onto these substrates cannot attach to the substrate surface. Instead, they aggregate with each other to form spheroids through cell-cell interaction. Low-adherence substrate can be obtained via in-house modification to normal cultureware substrates. Alternatively, commercial low-adherence cultureware are also available.

The most common method of substrate modification to obtain low-adherence substrate is through material coating. A wide range of materials have been used for this purpose, including agarose (Tevis, Cecchi et al. 2017, Abe-Fukasawa, Otsuka et al. 2018), poly(2-hydroxyethyl methacrylate) (poly-HEMA) (Kim, Yun et al. 2018), poly(ethylene glycol) (PEG) (Tong, Fang et al. 2016), galactose (Tong, Fang et al. 2016), polyvinyl alcohol (PVA) (Huang, Chan et al. 2013) and poly(N-isopropylacrylamide) (PNIPAAm) (Kim, Jung et

al. 2020). Other than material coating, substrate adherence can also be modified with topological nano-/micro-scale features (Takayama, Kawabata et al. 2013, Wang, Thissen et al. 2016, Kim, Park et al. 2019).

While customized modification to culture substrates gives researchers the flexibility and control over experimental design and cost, commercial low-adherence cultureware offers a ready-to-use solution that is neat and handy. These commercial products have been used for generating spheroids from a wide variety of cell types including cancer cells (Yoshii, Furukawa et al. 2015, Bayat, Ebrahimi-Barough et al. 2018), dental papilla cells (Yamamoto, Kawashima et al. 2014), mesenchymal stem cells (MSCs) (Yamaguchi, Ohno et al. 2014, Obara, Tomiyama et al. 2016), and mixture of heterogeneous cell types (Kim, Lee et al. 2015). The major market products include Nunclon™ Sphera™, Nunc™ UpCell™, Corning Ultra-Low Attachment surfaces, CELLSTAR®, Lipidure®-COAT and NanoCulture

Low-adherence substrates support the generation of cell spheroids in large quantities with minimal liquid handling requirement. However, it does not have good control over the spheroid size (Wang, Thissen et al. 2016), unless the substrates are patterned into small regions to produce one spheroid per region (Kim, Jung et al. 2020). Alternatively, low-adherence substrate can be combined with microwells (to be introduced in the next section) to produce spheroids of repeatable and consistent quality.

3. Microwells (Figure 1c)

Microwells confine cells in small physical compartments that are usually at a micrometer scale to promote cell-cell interaction and spheroid formation. Most

of the time, microwells are inter-connected with a shared medium reservoir. Cell loading and medium change to different microwells are performed through the shared medium reservoir. After the cell suspension is loaded to the shared reservoir, cells settle into different microwells under gravity, forming one spheroid per well.

Microwells can be designed with different sizes, shapes and depths. Their size can be used to control the size of the spheroids. Their surface is usually coated with low-adherence coating to prevent cell attachment to the wells. These coatings include Pluronic F127 (Liu, Winter et al. 2014, Sridhar, de Boer et al. 2014, Anada, Sato et al. 2016, Kamoya, Anada et al. 2016, Priwitaningrum, Blonde et al. 2016), bovine serum albumin (BSA) (Wong, No et al. 2011, Jun, Kang et al. 2013), poly(N-isopropylacrylamide) (PNIPAAm) (Shimizu, Kusamori et al. 2013, Kusamori, Nishikawa et al. 2014), poly (2-hydroxy-ethyl-methacrylate) (polyHEMA) (Markovitz-Bishitz, Tauber et al. 2010), and poly(vinyl alcohol) (PVA) (Huang, Chan et al. 2013).

Table 1 summarizes the research efforts going into microwell technology for cell spheroid generation, which mainly pertain to microwell moulding and designs. Despite the flexibility with microwell size and shape design, microwells might be difficult to adopt for researchers versed in cellular work as fabrication can be technically challenging and/or resource demanding. It is noted that some studies have used processes or materials that are non-biocompatible (Markovitz-Bishitz, Tauber et al. 2010), or hazardous (Lee, Cuddihy et al. 2009) to cells.

Commercial products with microwells are also available. The major ones include EZSPHERE™, AggreWell™, Elplasia plate, SpheroFilm and 3D Petri Dish® micro-mold. Most of the commercial products are designed in standard cultureware formats to facilitate common practices such as assay characterization and imaging. Some of them are also compatible with automated liquid handling stations.

Table 1: Research exploring microwell technology for cell spheroid generation

Material	**MouldingTechnique**	**Microwell Configuration**	**Reference**
PDMS*	Casting over frozen water droplets	Semi-spherical with Φ290-575 μm	(Liu, Winter et al. 2014)
	Casting over SU-8 based negative mold	Semi-spherical with Φ300-500 μm and 500 μm inter-well distance	(Wong, No et al. 2011, Jun, Kang et al. 2013)
	Casting over PDMS based negative mold	Round well with Φ1 mm, 1.05 mm pitch and 1.06 mm depth	(Anada, Sato et al. 2016, Kamoya, Anada et al. 2016)
	Casting over micropillar array	Round well with Φ300-432 μm and 310 μm height	(Shimizu, Kusamori et al. 2013, Kusamori, Nishikawa et al. 2014)
	Gas expansion molding over air bubbles in trenches upon rapid heating	Semi-spherical with Φ100-300 μm	(Giang, King et al. 2008, Chandrasekaran, Giang et al. 2011)

Agarose	Casting over PDMS mold with circular microwells	Round well with Φ2 mm	(Tang, Liu et al. 2016)
	Casting over silicon wafer with inverted pyramidal arrays	Inverted pyramidal well with sizes of 400 or 800 μm	(Hookway, Butts et al. 2016)
PS*	Hot embossing in petri dishes with PDMS stamp	Round well with Φ400 μm and 200 μm depth	(Sridhar, de Boer et al. 2014, Priwitaningrum, Blonde et al. 2016)
UV curable adhesive	Molded against PDMS stamp	Round well with Φ250 μm and 175 μm depth	(Markovitz-Bishitz, Tauber et al. 2010)
PEG*	Photolithography	Square well with 200 μm side and 300 μm depth	(Shin, Kook et al. 2016)
PEG*	Photolithography and micropatterning	Round well with Φ100 μm and 100 μm edge to edge distance	(Wang, Itaka et al. 2009)
PMMA*	Micromachining and microcontact printing	Round well with Φ 600 μm and 600 μm depth	(Yamazaki, Gotou et al. 2014)
Polyacrylamide hydrogel	Solvent casting and particulate leaching	Round well with Φ170 μm	(Lee, Cuddihy et al. 2009)

*PDMS: Polydimethylsiloxane; PS: Polystyrene; PEG: Poly ethylene glycol;

PMMA: Poly (methyl methacrylate)

Microwells can be integrated with automatic liquid handling stations to generate cell spheroids in large quantities and with uniform sizes and shapes, making them a good technology to adopt for large-scale manufacturing. They are also favored in terms of the small cultureware footprint. However, the main drawback is that spheroids may detach from the microwells during medium perturbation and fuse with each other to form irregular clumps, which may lead to reproducibility issues between runs (unpublished data).

A different product for spheroid generation that shares a similar working mechanism with microwells is microplates. Microplates are made of wells at a larger, minimeter scale and the microplate wells usually have U- or V-bottom for cell concentration. Typically, one spheroid is formed per well. Wells do not share media reservoir and liquid handling such as cell loading and medium transfer thus needs to be performed well by well. Microplates are usually available in 96-well formats, compatible with standard liquid handling station. Current commercial spheroid microplates include those from Corning, Nunclon™ Sphera™ from Thermo Fisher Scientific, and CELLSTAR® from Greiner Bio-One. The microplates from Corning are also available in 384- and 1536-well formats, and compatible with HTS instruments.

4. Microfluidics (Figure 1d)

Microfluidics manipulates and controls cell solutions and other liquids in flow channels with dimensions that range from tens to hundreds of micrometers. There are two main types of microfluidics used for cell spheroid generation. The first type produces droplets that encapsulate cells with an encapsulation medium such as alginate (Hidalgo San Jose, Stephens et al. 2018) and

polyethylene glycol (PEG) (Siltanen, Yaghoobi et al. 2016). This method has very good size control over the formed droplets and supports high throughput droplet generation. However, the encapsulation medium physically locks the cells in place, which retards cell-cell communication, limits cell movement, and hinders cell proliferation. The encapsulation medium also forms a barrier for nutrient supply and gas exchange. With double-emulsion method, it is possible to make the internal encapsulation matrix aqueous to allow cells to freely move and aggregate, which however still requires a separate outer encapsulation layer around the aqueous phase and the encapsulation layer still slows down the nutrient and metabolite waste exchange between the cells and the external bulk medium supply (Chan, Zhang et al. 2016). The second type of microfluidics directly aggregates cells without the use of any encapsulation medium and is more of interest for scaffold-free cell spheroid generation. Some studies have made use of cell trapping features such as micropillars or microwells along the flow path of the cells (Sakai and Nakazawa 2007, Okuyama, Yamazoe et al. 2010), while others have attempted to aggregate cells directly through shear flow (Ota, Yamamoto et al. 2010, Ota and Miki 2011) or acoustic forces (Chen, Wu et al. 2016).

Microfluidics chips can be designed to introduce feature specific controls over cell movement and spheroid formation. General concerns are however the limited throughput and tendency of flow channel clogging. In addition, microfluidics chips have very small culture volumes (Vadivelu, Kamble et al. 2017). To support longer term spheroid formation process, continuous medium perfusion with syringe pumps is usually necessary. Currently, there

are no major commercial microfluidics based products for cell spheroid generation.

It is difficult to adopt microfluidics technology in its current state into spheroid manufacturing workflow concerning its limited throughput and knowledge required for designing the microfluidics chips. Improving the scalability of the technology is necessary. Until then, microfluidics is likely to remain more as a research tool rather than a solution to spheroid manufacturing.

5.Bioreactor (Figure 1e)

To generate cell spheroids with bioreactors, cell solution is agitated and cells are brought together by bulk dynamic fluid flow. There are three main types of agitation bioreactors used for spheroid generation.

- Spinner flask

In a spinner flask, the cell solution is agitated by an internal impeller. By varying factors such as the impeller's design, impeller's position and vessel shape, and process parameters such as agitation speed, cell solution volume and cell density, flow shear forces exerted onto the cells can be adjusted and the density and size of spheroids can be controlled. Studies have used this method to produce spheroids of reproducible density and size. Importantly the agitation speed needs to be adjusted at different times of the culture to first initiate aggregate formation, then control spheroid size and at later stages, prevent spheroid fusion (Santos, Camões et al. 2015, Santo, Estrada et al. 2016). The optimum agitation speed to form compact spheroids are cell type specific (Lee, Yoon et al. 2004).

- Rotary shaker

A rotary shaker moves culture vessels on horizontal plane reciprocally, driving cell solutions to circulate within the vessels. It has been used for forming spheroids from primary rat hepatocytes (Inamori, Mizumoto et al. 2010), insulin secreting cells (Joo, Kim et al. 2010), and MSCs (Cha, Kim et al. 2015). The rotary shaker is compatible with common cultureware such as well plates and petri dishes. This allows the flexibility in handling cell solution of various volumes. Volumes as low as 1.5 mL have been successfully handled in 6-well plates for spheroid generation using this method (Inamori, Mizumoto et al. 2010).

- Microgravity bioreactor

In a microgravity bioreactor setup, culture vessel is rotated around a horizontal axis. As a result, cells are subjected to a balanced flow shear and gravity force, and are put in a constant “free-fall” environment. This environment stimulates gene expression changes that could favor spheroid formation (Kopp, Warnke et al. 2015, Strube, Infanger et al. 2020). Culturing cells in microgravity allows minimum collision between cells and has a reduced flow shear force compared to other bioreactor methods. It has been used successfully to generate spheroids from adipose-derived stem cells (ADSCs) (Zhang, Liu et al. 2015), cornea stroma cells (Li, Dai et al. 2015), and pancreatic β-cells (Tanaka, Tanaka et al. 2013). Seeding cells at a lower density in a microgravity bioreactor has led to bigger spheroids (Tanaka, Tanaka et al. 2013). Apart from commercially available bioreactors, such as Synthecon, microgravity in cell spheroid culture have also been explored

using self-assembled clinostat (Tanaka, Tanaka et al. 2013) and outer space (Pietsch, Ma et al. 2013). Spheroid size and gene expression of cells in the spheroids could be different when different methods of microgravity are used (Pietsch, Ma et al. 2013).

In general, bioreactors are a convenient technology to be used for manufacturing of spheroids in large quantities. They require minimum handling due to the large medium reservoir and motorized process. However, they do come with multiple variables, such as bioreactor design, and agitation speed, that require dedicated effort to optimize to obtain spheroids of desired characteristics (Massai, Isu et al. 2016, Yan, Song et al. 2018, Phelan, Gianforcaro et al. 2019). This technology also requires special equipment which could make the upfront cost prohibitive to some. Manufacturers would have to weigh the benefits of bioreactors against the cost of equipment and the effort needed for process development to determine if it is worth adopting this technology.

6. Magnetic manipulation (Figure 1f)

In this method, cells are first magnetized with paramagnetic nanoparticles and then rapidly aggregated by magnets. The magnet can be positioned either above or below the culture so that cells can be aggregated in levitation or at the bottom of the cultureware (Kim, Choi et al. 2013, Jafari, Han et al. 2019). The main advantages of this method include rapid cell aggregation, good control over spheroid sizes and minimal handling requirement. There is currently only one commercial product in the market, which is Magnetic 3D Cell Culture from Greiner Bio-One.

The main concern with this method is the internalization of paramagnetic nanoparticles, which might affect cellular function or behavior depending on various factors such as the surface charge, concentration of the nanoparticles and duration of incubation (Theumer, Grafe et al. 2015). To minimize the internalization of magnetic nanoparticles, Janus spheroid structure assembly which separates cells and nanoparticles could be considered (Mattix, Olsen et al. 2014).

Spheroid generation through magnetic manipulation is a promising method to be used in manufacturing of spheroids due to fast turnaround time, good process control and minimum labor requirement. However, the inclusion of magnetic nanoparticles poses a safety issue. Although it has been claimed the magnetic nanoparticles used are non-toxic, other non-toxic cellular changes are still unclear and should be considered as a risk if this technology is to be adopted for manufacturing of spheroids.

Other technologies for cell spheroid generation

New technologies for spheroid generation are being developed in laboratories. While they offer unique mechanisms and controls over the cell aggregation and spheroid formation process, overall they are still in the research stage and not ready to be adopted for large-scale manufacturing. This section introduces and comments on these new concepts to provide readers with a more thorough view on the current research landscape in spheroid generation technologies.

- Bioactive material

This approach makes use of active materials to promote spontaneous spheroid formation. For example, chitosan has been used for spheroid generation from melanocytes (Lin, Jee et al. 2005), keratocytes (Chen, Wang et al. 2009), periodontal ligament cells (Yan, van den Beucken et al. 2018) and ADSCs (Cheng, Wang et al. 2012). The spheroid formation on chitosan has been associated with cellular absorption of chitosan-bound calcium, which leads to elevated intracellular calcium levels of cells (Cheng, Wang et al. 2012) and expression of spheroid formation promoting genes (Yeh, Liu et al. 2012). Thicker chitosan membrane was found to be able to support formation of bigger spheroids due to its higher content of chitosan-bound calcium (Chou, Lai et al. 2016). Lower deacetylation level of chitosan has been related to quicker and bigger spheroid formation (Chou, Lai et al. 2016).

Similarly, hyaluronan (HA) has been used. HA with higher molecular weight, which is more negatively charged, roughened and less hydrophobic, could lead to formation of bigger spheroids (Carvalho, Costa et al. 2016).

As a method for cell spheroid generation, bioactive material is straightforward and can be performed fair easily as long as the material is available. It has minimum requirement on manual handling and laboratory tools. Its throughput can be controlled via the surface area of the material. Its major limitation however is the lack of good control over spheroid size, which makes it unsuitable for obtaining spheroids of consistent and repeatable quality in its current form. The technology however could be combined with other techniques such as substrate patterning to control the substrate area thus spheroid size.

- Cell membrane engineering

In this method, cells are tagged with intercellular linkers for speedy cell aggregation. The main chemical used for cell membrane modification is sodium periodate ($NaIO_4$) which produces aldehyde functionalities on the cell surface. Other chemicals that have been used for this method include polyethyleneimine-hydrazide (PEI-hy) (Ong, Zhang et al. 2008) and acrylic acid-modified chitosan (chitosan-AA) (Liu, Guan et al. 2015). As the linkers are non-degradable, this method poses concern for those who are interested in studying spheroids in their natural state. To overcome this issue, a biodegradable linker has been developed to contain an enzymatically-degradable peptide between two hydrophobic moieties (Rao, Sasaki et al. 2013).

Cell membrane engineering is advantageous for speedy cell aggregation. However, it is not able to control the cell formation process well. It is also concerned for the biodegradability of the cell linker. This method thus in our opinion can serve as an interesting research tool, but not suitable as a technology for the large-scale manufacturing of spheroids.

- Cell sheets

This approach cultures cell sheets and use the detached cell sheets as starting material to form cell spheroids (Kim, Park et al. 2019). This method preserves the structure of the cellular matrix, thus offers a quicker and more cell friendly spheroid formation process compared to those that use trypsinised cells. The complete release of cell sheets without leaving loose

cells behind on the substrate could be challenging however, even though chemical stimulants could be used to boost cellular production of collagen and obtain structurally stronger cell sheets (Yamauchi, Yamada et al. 2003). Releasing the cell sheet from culture substrate is often accomplished through the use of stimulus responsive hydrogels, which can be difficult to obtain or design for researchers in the laboratories (Yamauchi, Yamada et al. 2003).

In general, cell sheet based process is complex and not easy to master. It needs to be further developed and simplified before it can be considered as a candidate technology for spheroid manufacturing.

- Medium regulation

This line of thought increases the density of medium to above that of cells, forcing cells to float and aggregate. Cells have been floated within a two-phase system comprising Dextran and poly-ethylene glycol (PEG). As cells are denser than the PEG solution but less dense than the Dextran solution, they are trapped at the liquid boundary and aggregated with time. The release of the spheroids can be easily realized by washing the two-phase system with fresh medium (Han, Takayama et al. 2015). In another study, 3% methylcellulose (MC) has been used (Motoyama, Sayo et al. 2016).

Medium regulation based approach has special requirement for the materials, which poses concerns over the material effects on cell growth and behaviour. In addition, it requires deep understanding on the material properties and careful handling of fluid. All these requirements create barriers for the wide application of this method.

- Liquid droplets

This technology encapsulates cells in liquid droplets to facilitate cell spheroid formation. One example used solid alginate matrix and liquid gelatin cores (Leong, Kremer et al. 2016). It involves multiple handling steps and cannot support medium change for longer term spheroid culture. Together with the complexity of the process and the challenge in culture monitoring, this method is unlikely to gain popular adoption for spheroid manufacturing.

In another study, cell embedding liquid droplets were coated with hydrophobic powder. The droplets were then floated on a liquid bath. It was argued that compared to cells in hanging drop method, cells at the bottom of these liquid droplets were subjected to less external forces for aggregation due to buoyancy forces. (Vadivelu, Ooi et al. 2015). One big limitation with this method is the difficulty of spheroid retrieval at the end of the culture process. Nevertheless, it has the potential to be used similar to hanging drop method for spheroid manufacturing.

Desired characteristics of cell spheroid manufacturing process

The manufacturing process has a direct impact on the quality of the produced cell spheroid products. In our opinion, good cell spheroid manufacturing technologies designed for the future should equip the manufacturing process with characteristics including: tight process control, good reproducibility, fast turnaround, high throughput, good scalability, compatibility with upstream/downstream processes, and minimal requirement in labor and equipment.

Size and shape are two seemingly basic but very important physical attributes for cell spheroids. They exert great influence on the microenvironment inside the spheroid such as dissolved oxygen, nutrient, metabolic waste and pH, which can directly affect cellular functions such as metabolism, proliferation, and differentiation (Murphy, Hung et al. 2017, Nishikawa, Tanaka et al. 2017). To effectively design and manage the size and shape of the cell spheroids along with other quality attributes such as cell viability and function, tight manufacturing process control is essential. It ensures the produced spheroids have minimal quality variance, helping removing the need for pre-sorting or concern for dissimilarity in spheroid quality when spheroids are used for their intended applications.

Spheroids produced from different batches, at different sites, and by different operators from the same manufacturing system should possess the same quality. To achieve this, the spheroid manufacturing process should be made reproducible. The more reproducible the manufacturing process is, the easier the process transfer is and cell spheroids with more consistent properties could be attained (Das, Furst et al. 2016, Wiedemeier, Eichler et al. 2017, Hurrell, Ellero et al. 2018). It is noted that the reproducibility of the manufacturing process is directly related to the process control and the complexity of the manufacturing technology.

Fast turnaround time, high throughput and good scalability are also essential requirements for future cell spheroid manufacturing. Manufacturing technologies that present these process attributes could benefit from increased yield, shortened production cycle time and lower manufacturing

costs (Baillargeon, Shumate et al. 2019). Particularly, scalability is especially useful in allowing manufacturers to easily transit from small-scale processes in laboratories to large-scale commercial production.

When cell spheroids are manufactured, spheroid formation should be made compatible with different adjacent steps/processes such as medium change, spheroid transfer, imaging and various characterization and validation assays (Koudan, Gryadunova et al. 2020). This helps streamline the spheroid production workflow and improve the productivity. It also makes the manufacturing technology more adaptable for different types of spheroids and makes it easier for manufacturers to integrate the process into their existing workflow in a modular manner.

Finally, as human intervention not only increases the production cost, but also inclines to introduce handling error and contamination risk, manual handling and labor requirement should be reduced in manufacturing (Allen, Matyas et al. 2019). Requirements for special equipment would also raise the initial capital cost, complicate the manufacturing process, and deter manufacturers from potentially adopting the technology. Cell spheroid manufacturing technologies thus should attempt to avoid requiring special processing equipment.

Manufacturing capabilities of current mainstream cell spheroid generation technologies

Table 2 summarizes our views on the current mainstream cell spheroid generation technologies with regards to their capability to meet the process requirements of scaled spheroid manufacturing.

Table 2: Manufacturing capabilities of mainstream cell spheroid generation technologies

Cell spheroid generation technology	**Desired characteristics of cell spheroid manufacturing process**							**References**
	Tight process control	**Good reproducibility**	**Fast turnaround** **	**High throughput*** **	**Good scalability**	**Compatibility with adjacent processes**	**Minimum equirement for labor and special equipment**	
Hanging drop	Good	Good	Slow	Medium	Limited	Low	Low	(Naderi, Wilde et al. 2014, Raghavan, Ward et al. 2015)
Low adherence substrate	Low	Medium	Slow	High	Limited	Low	Low	(Sambale, Lavrentieva et al. 2015, Bayat, Ebrahimi-Barough et al. 2016, Obara, Tomiyama et al. 2016, Tong, Fang et al. 2016, Zhang, Li et al. 2016, Tevis, Cecchi et al. 2017)
Microwells	Good	Good	Slow	High	Limited	Medium	Low	(Anada, Sato et al. 2016, Kamoya, Anada et al. 2016, Shin, Kook et al. 2016, Wang, Kim et al. 2016)

Microfluidics *	Design dependent	Medium	Slow	Low	Limited	Low	High	(Cui, Liu et al. 2016, Siltanen, Yaghoobi et al. 2016)
Bioreactor	Low	Medium	Slow	High	Good	Medium	High	(Cha, Kim et al. 2015, Santos, Camões et al. 2015, Santo, Estrada et al. 2016)
Magnetic manipulation	Good	Good	Good	Medium	Limited	Medium	High	(Mattix, Olsen et al. 2014, Theumer, Grafe et al. 2015)

* Microfluidics for scaffold-free spheroid formation

**"Slow" if processes take>1 day in general

***Used with liquid handling station

Based on our evaluation, current mainstream cell spheroid generation technologies are in overall limited in their manufacturing capability. While different technologies vary in their process control and repeatability, the turnaround is generally slow taking more than one day. On the other hand, most of the technologies can actually be used to produce spheroids with reasonable throughput due to their compatibility with liquid handling stations. One common limitation shared by the different technologies is the poor compatibility with adjacent processes such as liquid change and spheroid characterization. For example, droplets from hanging drop are fragile to handle and the spheroids formed in microwells can be easily displaced.

Among the different technologies, the bioreactor is especially advantageous in scalability due to bulk medium supply and its capability of maintaining a suspension culture, the volume of which could be flexibly adjusted when needed. It is noted while technologies can be scalable, the starting cell numbers minimally required for each technology are different, which directly affect the time and effort needed to prepare the seed train before spheroid production process and thus the decision on the selection of specific spheroid generation technologies. In particular, among all, bioreactors require higher starting cell numbers due to their larger staring volumes. Finally, it is also of interest to note that half of the technologies have requirement for specialized equipment, which can be easily correlated to the level of technology adoption in research laboratories.

Gaps in current cell spheroid generation technologies and needs for future technology development

Overall, the current cell spheroid generation technologies are useful for research work at small scales. However, there are some important gaps to be addressed for large-scale manufacturing of cell spheroids. Some of the gaps are not solely on the spheroid formation, but also on the adjacent processes. This is because all these processes are closely connected and any missing or weak link in the process workflow would hinder the final delivery of spheroids for downstream applications. It is important that spheroid generation technologies are considered together with the other related process in the manufacturing context holistically.

- Liquid handling at the presence of spheroids

Liquid handling is needed almost at every step of the spheroid manufacturing process. Due to the small size of spheroids and suspension nature of spheroid culture, spheroids are easily displaceable with liquid perturbation even at mild flow turbulence. In platforms with smaller liquid volumes such as hanging drop plates and low-adherence substrates, liquid handling tends to dislodge the spheroids, leading to spheroid displacement, loss or agglomeration. In platforms with bigger volumes such as bioreactors, liquid exchange is often carried out after fluid flow is temporarily stopped and spheroids have settled to the bottom of the vessel. The temporary arrest of fluid flow reduces the risk of spheroid loss but increases that of spheroid fusion, leading to difficulty in attaining homogenous spheroid products. Finally, in most instances, liquid handling for spheroid related processes is performed manually. It requires great handling efforts to minimize disturbance to the spheroids and takes a long time.

Though automatic liquid handling stations and special design of spheroid holding well (e.g., deep well in iNCYTO) have already been made available to help ease some of the handling stress, new technologies that can yield better control over the liquid handling process are required. Ideally, new technologies should minimize disturbance to the spheroids and reduce labor handling requirement.

- Spheroid transfer

After spheroids are formed, they usually need to be transferred out of the spheroid generation cultureware and into different sets of vessels for downstream processing or applications. The current spheroid transfer process

relies mainly on manual pipetting and is laborious. It requires extensive attention and yet carries high risks of spheroid loss and property alteration. For example, spheroids could get stuck onto pipette tips or cultureware. The properties of the spheroids could be altered by the flow shear, or the physical contact with pipette tips or cultureware. The laborious and error-prone spheroid transfer process could thus easily lead to varied and unpredictable spheroid size and structure and cellular behaviors in the spheroids, which are undesirable in spheroid manufacturing where reproducibility is important.

New technologies that can reduce the need for manual pipetting and at the same time lower the risk of spheroid loss and damage, ideally through automation, are expected to help improve the spheroid transfer process.

- Spheroid characterization

Spheroid characterization is necessary for quality control of the produced spheroids. As of today, spheroids are most commonly characterized using physical size and shape, which can easily be discerned with the use of light microscopes. Other aspects, such as compactness of the spheroids, cellular material distribution inside of the spheroids, and state of cells that make up the spheroids, are much more technically challenging to characterize. Although proliferation and viability assays for monolayer cell characterization have been used for spheroid characterization, they may not be as effective. For example, spheroids present different levels of compactness depending on the cells, culture duration and culture environment. Penetration of the chemical reagents, which are originally designed for monolayer characterization assays, into the core of 3D spheroids can be difficult to

quantify and control (Lazzari, Vinciguerra et al. 2019). In addition, 3D structure of spheroids can be more challenging to assess using imaging techniques even though clarifying techniques could be used and helpful in some cases.

As spheroids vary in size, shape and compactness, sophisticated and dedicated spheroid characterization technologies that can be used for different types of spheroids should be developed. These new technologies should help better define the physical properties of spheroids (e.g., size, shape, compactness, uniformness, stiffness), detail cell-cell interaction within the spheroids and clarify extracellular network between cells in the spheroids. The characterization data would elucidate the relationship between the properties of spheroids and that of the constitutional cells.

- Process standardization, control and monitoring

Current spheroid generation methods produce spheroids without advanced process standardization, control and monitoring. This limits the process repeatability and robustness, directly contributing to difficulty in defining and controlling the quality of the end products, which are the cell spheroids.

Automation stations and close-loop feedback control could be integrated with the spheroid formation process. Advanced non-invasive and on-line monitoring technologies, which could be imaging or spectrum based, could be adapted to allow manufacturers to track and monitor spheroid development as the spheroids progress through the manufacturing workflow to ensure consistent product quality.

- Scalable manufacturing

Most of the existing spheroid generation methods have limited scalability for spheroid production. Some of the existing methods (e.g., hanging drop plates and microwells) can be scaled up through integration with automated liquid handling stations to improve manufacturing throughput. However, the cost of automated liquid handling stations can be prohibitive. Developments of new methods or adjustment of current methods should consider scalability and allow for the technology to be usable in both small-scale research settings and large-scale manufacturing settings. The flexibility of such a method would also be useful for process development for commercial manufacturing.

Conclusion

Cell spheroids have received increasing research interests in the past decades and are currently used in wide applications such as cell biology, drug screening and regenerative medicine. Various spheroid generation technologies have been developed in both research laboratories and the commercial field. These technologies are different in their mechanism and have their own advantages and limitations. In general, current technologies are highly manual and have slow turnaround time with limited process control, throughput and scalability. To meet increasing demands for spheroid based applications, future technological development has to be made to improve current technologies to make large-scale, consistent, and repeatable spheroid manufacturing possible. Such development efforts should focus on areas of liquid handling, spheroid transfer, spheroid characterization, process standardization, and process scalability.

Acknowledgement

This research is supported by Bioprocessing Technology Institute (BTI) under the Agency for Science, Technology and Research of Singapore. There is no grant applicable for the work.

Declaration of conflict of interest

The authors declare no conflict of interest for the manuscript submission.

Author Contribution Statement

Liu Dan: conceptualization, writing and editing. Chen Sixun: conceptualization and editing. May Win Naing: conceptualization.

References

Abe-Fukasawa, N., K. Otsuka, A. Aihara, N. Itasaki and T. Nishino (2018). "Novel 3D liquid cell culture method for anchorage-independent cell growth, cell Imaging and automated drug screening." Scientific Reports **8**(1): 3627.

Allen, L. M., J. Matyas, M. Ungrin, D. A. Hart and A. Sen (2019). "Serum-Free Culture of Human Mesenchymal Stem Cell Aggregates in Suspension Bioreactors for Tissue Engineering Applications." Stem Cells International **2019**.

Anada, T., T. Sato, T. Kamoya, Y. Shiwaku, K. Tsuchiya, T. Takano-Yamamoto, K. Sasaki and O. Suzuki (2016). "Evaluation of bioactivity of octacalcium phosphate using osteoblastic cell aggregates on a spheroid culture device." Regenerative Therapy **3**: 58-62.

Baillargeon, P., J. Shumate, S. R. Hou, V. Fernandez-Vega, N. Marques, G. Souza, J. Seldin, T. P. Spicer and L. Scampavia (2019). "Automating a magnetic 3D spheroid model technology for high-throughput screening." Slas Technology **24**(4): 420-428.

Bayat, N., S. Ebrahimi-Barough, A. Norouzi-Javidan, H. Saberi, M. M. M. Ardakan, A. Ai, M. Soleimannejad and J. Ai (2018). "Anti-inflammatory effects of atorvastatin by suppressing TRAF3IP2 and IL-17RA in human glioblastoma spheroids cultured in a three-dimensional model: possible relevance to glioblastoma treatment." Molecular Neurobiology **55**(3): 2102-2110.

Bayat, N., S. Ebrahimi-Barough, A. Norouzi-Javidan, H. Saberi, R. Tajerian, M. M. M. Ardakan, S. Shirian, A. Ai and J. Ai (2016). "Apoptotic effect of atorvastatin in glioblastoma spheroids tumor cultured in fibrin gel." Biomed Pharmacother **84**: 1959-1966.

Carvalho, M. P., E. C. Costa, S. P. Miguel and I. J. Correia (2016). "Tumor spheroid assembly on hyaluronic acid-based structures: A review." Carbohydr Polym **150**: 139-148.

Cha, H. M., S. M. Kim, Y. S. Choi and D. I. Kim (2015). "Scaffold-free three-dimensional culture systems for mass production of periosteum-derived progenitor cells." Journal of Bioscience and Bioengineering **120**(2): 218-222.

Chan, H. F., Y. Zhang and K. W. Leong (2016). "Efficient One-Step Production of Microencapsulated Hepatocyte Spheroids with Enhanced Functions." Small **12**(20): 2720-2730.

Chandrasekaran, S., U. B. T. Giang, M. R. King and L. A. DeLouise (2011). "Microenvironment induced spheroid to sheeting transition of immortalized human keratinocytes (HaCaT) cultured in microbubbles formed in polydimethylsiloxane." Biomaterials **32**(29): 7159-7168.

Chen, K. J., M. X. Wu, F. Guo, P. Li, C. Y. Chan, Z. M. Mao, S. X. Li, L. Q. Ren, R. Zhang and T. J. Huang (2016). "Rapid formation of size-controllable multicellular spheroids via 3D acoustic tweezers." Lab on a Chip **16**(14): 2636-2643.

Chen, Y. H., I. J. Wang and T. H. Young (2009). "Formation of keratocyte spheroids on chitosan-coated surface can maintain keratocyte phenotypes." Tissue Engineering Part A **15**(8): 2001-2013.

Cheng, N. C., S. Wang and T. H. Young (2012). "The influence of spheroid formation of human adipose-derived stem cells on chitosan films on stemness and differentiation capabilities." Biomaterials **33**(6): 1748-1758.

Chou, S. F., J. Y. Lai, C. H. Cho and C. H. Lee (2016). "Relationships between surface roughness/stiffness of chitosan coatings and fabrication of corneal keratocyte spheroids: Effect of degree of deacetylation." Colloids and Surfaces B-Biointerfaces **142**: 105-113.

Cui, X., Y. Liu, Y. Hartanto, J. Bi, S. Dai and H. Zhang (2016). "Multicellular spheroids formation and recovery in microfluidics-generated thermoresponsive microgel droplets." Colloid and Interface Science Communications **14**: 4-7.

Damman, R., A. Lucini Paioni, K. T. Xenaki, I. Beltrán Hernández, P. M. P. van Bergen en Henegouwen and M. Baldus (2020). "Development of in vitro-grown spheroids as a 3D tumor model system for solid-state NMR spectroscopy." Journal of Biomolecular NMR.

Das, V., T. Furst, S. Gurska, P. Dzubak and M. Hajduch (2016). "Reproducibility of Uniform Spheroid Formation in 384-Well Plates: The Effect of Medium Evaporation." Journal of Biomolecular Screening **21**(9): 923-930.

Ferreira, L. P., V. M. Gaspar and J. F. Mano (2018). "Design of spherically structured 3D in vitro tumor models -Advances and prospects." Acta Biomaterialia **75**: 11-34.

Giang, U. B. T., M. R. King and L. A. DeLouise (2008). "Microfabrication of bubbular cavities in PDMS for cell sorting and microcell culture applications." Journal of Bionic Engineering **5**(4): 308-316.

Han, C., S. Takayama and J. Park (2015). "Formation and manipulation of cell spheroids using a density adjusted PEG/DEX aqueous two phase system." Scientific reports **5**: 11891-11891.

Hidalgo San Jose, L., P. Stephens, B. Song and D. Barrow (2018). "Microfluidic Encapsulation Supports Stem Cell Viability, Proliferation, and Neuronal Differentiation." Tissue engineering. Part C, Methods **24**(3): 158-170.

Hookway, T. A., J. C. Butts, E. Lee, H. L. Tang and T. C. McDevitt (2016). "Aggregate formation and suspension culture of human pluripotent stem cells and differentiated progeny." Methods **101**: 11-20.

Huang, Y. C., C. C. Chan, W. T. Lin, H. Y. Chiu, R. Y. Tsai, T. H. Tsai, J. Y. Chan and S. J. Lin (2013). "Scalable production of controllable dermal papilla spheroids on PVA surfaces and the effects of spheroid size on hair follicle regeneration." Biomaterials **34**(2): 442-451.

Hurrell, T., A. A. Ellero, Z. F. Masso and A. D. Cromarty (2018). "Characterization and reproducibility of HepG2 hanging drop spheroids toxicology in vitro." Toxicology in Vitro **50**: 86-94.

Inamori, M., H. Mizumoto and T. Kajiwara (2010). "Investigation of medium perfusion through scaffold-free tissue constructs using endothelial cell-covered spheroids in vitro." Biochemical Engineering Journal **50**(3): 116-121.

Jafari, J., X. L. Han, J. Palmer, P. A. Tran and A. J. O'Connor (2019). "Remote control in formation of 3D multicellular assemblies using magnetic forces." Acs Biomaterials Science & Engineering **5**(5): 2532-2542.

Joo, D. J., J. Y. Kim, J. I. Lee, J. H. Jeong, Y. Cho, M. K. Ju, K. H. Huh, M. S. Kim and Y. S. Kim (2010). "Manufacturing of insulin-secreting spheroids with the RIN-5F cell line using a shaking culture method." Transplantation Proceedings **42**(10): 4225-4227.

Jun, Y., A. R. Kang, J. S. Lee, G. S. Jeong, J. Ju, D. Y. Lee and S. H. Lee (2013). "3D co-culturing model of primary pancreatic islets and hepatocytes in hybrid spheroid to overcome pancreatic cell shortage." Biomaterials **34**(15): 3784-3794.

Kamoya, T., T. Anada, Y. Shiwaku, T. Takano-Yamamoto and O. Suzuki (2016). "An oxygen-permeable spheroid culture chip (Oxy chip) promotes osteoblastic

differentiation of mesenchymal stem cells." Sensors and Actuators B: Chemical **232**: 75-83.

Kamoya, T., T. Anada, Y. Shiwaku, T. Takano-Yamamoto and O. Suzuki (2016). "An oxygen-permeable spheroid culture chip (Oxy chip) promotes osteoblastic differentiation of mesenchymal stem cells." Sensors and Actuators B-Chemical **232**: 75-83.

Kim, G., Y. Jung, K. Cho, H. J. Lee and W. G. Koh (2020). "Thermoresponsive poly(N-isopropylacrylamide) hydrogel substrates micropatterned with poly(ethylene glycol) hydrogel for adipose mesenchymal stem cell spheroid formation and retrieval." Materials Science & Engineering C-Materials for Biological Applications **115**.

Kim, J. A., J. H. Choi, M. Kim, W. J. Rhee, B. Son, H. K. Jung and T. H. Park (2013). "High-throughput generation of spheroids using magnetic nanoparticles for three-dimensional cell culture." Biomaterials **34**(34): 8555-8563.

Kim, M., H. W. Yun, D. Y. Park, B. H. Choi and B. H. Min (2018). "Three-dimensional spheroid culture increases exosome secretion from mesenchymal stem cells." Tissue Engineering and Regenerative Medicine **15**(4): 427-436.

Kim, S.-j., J. Park, H. Byun, Y.-W. Park, L. G. Major, D. Y. Lee, Y. S. Choi and H. Shin (2019). "Hydrogels with an embossed surface: An all-in-one platform for mass production and culture of human adipose-derived stem cell spheroids." Biomaterials **188**: 198-212.

Kim, S. A., E. K. Lee and H. J. Kuh (2015). "Co-culture of 3D tumor spheroids with fibroblasts as a model for epithelial-mesenchymal transition in vitro." Experimental Cell Research **335**(2): 187-196.

Kopp, S., E. Warnke, M. Wehland, G. Aleshcheva, N. E. Magnusson, R. Hemmersbach, T. J. Corydon, J. Bauer, M. Infanger and D. Grimm (2015). "Mechanisms of three-dimensional growth of thyroid cells during long-term simulated microgravity." Scientific Reports **5**(1): 16691.

Koudan, E. V., A. A. Gryadunova, P. A. Karalkin, J. V. Korneva, N. Y. Meteleva, Babichenko, II, A. V. Volkov, S. A. Rodionov, V. A. Parfenov, F. Pereira, Y. D. Khesuani, V. A. Mironov and E. A. Bulanova (2020). "Multiparametric Analysis of Tissue Spheroids Fabricated from Different Types of Cells." Biotechnology Journal **15**(5).

Kunz-Schughart, L. A., J. P. Freyer, F. Hofstaedter and R. Ebner (2004). "The use of 3-D cultures for high-throughput screening: The multicellular spheroid model." Journal of Biomolecular Screening **9**(4): 273-285.

Kusamori, K., M. Nishikawa, N. Mizuno, T. Nishikawa, A. Masuzawa, K. Shimizu, S. Konishi, Y. Takahashi and Y. Takakura (2014). "Transplantation of insulin-secreting multicellular spheroids for the treatment of type 1 diabetes in mice." Journal of Controlled Release **173**: 119-124.

Langhans, S. A. (2018). "Three-dimensional in vitro cell culture models in drug discovery and drug repositioning." Frontiers in Pharmacology **9**.

Lazzari, G., D. Vinciguerra, A. Balasso, V. Nicolas, N. Goudin, M. Garfa-Traore, A. Feher, A. Dinnyes, J. Nicolas, P. Couvreur and S. Mura (2019). "Light sheet fluorescence microscopy versus confocal microscopy: in quest of a suitable tool to assess drug and nanomedicine penetration into multicellular tumor spheroids." European Journal of Pharmaceutics and Biopharmaceutics **142**: 195-203.

Lee, D. H., H. H. Yoon, J. H. Lee, K. W. Lee, S. K. Lee, S. K. Kim, J. E. Choi, Y. J. Kim and J. K. Park (2004). "Enhanced liver-specific functions of endothelial cell-covered hepatocyte hetero-spheroids." Biochemical Engineering Journal **20**(2-3): 181-187.

Lee, J., M. J. Cuddihy, G. M. Cater and N. A. Kotov (2009). "Engineering liver tissue spheroids with inverted colloidal crystal scaffolds." Biomaterials **30**(27): 4687-4694.

Leong, W., A. Kremer and D. A. Wang (2016). "Development of size-customized hepatocarcinoma spheroids as a potential drug testing platform using a sacrificial gelatin microsphere system." Mater Sci Eng C Mater Biol Appl **63**: 644-649.

Li, H. Y., Y. Dai, J. C. Shu, R. J. Yu, Y. L. Guo and J. S. Chen (2015). "Spheroid cultures promote the stemness of corneal stromal cells." Tissue & Cell **47**(1): 39-48.

Lin, B. J., Y. Miao, J. Wang, Z. X. Fan, L. J. Du, Y. S. Su, B. C. Liu, Z. Q. Hu and M. Xing (2016). "Surface tension guided hanging-drop: producing controllable 3D Spheroid of high-passaged human dermal papilla cells and forming inductive microtissues for hair-follicle regeneration." Acs Applied Materials & Interfaces **8**(9): 5906-5916.

Lin, S. J., S. H. Jee, W. C. Hsaio, S. J. Lee and T. H. Young (2005). "Formation of melanocyte spheroids on the chitosan-coated surface." Biomaterials **26**(12): 1413-1422.

Liu, T. Q., M. Winter and B. Thierry (2014). "Quasi-spherical microwells on superhydrophobic substrates for long term culture of multicellular spheroids and high throughput assays." Biomaterials **35**(23): 6060-6068.

Liu, Y., Y. Guan and Y. Zhang (2015). "Chitosan as inter-cellular linker to accelerate multicellular spheroid generation in hydrogel scaffold." Polymer **77**: 366-376.

Markovitz-Bishitz, Y., Y. Tauber, E. Afrimzon, N. Zurgil, M. Sobolev, Y. Shafran, A. Deutsch, S. Howitz and M. Deutsch (2010). "A polymer microstructure array for the formation, culturing, and high throughput drug screening of breast cancer spheroids." Biomaterials **31**(32): 8436-8444.

Massai, D., G. Isu, D. Madeddu, G. Cerino, A. Falco and C. Frati (2016). "A Versatile Bioreactor for Dynamic Suspension Cell Culture. Application to the Culture of Cancer Cell Spheroids (vol 11, e0154610, 2016)." Plos One **11**(6).

Mattix, B. M., T. R. Olsen, M. Casco, L. Reese, J. T. Poole, J. Zhang, R. P. Visconti, A. Simionescu, D. T. Simionescu and F. Alexis (2014). "Janus magnetic cellular spheroids for vascular tissue engineering." Biomaterials **35**(3): 949-960.

Motoyama, W., K. Sayo, H. Mihara, S. Aoki and N. Kojima (2016). "Induction of hepatic tissues in multicellular spheroids composed of murine fetal hepatic cells and embedded hydrogel beads." Regenerative Therapy **3**: 7-10.

Murphy, K. C., B. Hung, S. Browne-Bourne, D. J. Zhou, J. Yeung, D. C. Genetos and J. K. Leach (2017). "Measurement of oxygen tension within mesenchymal stem cell spheroids." Journal of the Royal Society Interface **14**(127).

Naderi, N., C. Wilde, T. Haque, W. Francis, A. M. Seifalian, C. A. Thornton, Z. Xia and I. S. Whitaker (2014). "Adipogenic differentiation of adipose-derived stem cells in 3-dimensional spheroid cultures (microtissue): Implications for the reconstructive surgeon." Journal of Plastic, Reconstructive & Aesthetic Surgery **67**(12): 1726-1734.

Nishikawa, T., Y. Tanaka, K. Kusamori, N. Mizuno, Y. Mizukami, Y. Ogino, K. Shimizu, S. Konishi, Y. Takahashi, Y. Takakura and M. Nishikawa (2017). "Using size-controlled multicellular spheroids of murine adenocarcinoma cells to efficiently establish pulmonary tumors in mice." Biotechnology Journal **12**(8).

Obara, C., K. Tomiyama, K. Takizawa, R. Islam, T. Yasuda, T. Gotoh and K. Tajima (2016). "Characteristics of three-dimensional prospectively isolated mouse bone marrow mesenchymal stem/stromal cell aggregates on nanoculture plates." Cell and Tissue Research **366**(1): 113-127.

Okudaira, T., R. Yabuta, H. Mizumoto and T. Kajiwara (2017). "Fabrication of a fiber-type hepatic tissue by bottom-up method using multilayer spheroids." Journal of Bioscience and Bioengineering **123**(6): 739-747.

Okuyama, T., H. Yamazoe, N. Mochizuki, A. Khademhosseini, H. Suzuki and J. Fukuda (2010). "Preparation of arrays of cell spheroids and spheroid-monolayer cocultures within a microfluidic device." Journal of Bioscience and Bioengineering **110**(5): 572-576.

Ong, S. M., C. Zhang, Y. C. Toh, S. H. Kim, H. L. Foo, C. H. Tan, D. van Noort, S. Park and H. Yu (2008). "A gel-free 3D microfluidic cell culture system." Biomaterials **29**(22): 3237-3244.

Ota, H. and N. Miki (2011). "Microfluidic experimental platform for producing size-controlled three-dimensional spheroids." Sensors and Actuators a-Physical **169**(2): 266-273.

Ota, H., R. Yamamoto, K. Deguchi, Y. Tanaka, Y. Kazoe, Y. Sato and N. Miki (2010). "Three-dimensional spheroid-forming lab-on-a-chip using micro-rotational flow." Sensors and Actuators B-Chemical **147**(1): 359-365.

Phelan, M. A., A. L. Gianforcaro, J. A. Gerstenhaber and P. I. Lelkes (2019). "An Air Bubble-Isolating Rotating Wall Vessel Bioreactor for Improved Spheroid/Organoid Formation." Tissue Engineering Part C-Methods **25**(8): 479-488.

Pietsch, J., X. Ma, M. Wehland, G. Aleshcheva, A. Schwarzwalder, J. Segerer, M. Birlem, A. Horn, J. Bauer, M. Infanger and D. Grimm (2013). "Spheroid formation of human thyroid cancer cells in an automated culturing system during the Shenzhou-8 Space mission." Biomaterials **34**(31): 7694-7705.

Prestigiacomo, V., A. Weston and L. Suter-Dick (2020). "Rat multicellular 3D liver microtissues to explore TGF-beta 1 induced effects." Journal of Pharmacological and Toxicological Methods **101**.

Priwitaningrum, D. L., J. B. G. Blonde, A. Sridhar, J. van Baarlen, W. E. Hennink, G. Storm, S. Le Gac and J. Prakash (2016). "Tumor stroma-containing 3D spheroid

arrays: A tool to study nanoparticle penetration." Journal of Controlled Release **244**: 257-268.

Raghavan, S., M. R. Ward, K. R. Rowley, R. M. Wold, S. Takayama, R. J. Buckanovich and G. Mehta (2015). "Formation of stable small cell number three-dimensional ovarian cancer spheroids using hanging drop arrays for preclinical drug sensitivity assays." Gynecol Oncol **138**(1): 181-189.

Rao, Z., M. Sasaki and T. Taguchi (2013). "Development of amphiphilic, enzymatically-degradable PEG-peptide conjugate as cell crosslinker for spheroid formation." Colloids and Surfaces B: Biointerfaces **101**: 223-227.

Sakai, Y. and K. Nakazawa (2007). "Technique for the control of spheroid diameter using microfabricated chips." Acta Biomaterialia **3**(6): 1033-1040.

Sambale, F., A. Lavrentieva, F. Stahl, C. Blume, M. Stiesch, C. Kasper, D. Bahnemann and T. Scheper (2015). "Three dimensional spheroid cell culture for nanoparticle safety testing." J Biotechnol **205**: 120-129.

Santo, V. E., M. F. Estrada, S. P. Rebelo, S. Abreu, I. Silva, C. Pinto, S. C. Veloso, A. T. Serra, E. Boghaert, P. M. Alves and C. Brito (2016). "Adaptable stirred-tank culture strategies for large scale production of multicellular spheroid-based tumor cell models." Journal of Biotechnology **221**: 118-129.

Santos, J. M., S. P. Camões, E. Filipe, M. Cipriano, R. N. Barcia, M. Filipe, M. Teixeira, S. Simões, M. Gaspar, D. Mosqueira, D. S. Nascimento, P. Pinto-do-Ó, P. Cruz, H. Cruz, M. Castro and J. P. Miranda (2015). "Three-dimensional spheroid cell culture of

umbilical cord tissue-derived mesenchymal stromal cells leads to enhanced paracrine induction of wound healing." Stem Cell Research & Therapy **6**(1): 90.

Shimizu, K., K. Kusamori, M. Nishikawa, N. Mizuno, T. Nishikawa, A. Masuzawa, S. Katano, Y. Takahashi, Y. Takakura and S. Konishi (2013). "Poly(N-isopropylacrylamide)-coated microwell arrays for construction and recovery of multicellular spheroids." Journal of Bioscience and Bioengineering **115**(6): 695-699.

Shin, H. S., Y. M. Kook, H. J. Hong, Y. M. Kim, W. G. Koh and J. Y. Lim (2016). "Functional spheroid organization of human salivary gland cells cultured on hydrogel-micropatterned nanofibrous microwells." Acta Biomaterialia **45**: 121-132.

Shin, H. S., Y. M. Kook, H. J. Hong, Y. M. Kim, W. G. Koh and J. Y. Lim (2016). "Functional spheroid organization of human salivary gland cells cultured on hydrogel-micropatterned nanofibrous microwells." Acta Biomater **45**: 121-132.

Siltanen, C., M. Yaghoobi, A. Haque, J. You, J. Lowen, M. Soleimani and A. Revzin (2016). "Microfluidic fabrication of bioactive microgels for rapid formation and enhanced differentiation of stem cell spheroids." Acta Biomaterialia **34**: 125-132.

Sridhar, A., H. L. de Boer, A. van den Berg and S. Le Gac (2014). "Microstamped Petri Dishes for Scanning Electrochemical Microscopy Analysis of Arrays of Microtissues." Plos One **9**(4).

Strube, F., M. Infanger, M. Wehland, X. Delvinioti, A. Romswinkel, C. Diet and A. Kraus (2020). "Alteration of cytoskeleton morphology and gene expression in human breast cancer cells under simulated microgravity." Cell Journal **22**(1): 106-114.

Takayama, K., K. Kawabata, Y. Nagamoto, K. Kishimoto, K. Tashiro, F. Sakurai, M. Tachibana, K. Kanda, T. Hayakawa, M. K. Furue and H. Mizuguchi (2013). "3D spheroid culture of hESC/hiPSC-derived hepatocyte-like cells for drug toxicity testing." Biomaterials **34**(7): 1781-1789.

Tanaka, H., S. Tanaka, K. Sekine, S. Kita, A. Okamura, T. Takebe, Y. W. Zheng, Y. Ueno, J. Tanaka and H. Taniguchi (2013). "The generation of pancreatic beta-cell spheroids in a simulated microgravity culture system." Biomaterials **34**(23): 5785-5791.

Tang, Y. D., J. M. Liu and Y. Chen (2016). "Agarose multi-wells for tumour spheroid formation and anti-cancer drug test." Microelectronic Engineering **158**: 41-45.

Tevis, K. M., R. J. Cecchi, Y. L. Colson and M. W. Grinstaff (2017). "Mimicking the tumor microenvironment to regulate macrophage phenotype and assessing chemotherapeutic efficacy in embedded cancer cell/macrophage spheroid models." Acta Biomaterialia **50**: 271-279.

Theumer, A., C. Grafe, F. Bahring, C. Bergemann, A. Hochhaus and J. H. Clement (2015). "Superparamagnetic iron oxide nanoparticles exert different cytotoxic effects on cells grown in monolayer cell culture versus as multicellular spheroids." Journal of Magnetism and Magnetic Materials **380**: 27-33.

Tong, W. H., Y. Fang, J. Yan, X. Hong, N. H. Singh, S. R. Wang, B. Nugraha, L. Xia, E. L. S. Fong, C. Iliescu and H. Yu (2016). "Constrained spheroids for prolonged hepatocyte culture." Biomaterials **80**: 106-120.

Vadivelu, R. K., H. Kamble, M. J. A. Shiddiky and N.-T. Nguyen (2017). "Microfluidic Technology for the Generation of Cell Spheroids and Their Applications." Micromachines **8**(4): 94.

Vadivelu, R. K., C. H. Ooi, R.-Q. Yao, J. Tello Velasquez, E. Pastrana, J. Diaz-Nido, F. Lim, J. A. K. Ekberg, N.-T. Nguyen and J. A. St John (2015). "Generation of three-dimensional multiple spheroid model of olfactory ensheathing cells using floating liquid marbles." Scientific Reports **5**(1): 15083.

Verjans, E. T., J. Doijen, W. Luyten, B. Landuyt and L. Schoofs (2018). "Three-dimensional cell culture models for anticancer drug screening: Worth the effort?" Journal of Cellular Physiology **233**(4): 2993-3003.

Wang, P. Y., H. Thissen and P. Kingshott (2016). "Stimulation of Early Osteochondral Differentiation of Human Mesenchymal Stem Cells Using Binary Colloidal Crystals (BCCs)." Acs Applied Materials & Interfaces **8**(7): 4477-4488.

Wang, W. J., K. Itaka, S. Ohba, N. Nishiyama, U. I. Chung, Y. Yamasaki and K. Kataoka (2009). "3D spheroid culture system on micropatterned substrates for improved differentiation efficiency of multipotent mesenchymal stem cells." Biomaterials **30**(14): 2705-2715.

Wang, Y., M. H. Kim, S. R. Tabaei, J. H. Park, K. Na, S. Chung, V. P. Zhdanov and N. J. Cho (2016). "Spheroid formation of hepatocarcinoma cells in microwells: experiments and Monte Carlo simulations." PLoS One **11**(8): e0161915.

Wiedemeier, S., M. Eichler, R. Romer, A. Grodrian, K. Lemke, K. Nagel, C. P. Klages and G. Gastrock (2017). "Parametric studies on droplet generation reproducibility for applications with biological relevant fluids." Engineering in Life Sciences **17**(12): 1271-1280.

Wong, S. F., D. Y. No, Y. Y. Choi, D. S. Kim, B. G. Chung and S. H. Lee (2011). "Concave microwell based size-controllable hepatosphere as a three-dimensional liver tissue model." Biomaterials **32**(32): 8087-8096.

Yamaguchi, Y., J. Ohno, A. Sato, H. Kido and T. Fukushima (2014). "Mesenchymal stem cell spheroids exhibit enhanced in-vitro and in-vivo osteoregenerative potential." Bmc Biotechnology **14**.

Yamamoto, M., N. Kawashima, N. Takashino, Y. Koizumi, K. Takimoto, N. Suzuki, M. Saito and H. Suda (2014). "Three-dimensional spheroid culture promotes odonto/osteoblastic differentiation of dental pulp cells." Archives of Oral Biology **59**(3): 310-317.

Yamauchi, N., O. Yamada, T. Takahashi, K. Imai, T. Sato, A. Ito and K. Hashizume (2003). "A three-dimensional cell culture model for bovine endometrium: regeneration of a multicellular spheroid using ascorbate." Placenta **24**(2-3): 258-269.

Yamazaki, H., S. Gotou, K. Ito, S. Kohashi, Y. Goto, Y. Yoshiura, Y. Sakai, H. Yabu, M. Shimomura and K. Nakazawa (2014). "Micropatterned culture of HepG2 spheroids using microwell chip with honeycomb-patterned polymer film." Journal of Bioscience and Bioengineering **118**(4): 455-460.

Yan, X. Z., J. van den Beucken, C. Yuan, J. A. Jansen and F. Yang (2018). "Spheroid formation and stemness preservation of human periodontal ligament cells on chitosan films." Oral Dis **24**(6): 1083-1092.

Yan, Y. W., L. Q. Song, J. Madinya, T. Ma and Y. Li (2018). "Derivation of Cortical Spheroids from Human Induced Pluripotent Stem Cells in a Suspension Bioreactor." Tissue Engineering Part A **24**(5-6): 418-431.

Yeh, H. Y., B. H. Liu and S. H. Hsu (2012). "The calcium-dependent regulation of spheroid formation and cardiomyogenic differentiation for MSCs on chitosan membranes." Biomaterials **33**(35): 8943-8954.

Yoshii, Y., T. Furukawa, A. Waki, H. Okuyama, M. Inoue, M. Itoh, M. R. Zhang, H. Wakizaka, C. Sogawa, Y. Kiyono, H. Yoshii, Y. Fujibayashi and T. Saga (2015). "High-throughput screening with nanoimprinting 3D culture for efficient drug development by mimicking the tumor environment." Biomaterials **51**: 278-289.

Zhang, S. C., P. Liu, L. Chen, Y. J. Wang, Z. G. Wang and B. Zhang (2015). "The effects of spheroid formation of adipose-derived stem cells in a microgravity bioreactor on sternness properties and therapeutic potential." Biomaterials **41**: 15-25.

Zhang, W., C. Li, B. C. Baguley, F. Zhou, W. Zhou, J. P. Shaw, Z. Wang, Z. Wu and J. Liu (2016). "Optimization of the formation of embedded multicellular spheroids of MCF-7 cells: How to reliably produce a biomimetic 3D model." Anal Biochem **515**: 47-54.

List of Figures

Figure 1: Schematic of different spheroid generation technologies. (a) Hanging drop, (b) Low-adherence substrate, (c) Microwells, (d) Microfluidics, (e) Bioreactor, (f) Magnetic manipulation