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Current approaches and techniques of 3D cell culture systems: a review

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Abstract

Presently, the majority of cells are cultivated by two-dimensional (2D) methods; however, latest and enhanced procedures employing three-dimensional (3D) cell culturing techniques provide strong indications that significantly more sophisticated studies can be carried out, providing invaluable insights. Recent years have seen a rapid development of 3D cell cultures since cells grown in a static environment on a flat substrate are far from reaching an *in vivo* condition. Currently, scientists are gradually realising that *in vitro* cell shape, structure, and physiological activities may be achieved. As a resolution, a three-dimensional matrix-like framework for cell attachment, proliferation, differentiation, and communication in both static and dynamic culture conditions is what three-dimensional cell carriers have gradually come to offer. Different mechanical stimulations that more closely resemble the genuine *in vivo* microenvironment could be the main function of 3D cell carriers in dynamic culture systems. Current developments in 3D dynamic cell culture techniques have been presented in this review, along with a discussion of their benefits and drawbacks when compared to conventional 2D cell growth under static settings.

1. Introduction

In order to understand the basic biophysical and biomolecular principles by which cells aggregate into tissues and organs, how these tissues work, and that function is disturbed in disease, cell culture has become an essential tool. Nowadays, tissue engineering, regenerative medicine, biomedical research, and industrial processes all make extensive use of cell culture. To learn more about the mechanisms behind cell behaviour *in vivo*, *in vitro* cell cultures are widely used. These behaviours, which are influenced by their biochemical and biomechanical surroundings, include mechanics, growth, migration, and differentiation of cells (**Huh *et al.*, 2011**). As the number of cell lines rises, so too are the technologies for cell culture, imaging, data collecting, and analysis growing in tandem. Cell cultures refer to the overall process of removing organs, tissues, or cells from an animal or plant, and place them in a synthetic setting that will allow them to survive and/or proliferate. There are two types of established cell lines: suspension and monolayer types. Cultured cells have a distinct doubling time that is governed by the length of the cell cycle and other physiological processes (**Ravi *et al.*, 2014**).

In 1907, Harrison conducted the first cell cultures as part of his investigation into the development of nerve fibres (**Harrison *et al.*, 1907**). Since then, the technique has been refined and applied to the study of cell division and growth outside the body (**Harrison, 1910**; **Scudiero *et al.*, 1988**). Fo experiments can be used primary cells that have been extracted straight from donor tissue or established cultures kept in cell banks. Primary cultures are created by isolating live things and typically comprise populations of various cell types found in the original tissue. It´s critical to isolate the right cell type in this situation (**Jakoby and Pasten, 1979**). Primary cell lines are characterised by two things: (a) challenges with isolation and (b) limited life span. However, they closely resemble the genetic characteristics of tumours *in vivo*, which allows for the execution of certain useful investigations. Using a proven cell line is an additional choice. Characterised models of different cancer cell lines are available from bioresource centres like the American Type Culture Collection (ATCC), which are frequently employed in research. In adherent conditions, the cells are adhered to a glass or plastic dish; in other cases, the cultures can be performed in a suspension, which more closely resembles the natural environment (**Ryan, 2003**). The 2D model is the most often utilised type of cell culture, although 3D culture is becoming more and more popular lately (**Pampaloni *et al.*, 2007**).

2. Comparison of 3D cell development and 2D cultures

In terms of cellular dynamics, nutrition, and cell-cell contact, 3D cell cultures diverge significantly from conventional 2D cultures (**Figure 1**) (**Edmondson *et al.*, 2014**). Because 3D culture methods can reasonably replicate the in vivo environment, they are becoming more and more common. Nonetheless, even when compared to in vivo systems, many epithelial systems may be accurately modelled in two dimensions. For instance, in air-liquid interface culture on two-dimensional surfaces, lung airway epithelia will grow normally (**Kesimer *et al.*, 2009**;

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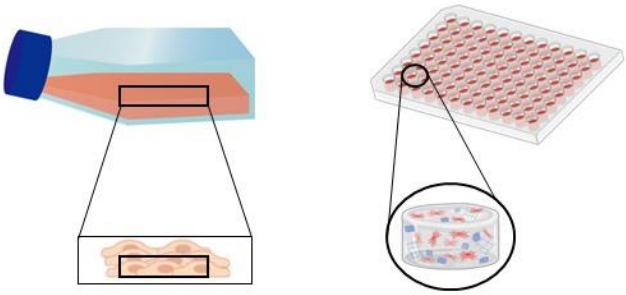
Nalayanda *et al.*, 2009). Nevertheless, because of their simplicity, 2D monolayer cell culture techniques might occasionally fall short of exhibiting the cell development process observed in the physiological environment in vivo. The reason for this discrepancy may be that these 2D systems lack a complex and biologically rich environment. In contrast to 2D planar cell cultures, both 2D and 3D cell culture methods have distinct effects on behaviours associated with in vivo-like cell development, including cell motility, apoptosis, differentiation, and proliferation (**Baker and Chen, 2012**; **Friedl *et al.*, 2012**; **Bonnier *et al.*, 2015**).

In the case of apoptosis, recent research has shown that when some breast cancer cell lines are exposed to cancer medications like paclitaxel, the development of dense spheroids can stop the cell lines from going through apoptosis (**Imamura *et al.*, 2015**). Within 2D cultivation, gradient of cells has no effect on nutrients access, unlike those in 3D cultures, since necrotic cells separate into the medium and only viable cells remain exposed on the culture surface. In aggregated spheroids, for example, the surface exhibits the highest levels of proliferation, whereas the core of the 3D cell bodies contains the biggest amount of quiescent or necrotic cells (**Edmondson *et al.*, 2014**). This shows that geometry-induced alterations in cell behaviour can have far-reaching effects on understanding physiological response, going beyond simple diffusion constraints.

The differences in cell migration between dimensionalities may be attributed to the intricate connections between cells in a three-dimensional substrate. 3D cells are encircled and adhered to one another on all sides, which obstructs migration and, as a result, modifies cell motility and the mechanisms cells employ (**Grinell, 2003**; **Yoshii *et al.*, 2011**; **Gjorevski *et al.*, 2015**).



2D cell cultures	3D cell cultures
- soluble gradients absent	Soluble gradients present
- forced apical-basal polarity	No prescribed polarity
- continuous layer of matrix	Discrete matrix fibrils
- unconstrained spreading and migration in x-y	Spreading and migration sterically hindered
- adhesions restricted to x-y plane	adhesions distributed in all 3 dimensions
- high substrate stiffness	Low substrate stiffness

Figure 1 Illustration showing the variations between 3D and 2D cell cultures (**Charwat and Egger, 2018**).

3. 3D culture models

Growing cells on a flat surface is known as a 2D cell culture, a technique that has been utilised for a long time in the life sciences (**Jensen and Teng, 2020**). Through the use of this technique, people can investigate the physiological and pathological functions of cells in vitro (**Duval *et al.*, 2017**). The physiological state and activity of cells in the 2D culture growth environment, however, are not entirely consistent with the cells in vivo, as scientists have gradually come to appreciate the idea of cell microenvironment (**Fatehullah et al., 2016**; **Riedl et al., 2017**; **Kapałczyńska et al., 2018**). Therefore, researchers have spent the last ten years developing a variety of 3D culture methods to give cells a culture environment that is more like an in vivo milieu (**Knight and Przyborski, 2014**).

According to the previous sections, 3D and dynamic cell culture can fill in the gaps between in vitro cell culture and animal models by offering a reproducible and regulated environment that replicates the conditions in vivo, as opposed to the conventional 2D/static culture. Various 3D cell culture techniques have been developed up to this point with the goal of simulating in vivo cell interactions in tissues and organs. The use of these systems has enabled more in-depth studies of biochemical and biomechanical signals (**Banerjee and Bhonde, 2006**). The study of complete animals, organotypic explant cultures (including embryos), cell spheroids, microcarrier cultures, and tissue-engineered models are the categories into which three-dimensional culture models can be divided (**Pampaloni *et al.*, 2007**). Microcarrier- and bioreactor-based systems make up the majority of the development of 3D dynamic cell culture systems, while microfluidic technologies have provided new insights into cell culture technology that may eventually eliminate the use of laboratory animal models.

3.1 Static Methods for 3D Cell Culture

In bioengineering, a well-designed three-dimensional culture system can help to promote several cell behaviours, including gene expression, differentiation, proliferation, and organisation of the cytoskeleton. The two categories of static 3D culture approaches—scaffold-free and scaffold-dependent (**Figure 2**)—will be covered in the ensuing sections.

3.1.1 Scaffold-Free 3D cell cultures

The term "scaffold-free 3D static cell culture systems" refers to techniques for growing spheroids of cells in a 3D static environment without the need for a scaffold for growth surface attachment. The spheroid culture methods, which primarily consist of the low adhesion surface modification approach and the hanging drop method, rely on a different methodology to collect the cells together, generating a spheroid-like cell aggregation (**Wu *et al.*, 2008**; **Tung *et al.*, 2010**). Nevertheless, single cell suspension cultures can be used to create 3D aggregate cultures. Scaffold-free 3D culture uses the cellular capacity for self-assembly to generate 3D cultures without the guidance of a supporting structure, keeping cells adherent close to each other or guiding them towards each other. Additionally, cells often release their own aggregates of extracellular matrix proteins to create their own milieu. This has a reciprocal effect on the fate and functionality of the cells as well as their subsequent extracellular matrix deposition (**Li *et al.*, 2014**).

Low adhesion technique for surface modification

In general, low adhesion surface modification uses a rather straightforward tactic to stop cells from adhering to the culture surface. As a result, cells would need to bind and produce three-dimensional spheroids on their own. Numerous methods for culture surface modification can be used to accomplish this. In the past, 96-well plates were modified with 0.5% poly-2-hydroxyethyl methacrylate (poly-HEMA) to prevent cells from adhering to the plate surface. This resulted in the formation of 3D cell spheres in a variety of malignant and non-cancerous cell lines. Likewise, the cell culture disc might be coated with 1.5% agarose (**Ivascu and Kubbies, 2006**; **Hampel *et al.*, 2018**). Additionally, the microchip approach was created to control the

size of the cell aggregation and maintain the cell spheres for a minimum of two weeks (**Tibbitt and Anseth, 2009**).

The hanging drop technique

Another culture technique is called droplets, where cells use the hanging drop technique to aggregate and produce tissues resembling spheres. This approach consists of placing droplets of the cell suspension on the culture dish lid. Subsequently, the cover is cautiously turned upside down and positioned over the culture dish, which holds a culture medium to maintain a growth-promoting environment for the cells. Suspended cells will assemble into a miniature three-dimensional aggregate at the top of droplets. It keeps cells in their proper phenotype and permits long-term cell survival (**Keller, 1995**; **Banerjee and Bhonde, 2006**).

3.1.2 3D Static Cell Culture Dependent on Scaffold

Another 3D static cell culture is scaffold-dependent method, which is created as environment similar to the extracellular matrix. The scaffold-dependent culture system provides a 3D scaffold with surfaces for cell attachment and growth, in contrast to the automatically produced 3D cell aggregation previously described. Furthermore, effective interchange of waste, nutrients, and oxygen between the inside and outside of the culture environment may be made possible by the scaffold's porosity (**Antoni et al., 2015**; **Ravi *et al.*, 2015**). Researchers create solid scaffolds, which are primarily divided into four categories as explained below, using a variety of biological materials, including polymers, bioceramics, and bimetals, such as fibrin, bioactive glasses, and titanium (**Dolder *et al.*, 2003**).

Natural scaffolds derived from the extracellular matrix

Natural-derived extracellular matrix mjay create favourable conditions for human tissue cell development. Thus, native extracellular matrix (ECM) proteins are regarded as an appropriate biomaterial that may stimulate advantageous cellular behaviours. To protect the underlying cellular environment, an early commercial wound healing treatment, for instance, used synthetic mesh conjugated with porcine collagen to provide a temporary barrier between the wound bed and the air (**Smith, 1995**). Tissue regeneration can be accelerated by biopolymers like collagen and fibronectins that are taken from animal extracellular matrix (ECM) because they share biochemical components with real tissues and organs. The highly open porosity features of the biopolymer-based scaffold enable cell seeding while preserving the microstructure and preventing cell flattening. Additionally, the scaffold permits cells to attach and lengthen, enabling guided culture and cell alignment (**Fischbach *et al.*, 2007**).

Hydrogels scaffolds

Both natural and synthetic raw materials can be used to create hydrogels scaffolds, including as cellulose, alginate, hyaluronic acid, collagen, and polyethylene glycol. Hydrogels can also be compounded or assembled using these natural raw ingredients. Natural materials are mostly sourced from animals, which means they offer exceptional cell adhesion, hydrophilicity, biocompatibility, and bioactivity (**Tibbith and Anseth, 2009**). Higher in vivo relevance can result from the hydrogel's ability to give the cells a 3D and possibly dynamic environment. But there are some restrictions. In a hydrogel environment, for instance, it is feasible to co-cultivate different cell types, but harvesting and analysing each one separately is typically not feasible. Even though hydrogels have outstanding mechanical qualities and can stretch in three dimensions, most of them contain cross-linking agents that might induce DNA or cell mutation or death (**Nicodemus and Bryant, 2008**).

Scaffolds made of synthetic polymers

Scaffolds are commonly made using synthetic polymers such as polycaprolactone (PCL), polylactic acid (PLA), and polyurethane as basic materials. The mechanical properties of synthetic polymer scaffolds are significantly stronger than those of natural and hydrogel scaffolds (**Caetano *et al.*, 2015**; **Rodrigues *et al.*, 2016**). In order to accelerate the healing of injuries and offer mechanical support while awaiting the integration of newly produced tissue and cells with native tissue, synthetic polymer scaffolds have been extensively employed in tissue engineering. The physio-chemical characteristics of synthetic polymers are detrimental because they are unsuitable for cell adhesion and growth (**Milovac *et al.*, 2014**).

Ceramic and metal scaffolds

In addition to polymers, titanium, magnesium, and tricalcium phosphate are among the metals and ceramics that are commonly utilised as raw materials to make scaffolds. In contrast to polymers, they could offer more mechanical strength for a specific application, like bone replacements. Bone regrowth is supported by ceramics. In particular, tricalcium phosphate can imitate the composition and structure of bone, and it has been discovered that the related ceramics 3D scaffold helps bone tissue engineering by promoting the mineralization of bone (**Thimm *et al.*, 2013**; **Surmeneva *et al.*, 2017**; **Ghassemi *et al.*, 2018**).

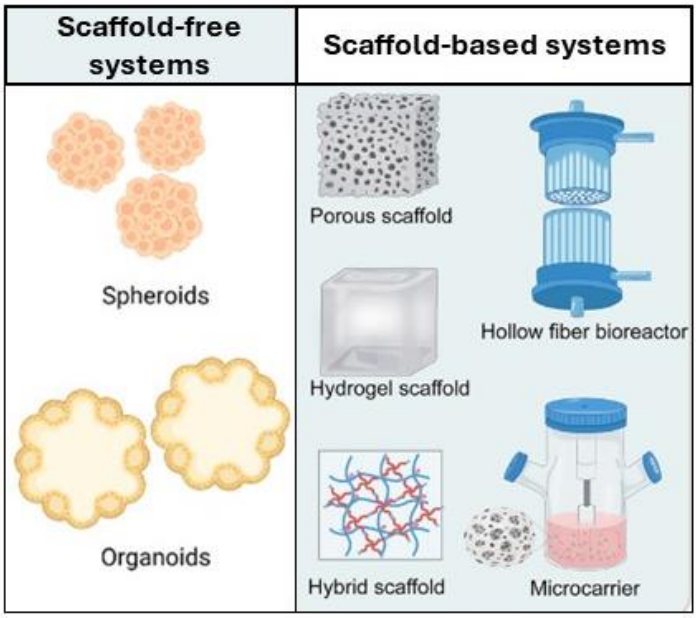


Figure 2 Types of Scaffold-free systems and Scaffold-based systems (**Chen *et al.*, 2024; de Dios Figueroa *et al.*, 2021**)

3.1.3 Current Applications and Systems for 3D Dynamic Cell Culture

Microcarrier-Based Culture System and Bioreactors

In order to cultivate cells in a three-dimensional dynamic environment, researchers have lately employed bioreactor-based devices including rotating wall bioreactors and spinning flasks (**Yeatts *et al.*, 2013**; **Tsimbouri *et al.*, 2017**). By creating

a dynamic environment, these devices hope to improve the circulation of nutrients, provide a uniform oxygen gradient, and provide a minimal amount of shear force to the cells. Rotating wall bioreactors rotate and circulate the medium in a circulatory fashion, while spinning flask bioreactors accomplish this by producing medium movement horizontally using a stirring bar in the centre. Numerous indicators can be examined using bioreactor systems, such as the gas content of cells, which can be used for a number of tasks such as measuring the oxygen content, transmission of biosignals, and differentiation of vascular branching (**Al-Quodah et al., 2017**). One of the most significant advantages of bioreactors is widely thought to be the enhancement of cell differentiation and proliferation among these uses. There are hundreds of distinct-designed microcarriers available nowadays. These microcarriers fall into many categories according to their form and substance. Due to their high repeatability and good biocompatibility, natural polymers (such as crosslinked dextran, collagen, gelatin, and cellulose) or synthetic polymers (such as polystyrene, polyacrylamide, and poly hydroxyethyl methacrylate) are used to make the majority of microcarriers (**Li *et al.*, 2015**; **Tavassoli *et al.*, 2018**). The microcarriers can be divided into two groups based on their morphology: (1) solid with a smooth surface, and (2) porous structure (**Tavassoli *et al.*, 2018**). The microcarrier's primary benefit is its cost-effectiveness in large-scale cell culturing. The creation of inactivated or live attenuated virus vaccines using the Vero cell line, which has been widely utilised to produce numerous vaccines, is a well-known example of microcarriers in large-scale production (**Kiesslich and Kamen, 2020**).

Organ-on-a-Chip Microfluidic Cell Culture System

The microfluidic cell culture system is a collection of apparatuses designed to give cells a dynamic culture environment by creating fluidic shear stress (**Gao, 2022**). In recent years, the idea of an organ-on-a-chip has been created, based on the microfluidic technology. It defines a combination system designed to resemble the form and function of an organ, with several cell types connected via microfluidics devices. The organ-on-a-chip carrier could comprise the hydrogel and transwell membrane that were previously stated, along with a particularly shaped culture chamber and low-binding chambers for organoid cultivation. Glass, silicon, and thermoplastics are common materials used in the fabrication of the organ-on-a-chip (**Low *et al.*, 2020**). Furthermore, the majority of microfluidic devices can regulate the mechanical force, making organ-on-chips an effective tool for examining how the dynamic environment—specifically, shear stress—affects cell behaviours. An intestine-on-a-chip apparatus, for instance, showed notable variations in intestinal epithelial function in response to varying fluid shear stress levels, including the development of tight junctions and mucus formation (**Delon *et al.*, 2019**). The organ-on-a-chip system has significant drawbacks in addition to its advantages over traditional cell culture apparatuses. Firstly, the establishment of an organ-on-a-chip system is a challenging procedure that involves several stages of fabrication, assembly, and cell seeding. Each step's deviations could make the process less repeatable. Secondly, microfluidic devices often have a modest number of cells, which may not be applicable to large assays like proteomics. Third, there isn't enough information to say that an organ-on-a-chip is functionally superior to other systems—that is, if it can predict in vivo medication efficacy more accurately—despite the fact that it resembles several aspects of in vivo organs, such as cell type and arrangement (**Huang *et al.*, 2022**).

4. Future remarks and conclusion

The need for 3D cell culture technologies is growing as a result of recent advancements in the biological sciences. 2D cell culture technique cannot match the benefits of the 3D cell culture system created by encasing cells in hydrogels. However, both 2D and 3D cell culture techniques provide methods which are necessary for advancing research. Effective coordination between diverse technologies is also necessary for the widespread deployment of 3D cell culture technology. The first is material science, where 3D cell culture technology is developed on the foundation of producing superior hydrogel culture media. Second, the *in vivo* simulation of tissues and organs opens the door to the possibility of 3D cell culture when paired with materials science and biological manufacturing technology. Among the various production techniques, bioprinting offers special benefits. Lastly, a key strategy for developing organs on a chip is the advancement of microfluidic technology, which combines detection and culture.

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Declaration of interest

The authors report no conflicts of interest.

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