Drug Delivery and Translational Research (2023) 13:2239–2253
https://doi.org/10.1007/s13346-023-01327-6

REVIEW ARTICLE

Role of three-dimensional cell culture in therapeutics and diagnostics: an updated review

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Accepted: 6 March 2023 / Published online: 27 March 2023

Abstract

Drug development and testing are a tedious and expensive process with a high degree of uncertainty in the clinical success and preclinical validation of manufactured therapeutic agents. Currently, to understand the drug action, disease mechanism, and drug testing, most therapeutic drug manufacturers use 2D cell culture models to validate the drug action. However, there are many uncertainties and limitations with the conventional use of 2D (monolayer) cell culture models for drug testing that are primarily attributed due to poor mimicking of cellular mechanisms, disturbance in environmental interaction, and changes in structural morphology. To overcome such odds and difficulties in the preclinical validation of therapeutic medications, newer in vivo drug testing cell culture models with higher screening efficiencies are required. One such promising and advanced cell culture model reported recently is the "three-dimensional cell culture model." The 3D cell culture models are reported to show evident benefits over conventional 2D cell models. This review article outlines and describes the current advancement in cell culture models, their types, significance in high-throughput screening, limitations, applications in drug toxicity screening, and preclinical testing methodologies to predict in vivo efficacy.

Keywords Three-dimensional cell culture models · Drug testing · Preclinical validation · Drug toxicity screening · High-throughput screening · 2D cell culture

Introduction

Developing a drug is bringing a novel drug molecule into clinical use. It is a tedious process starting from basic research to finding the active compound that targets a particular molecule to its commercial launch in the country. The cost of processing the development of the drug till lead discovery and optimization is also high [1]. An important step in screening the new drug is the high-throughput screening (HTS) of the potential drug molecule, which is currently based on the 2D cell culture technique on a flat plastic surface. Unfortunately, this 2D cell culture technique has its disadvantages such as it does not represent the exact physiological conditions, its lack of certainty, high cost for maintenance and rate of failure, and issues caused by the media used for culture and growth of the cells. Researchers are now focusing on overcoming these limitations by using a 3D culture technique that can closely replicate the in vivo conditions [2]. This technique is now focused on as it gives accuracy in drug discovery and development. The importance of this technology to understanding the tissue microenvironment was proposed as early as the 1980s by Bissell, a research scholar at Lawrence Berkeley National Laboratory [3]. But recently, this technique is more focused on 3D culture as it represents the three-dimensional physiological condition and its application in drug discovery is making a rapid process.

The cell-based assay is very crucial in the field of drug discovery when compared to cost-intensive animal models and their related ethical issues. It can provide a well-established model which can mimic cellular interactions. It overcomes the ethical issues raised by using animal models used for drug testing. 2D cell cultures are the most understood technique and have a well-established protocol. All the drugs will undergo an extensive screening process before their launch of the drug in the market. Therefore, this 2D culture platform has rendered an efficient way to screen

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potential drug compounds. However, it has its limitations such as changes in the morphology and genotype of the cells from that of the parent cell and loss of interaction between the cellular and extracellular environment which are discussed in detail below.

Recently, the technique is being replaced with a three-dimensional cell culture model. The primary benefit of using a 3D culture over a 2D culture is the production of a more relevant cell model that resembles the 3D physiological environment. This model has high structural complexity and the ability to retain a steady state. The integration of the flow of fluids, that is, body fluids such as blood, plasma, and urine, is well maintained [4]. It can provide a well-established model which can mimic cellular interactions. It overcomes the ethical issues raised by using animal models used for drug testing [5]. Overall, this technique provides a much better simulation of cells in a more realistic way to grow and understand the physiological process and the response of a cell. Although this technique outweighs the limitations of a 2D cell culture, it has its limitations which are discussed below.

Different types of preclinical cell culture models

In 1906, Harrison carried out a research where he used the first cell culture using nerve fibers [6]. Since then, various researches have been conducted to understand the physiological, pathological, and molecular response of cells to various external stimuli, drugs, etc. and its interactions between other cells and the external environment. Cell culture now has an established protocol and is the most understood technique for drug testing. Therefore, all drugs that come into clinical use will undergo high-end screening of their potential active molecule for which 2D cell cultures are routinely used to screen its efficacy before proceeding to human clinical trials. The decision on whether to move the drug to the next phase of the study is made with the results of these initial screening processes with cell culture. It is known that only approximately 10% of these drugs make it to clinical trials. The most researched and understood cell culture model is the two-dimensional cell culture model. However, it has its limitations such as changes in the morphology and genotype of the cells from that of the parent cell and loss of interaction between the cellular and extracellular environment [2]. Therefore, this limitation has led to the discovery of three-dimensional cell culture models which can closely mimic the physiological cells which are in a three-dimensional structure. This method has optimized the understanding of the physiological response of the cells during drug testing. The most frequently used cell culture techniques are discussed below.

Two-dimensional cell culture

2D culture is a technique to grow cells as an adherent monolayer on a horizontal surface either in a cell culture flask or Petri dishes. It has been used for decades because of its ease of use and low-cost maintenance. The duration for the culture to form takes minutes to a few hours. It has high performance and can produce long-term cultures, and it is simple and easy to infer. This type of culture will have unrestricted access to oxygen, nutrients and metabolites, and other signaling molecules which are in divergence with that of the in vivo conditions [2]. The maintenance expenditure of a 2D cell culture is relatively low when compared to 3D culture, and the medium is also commercially readily available. Unfortunately, most studies and understood techniques also have their disadvantages. The first and foremost is that it does not imitate the natural physiological structure of the tissues or cells. In tumor cells, the cell–cell interaction and cell-external environment communications are not well represented and subsequently, it loses their polarity. After initiating the tissue in a cell culture flask, the cells are prone to changes in morphology and the division of the cells eventually affecting its function, structural organization, secretion, and cell signaling. Another major limitation of this technique is the unrestricted access to oxygen, nutrients, and metabolites which is in dissimilarity with the natural environment of the cells which is subjected to variation of these factors [2]. In addition to this, the cancer 2D cell monoculture will only allow one type of cells to grow which can result in the lack of tumor microenvironment or niches. Therefore, owing to these disadvantages, there is a need to overcome them by finding alternative models which can impersonate the natural physiological state of the cells. This gave rise to the 3D cell culture system. Figure 1 describes the 2D and 3D cell culture models.

Three-dimensional cell culture

In 1970, Hamburg and Salmon carried out the first three-dimensional cell culture on soft agar gel which showed the potential of 3D culture to mimic the morphological nature and the behavior of the cells in in vivo conditions. On the basis of the method opted for preparation, 3D models can be classified into three categories as follows: (i) suspension cultures on non-adherent plates, (ii) cultures in concentrated medium or gel-like structures, and (iii) cultures in scaffolds [8]. Here, the cells harvested from the donor tissue can be cultured in a multicellular 3D structure that can imitate the parent cell more accurately than the traditional 2D cell culture. These 3D models are capable of

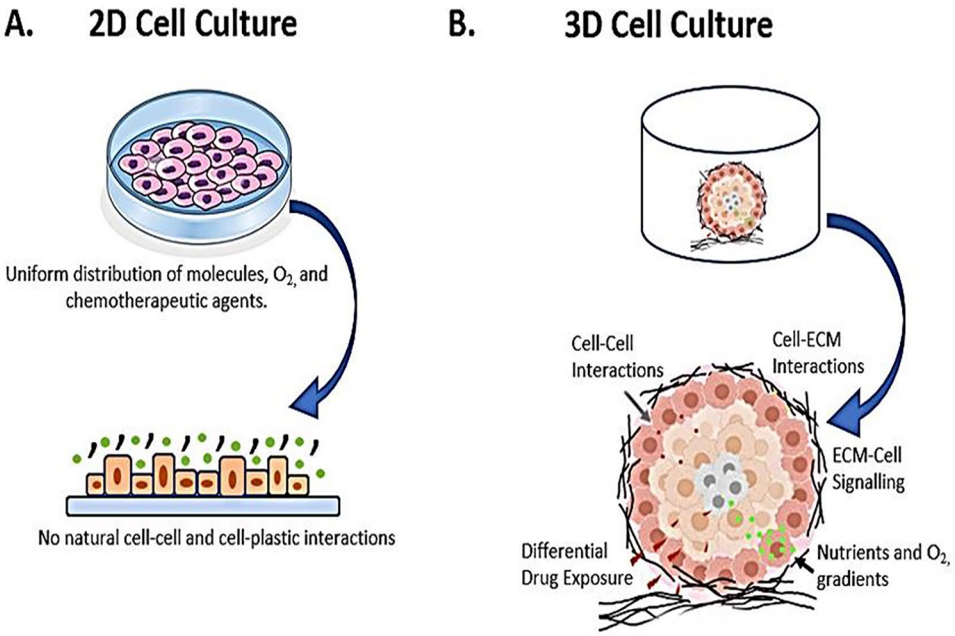

Fig. 1 2D (**A**) vs 3D cell culture (**B**) (modified from Salina-Vera et al. 2022 [7])

cell-to-cell interactions and also with the external environment similar to that of the in vivo conditions. Moreover, the morphology of the cells is well maintained here; the polarity is also retained. Factors such as oxygen supply and nutrients can be determined for a given experimental procedure but data are being optimized for the production rate and consumption of growth factors, cytokines, and other effector molecules [8]. The proliferation of the cells in the culture vessel is dependent on the location of the cell, and it is now found that it is higher in the peripheral part of the 3D structure. The time of culture formation in 3D culture ranges from a few hours to a few days. In this type of culture, expression of the genes, splicing, and topology as well as biochemistry is also well maintained. There are different types of 3D cell culture techniques, some of which are discussed below.

Spheroids

A type of 3D culture was initially developed in the 1970s to understand the phenotype of tumors and their response to chemotherapy [9]. Since then, spheroid cultures are extensively used to harvest many cell types including neuronal cells, hepatocytes, and even stem cells. Spheroids have the competence to develop gradients of oxygen, nutrients, metabolites, and soluble signals eventually producing a heterogeneous cell population. It also has a defined geometry possessing a cell-to-cell and cell-to-ECM interaction. Spheroids are formed because of integrin—a type of membrane protein. The spheroid formation can take place due to the following factors: (i) dispersion of cell aggregates due to a long chain of ECM fiber consists of RGD motifs that can allow the binding of the integrin leading to the upregulation of cadherins; (ii) cadherin aggregates on the surface of the cell; and (iii) the hemophilic cadherin-to-cadherin binding takes place between nearby cells allowing it to strengthen the connection between cells and spheroids. There are four different approaches to enable this type of culture: the first one is by using low-adhesion plates for the self-aggregation of cells into spheroids. This technique used agarose hydrogel which does not interfere with the ECM. The cell–cell interactions are higher when using hydrogels because of their defined shape. Therefore, it is versatile and can control microtissue production [10]. The second one is to use hanging drop plates to drive the spheroid formation. It is a type of scaffold-free culture technique and has certain limitations such as low throughput. The cells are suspended onto plates with wells, which are then turned upside down such that it becomes a hanging drop model which is held due to the surface tension [10]. The third approach is to use a bioreactor to promote the cells to self-aggregate into the formation of spheroids. This is due to which the cells cannot aggregate on the vessel; instead, they will start aggregating and assembling. This method has certain disadvantages such as mechanical damage to the cells, spheroid structure variation, and longevity of the cells. Therefore, this method is generally not preferred. The fourth one is by using micro-/nanopatterned surfaces as the scaffolds to promote spheroid formation. For example, nanofibers are shown to increase spheroid formation and their tendency to not aggregate prevents cell death. When there is an absence of nanofibers, the cell will interact because of the presence of cadherins. Cell binding is done by adding proteins such as fibronectin which will facilitate the binding of the cells onto these nanofibers

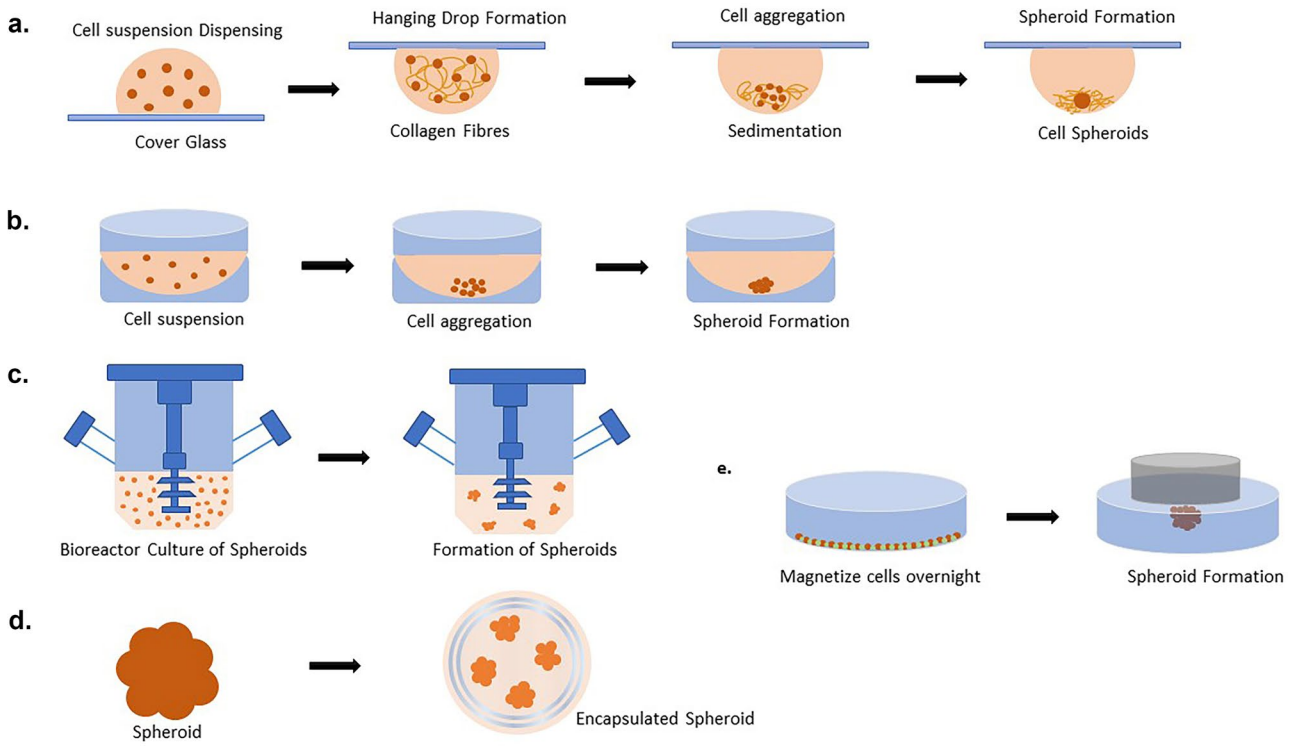

Fig. 2 Approaches in the formation of a multicellular spheroid. **a** Hanging drop method. **b** Liquid overlays. **c** Bioreactor system. **d** Microencapsulation. **e** Magnetic levitation

which leads to the aggregation of cells on nanofibers [10, 11]. The different approaches involved in the multicellular spheroid formation are explained in Fig. 2.

Spheroids are generally developed based on the anchorage-independent methodology. In spheroid formations, the cells in the culture aggregate to form spheroids instead of adhering to the plate as in 2D culture [12]. The most commonly employed method for spheroid 3D culture is the hanging drop method and liquid overlay. Other methods include the agitation/rotator system, microencapsulation, and magnetic levitation. The methods for the development of these spheroid 3D cultures are briefly discussed below.

(i) Hanging drop method: this method does not require any special equipment and can be modified based on the cell type used for culturing. The spheroids will be formed within 24 h of culture and may differ with the type of cells used [13]. In a study, the hanging drop method was employed by using collagen-coated plates to establish the 3D culture of hepatocytes. This study reported the first-time use of the hanging drop method for the formation of 3D hepatocytes and showed that this technique can be used for routine screening for toxicity studies by easily obtaining tissues from the slaughterhouse [14]. Other studies showed breast cancer tissue derived from patients to develop spheroid culture. They successfully developed a 3D model using patients' tissue and confirmed the recapitulation of the tissue's original histopathology by techniques such as immunohistochemistry. Moreover, a standard chemotherapeutic drug panel was also tested on these cell cultures and showed its tremendous efficacy and proved the variation in different cancer cells [15]. A recent study also showed the efficacy of this technique which produced pluripotent stem cells which had uniform size and shape and also increased gene expression and functionality [16].

(ii) Liquid overlays: it is another common and effective method for the development of the 3D spheroid culture. The cells are grown to form spheroids by using suspension culture which prevents cell attachment which is also known as liquid overlay culture. It can be grown on the bottom of the normal cell culture vessel which is then covered with a thin layer of agarose. This method has proved to facilitate the development of many types of spheroids [17]. It was found in studies that these types of spheroids show variations in cancer cells such as growth rate, morphology, and thickness of the cell wall which correlates with the characteristics of those cells in in vivo conditions. This technique's major limitation can be resolved by using 96-well plates where single spheroid cells can be obtained [18].

(iii) Bioreactor system: this type of cell culture can be employed to produce the mass quantity of cells where the spheroids produced are heterogenous in size, shape, and cell population. The major limitation of this type of culture is the requirement of large instruments and trained professionals to operate and a large quantity of culture media required [18]. This type of culture is in continuous motion and hence prevents the cells from attaching to the surface of the culture vessel.

(iv) Microencapsulation: this method of cell culture has proven to be more suitable for cell growth with precise control of the cell shape and size. This method can be used to manipulate different types of cells with diverse physical and chemical characteristics for the development of microcapsule core shell. Recent research explored the cell culture by alginate encapsulation which employed the co-culture of tumor cell spheroids of non-small cell lung carcinoma, fibroblasts, and monocytes and showed that the 3D culture reconstructs an invasive and immunosuppressive tumor microenvironment along with the build-up of cytokines/chemokines and other ECM elements, and matrix metalloproteinase was shown to promote cell migration and cell to cell interactions within this alginate microcapsule [19]. This method is not commonly employed because of its disadvantages such as reduced oxygen and nutrient supply and the contact between the cells due to its isolation inside the core shell.

(v) Magnetic levitation: this system involves mixing the cells with magnetic particles and exposing them to a magnetic force during the culturing process. In 2010, Souza et al. first developed this model to develop a hydrogel-based 3D culture model using gold magnetic iron nanoparticles. These magnetically levitated human glioblastoma cells showed similar protein expression to that of those observed in vivo tumors. Their results indicated that a magnetically levitated 3D model can closely recapitulate in vivo protein expression [20]. It is found that magnetic levitation by negative magnetophoresis is suitable for long-term cell culture [21].

Organoids

Organoids originate from a self-renewing tissue that can self-organize in in vitro conditions imitating the in vivo–like organ complexity. Organoids can recapitulate development and tissue organization and resemble organs in the body. They are generally developed from tissues such as induced pluripotent stem cells, embryonic stem cells, and even tissue-specific stem cells. This has insight into the human model that can replicate the natural physiological state of the cell. This technique of 3D culture has various applications in studying human biology such as studying the morphological events of human cells from development to organ formation and studying the mechanism of diseases which qualifies it as a valuable tool for drug discovery and development and in preclinical studies, to study the variability of cells among different individuals and understanding its phenotype, and other applications in the field of tissue engineering as well as regenerative medicine [22]. Organoids can be developed in different ways either by developing an organ or regenerating them. Most of them are generated from adult tissue stem cells which are then cultured in a matrix supplemented with growth factors similar to that of the stem cell niche. Another method by which organoids could be developed is the pluripotent stem cells/induced pluripotent stem cells and also the embryonic stem cells. This type of cell is cultured by using a series of growth factors and media that can induce organ development that mimics its normal development. The final amalgamation of organ-specific cell types will have the ability to arrange itself in a way similar to that found in the original organ [22]. The schematic representation of steps followed during organoid 3D culture development is given in Fig. 3.

The new developments in organoid culture techniques have made this technique advance its molecular and physical similarities to its tissue origin. Some of the bioengineered approaches are briefly discussed below:

(i) Bioreactor system: the continuous growth and supply of nutrients and oxygen are the major limitation in the culturing of organoids which can be overcome by the use of stirred tank bioreactors. In this technique, the organoids can be supplied with improved aeration and nutrient uptake. The culturing of brain organoids using bioreactors showed improved reproductivity, lesser culture volume, and recapitulate dynamic features of the brain at the molecular level and can be grown in large quantities despite the lack of vasculature [24]. Another study showed that bioreactors can be a promising tool to culture retinal cells for modeling and drug testing. Their results showed improved stratification of retinal cells and increased yield of photoreceptor cells [25]. In 2018, Przepiorski et al. developed a simple and cost-effective method to generate renal organoids using human induced pluripotent stem cells (iPSC) to differentiate into kidney organoids [26]. A novel 3D-printed bioreactor was developed when a 12-well cell culture plate was turned into a miniature stirred flask bioreactor. Other miniature bioreactors are invented to produce large human brain organoids using calcium alginate hollow fiber [27].

(ii) Air liquid interface: a recent research showed that co-culturing of primary tumor epithelia with lymphocytes with an air–liquid interface using patients'

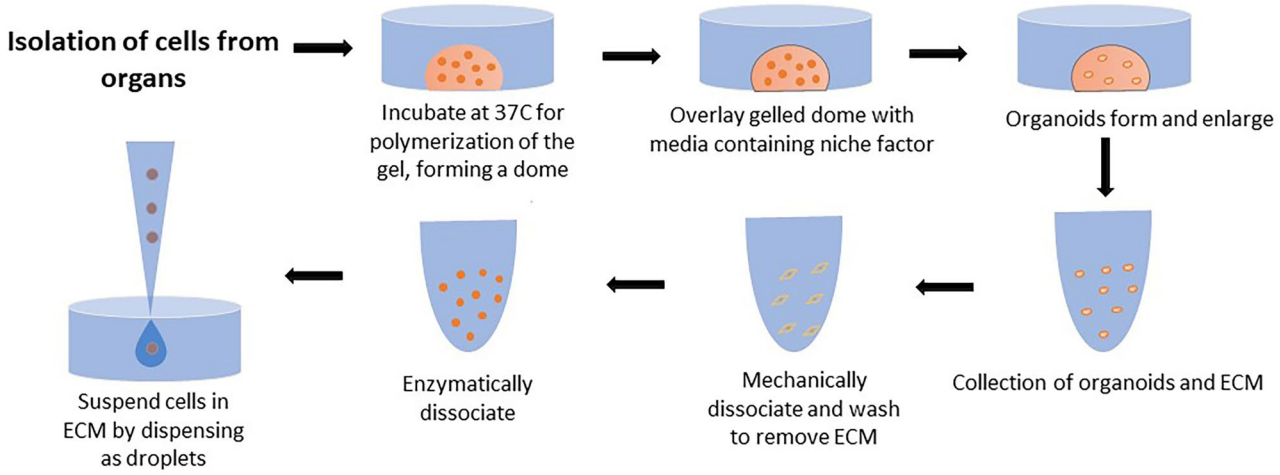

Fig. 3 Workflow involved in the production of an organoid 3D culture modified from ATCC organoid culture guide [23]

biopsies accurately preserved the original tumor's T-receptor cells [28]. This technique is also applied to the 3D culturing of other organs. The advantage of this method is that it can incorporate the epithelial and the stromal cells thereby accurately recapitulating the stem cell niche in the culture system. Therefore, this technique is used to understand the in vitro interaction of the tumor cells and the immune cells [29]. This method is also known to maintain the phenotype and the genetic makeup of the patient's tissue allowing precision in the drug response. Its main advantage is that it requires only a small amount of samples to start the culture and it can be maintained for a very long time [30]. In 2019, research was done on developing brain organoids using an air–liquid interface which showed improved neural survival and axon outgrowth and their results showed tremendous self-organization of the callosal tract [31]. One of the challenges in maintaining organoids is the supply of nutrients and the removal of waste from the media. This can be overcome by the use of the perfusion culture system which was developed by Sekiya et al., in 2019. This method accelerated the organization of the renal epithelia and the diffusion rate was also increased. This was achieved by the use of perfusion culture systems by air–liquid interface along with the fabrication using 3D bioprinting technology [32].

(iii) Vascularization: the major limitation of using cell culture is the lack of vasculature which is discussed in detail below. In culturing organoids, a lack of vasculature might lead to the development of immature organoids and their premature differentiation. Various techniques were developed to vascularize these organoids such as sacrificial molding and laser ablations [33]. This technique can help these organoids to build a microvascular structure. Another research used a co-culturing technique where the organ of interest is co-cultured with vascular endothelial cells [34]. Various techniques have been employed to improve vasculature in in vitro models such as bioprinting, where the cells of interest are imparted in hydrogels and deposited layer by layer; approaches based on photopolymerization using stereolithography enabled formation of the endothelial network and anastomosis of immunodeficiency mouse, a sacrificial network which focuses upon removal leaving the perfusable channels to be seeded with endothelial cells, inducing angiogenesis in engineered tissues, etc. [35]. A study used embryonic stem cells to express the ETS variant 2 protein that plays a role in the development of vascular endothelial cells which resulted in the increased expression, maturation of organoids, and acquired blood–brain barrier [36].

(iv) CRISPR-Cas 9 editing: researchers are at the very initial stage of the development of 3D organoids using CRISPR-Cas 9 technology to knock out the genes from the organoids to understand the role of the gene in disease pathology. This technique is also used to introduce a mutation into organoids such as KRAS and p53. [37]. This technology has found its application in various fields of biotechnology and is currently widely investigated for its potential to be used in 3D cell culture technology to study the cells in real time in vitro for disease modeling, drug testing, targeted therapy, and many more. CRISPR/Cas 9 can be employed in 3D culture in many ways such as modifying the genome of the cells before encapsulating it in the matrix and introducing Cas9-sgRNA complex in the target organoid [38]. In 2021, a study showed that genetically engineering an organoid

using CRISPR/Cas 9 technology reveals the essential host factors for coronavirus and demonstrated which receptors may or may not play a role in the entry and the pathogenesis of the virus, thus, rendering its value in the field of disease pathology [39]. Roper and Yilmaz demonstrated that the CRISPR/Cas 9 technology can be used to treat genetic diseases using organoids that are derived from patients. Although there are limitations of using gene editing technology in human cells, it can treat various incurable genetic disorders that can be patient-specific [40].

Scaffolds

Scaffolds are biopolymers used in the 3D cell culture technique that are arranged to imitate the physiological ECM. Figure 4, describes the different types of scaffolds used for the 3D culture model. 3D culture is used as a starting material for developing artificial organs, producing cellular products in huge quantities, developing lab-grown meat, etc. Biological scaffolds use naturally derived substances such as chitosan, hyaluronic acid, and collagen, whereas polymeric scaffolds include hydrogels such as polyethylene glycol (PEG), polyvinyl alcohol, and poly 2-hydroxyethyl methacrylate [41]. Types of 3D scaffolds include the (i) nanofiber which is cultured in culture vessels and can recapitulate the 3D architecture in an in vivo state. In this method, the cells can be removed easily from nanofibers and can be used for further downstream analysis. (ii) Collagen scaffolds have a porous architecture and have unique columns present in these pores that facilitate the movement of cells and nutrients in and out in all directions. This provides an enhanced surface area that can facilitate better cell attachment, growth, and migration. (iii) Polystyrene and polycaprolactone scaffold is a porous scaffold and can promote the growth of cells, and it is optically clear for imaging by microscopy. PCL is mostly used in the fabrication of surgical implants because of its biodegradable properties and is also used in tissue engineering applications. (iv) Cell-grown culture inserts are grown on well plates such as 6, 12, 24, 28, and 96. They are inserted in a stable position inside the wells which aids during the exchange of media by pipetting between the well and the insert.

Hydrogel scaffolds Hydrogens are a hydrophilic polymer that is cross-linked to form a 3D structure and has a structural similarity to that of the natural ECM. It possesses excellent water retention, biocompatibility, self-healing properties, etc., but it has low structural stability. Chemically cross-linked hydrogels have better structural stability than physical cross-linking both in in vitro and in vivo conditions [43]. These hydrogels have proven their usefulness in cell culture applications. In a study, a high-throughput screening platform–based hydrogel system was used to screen the chemotherapeutic drugs which showed that when cells are cultured in the stiff collagen-rich substrate, they showed resistance to those drugs compared to when used in softer substrates [44]. There are other materials other than collagen and Matrigel that are routinely used in hydrogel systems such as fibrin

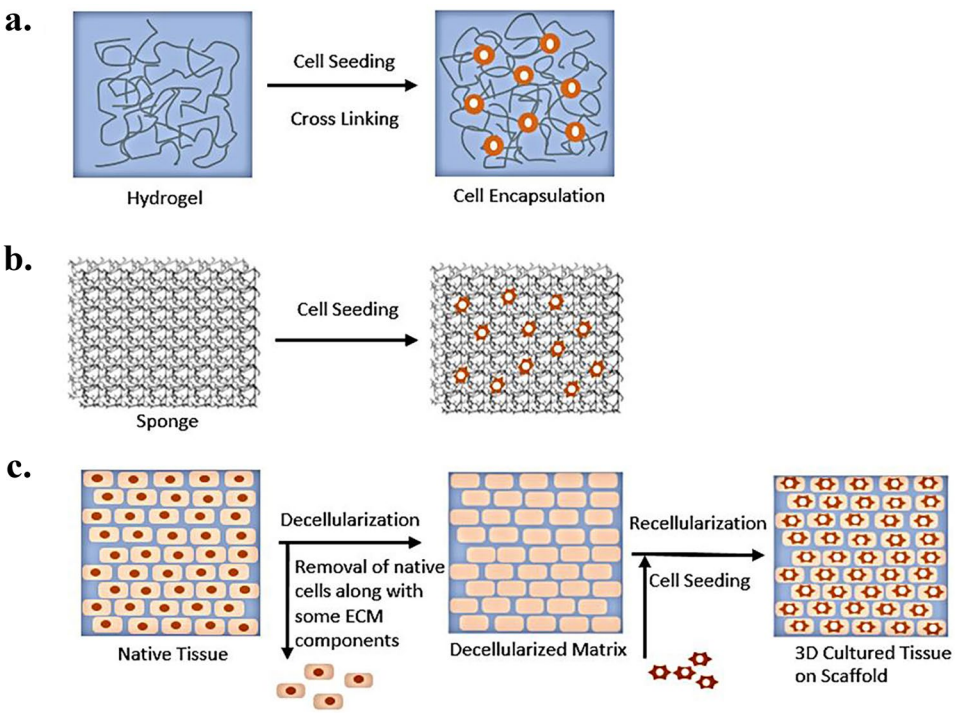

Fig. 4 The different types of scaffolds used for 3D culture. **a** Hydrogel. **b** Solid scaffold. **c** Decellularized native tissue (modified from Park et al. [42])

a natural polymer, alginate a polysaccharide obtained from brown algae, polyacrylamide which is produced by reacting acrylamide monomer and bisacrylamide in the presence of ammonium persulfate and tetramethyl ethylenediamine, polyethylene glycol a synthetic polymer, hyaluronic acid a non-sulfated glycosaminoglycan composed of a repeating disaccharide unit of glucuronate and N-acetylglucosamine, and polypeptides [45]. Hydrogels can be manipulated to investigate the diverse range of cell properties such as the migration rate of cells in microfluidic hydrogels, cell-to-material interaction, and high-throughput screening. Hydrogels possess dynamic properties that can precisely recreate the complex structural and mechanical environments found in natural tissues and have immense application as cell culture substrates. The different types of materials used for the fabrication of scaffolds are briefed below:

(i) Natural polymer material: natural polymer materials are generally used for engineering biomedical and pharmaceutical products as they exhibit excellent properties such as biocompatibility and degradability. Natural polymers have many advantages over synthetic polymers making them suitable to act as a substitute for ECM. The main advantage of using a natural polymer system is that during enzymatic degradation, the byproducts do not cause any toxic effects on humans but the high degradation rate of natural polymers is difficult to control [46]. It is used for various biomedical applications such as wound healing, soft tissue repair, and blood vessel grafting and possesses its importance in the field of tissue engineering and regenerative medicine. The most studied and used natural polymers include alginate, fibrinogen, collagen, and other polysaccharide and proteins which include chitosan and hyaluronic acid. Various methods are employed in the fabrication of such natural polymers to be used as scaffolds such as electrospinning, freeze-drying, and 3D printing [47].

(ii) Synthetic material: although natural polymers have certain advantages, they cannot fulfill all the requirements to fabricate scaffolds for wound healing applications. Synthetic materials such as PLGA and PCL have been extensively studied and tuned to be used for engineering scaffolds for tissue engineering applications [48]. A 2020 study by Li et al. reported that a silica-based nanocomposite hydrogel scaffold can significantly enhance the healing properties of diabetic wounds by promoting angiogenesis [49]. Ceramics is also extensively studied for the repair of hard tissue since it is found to regulate cell proliferation, migration, and growth factors which ultimately promoted tissue repair and regeneration [50]. This type of scaffold's major disadvantage is the stimulation of immune reaction to the material as a foreign body that is regulated by cytokines, matrix metalloproteinases, and chemokines [51].

(iii) Natural-synthetic hybrid material: both natural and synthetic scaffolds have their advantages and disadvantages. Thereby, this technology is developed to use the advantages of both types of scaffolds to produce desirable scaffolds which have found greater application in wound healing and also in the field of tissue engineering and regenerative medicine. Materials such as chitosan-PLGA are chemically cross-linked and are developed to have low toxicity and inflammation which was studied to improve the wound healing properties in a rat model [52]. Alginate-PVA is developed using a solvent casting method to produce scaffolds of good mechanical properties, sustained release, and granulation tissue formation for efficient and fast wound healing [53]. Freeze–thaw method is employed to produce large exudate absorption, suitable transmission rate, and absorption by using PVA-dextran aldehyde and is found to accelerate wound healing in full-thickness skin defect model [54]. On the whole, this model is shown to have improved potential in the engineering of scaffolds for wound healing and tissue regeneration.

(iv) Decellularized materials: certain cellular components can cause an immunological response in the host body which can be resolved by removing those cellular components but preserving the 3D structure, EMC, and its morphology. This technique is called decellularization and is now known for its immense capability in tissue repair and regeneration. The decellularization procedure is generally done through physical, chemical, or enzymatic methods. In 2019, Wang et al. developed a hydrogel scaffold by chemically modifying hyaluronic acid and dextran by UV irradiation method to heal burn wounds. This type of VEGF gene–loaded hydrogel showed excellent mechanical properties, pore size, and water retention ability facilitating an anti-inflammatory and pro-angiogenic compartment for efficient and fast healing of burn wounds [55]. Decellularized materials can also be used along with other materials for full-thickness skin wound healing and chronic wound healing using adipocytes for skin regeneration.

Organ-on-a-chip

Organ-on-a-chip is a biomimetic system that represents a physiological organ built on a microfluidic chip (Fig. 5). It stimulates the structural and functional characteristics of human tissue and can predict various stimuli such as drug response and other environmental influences. It

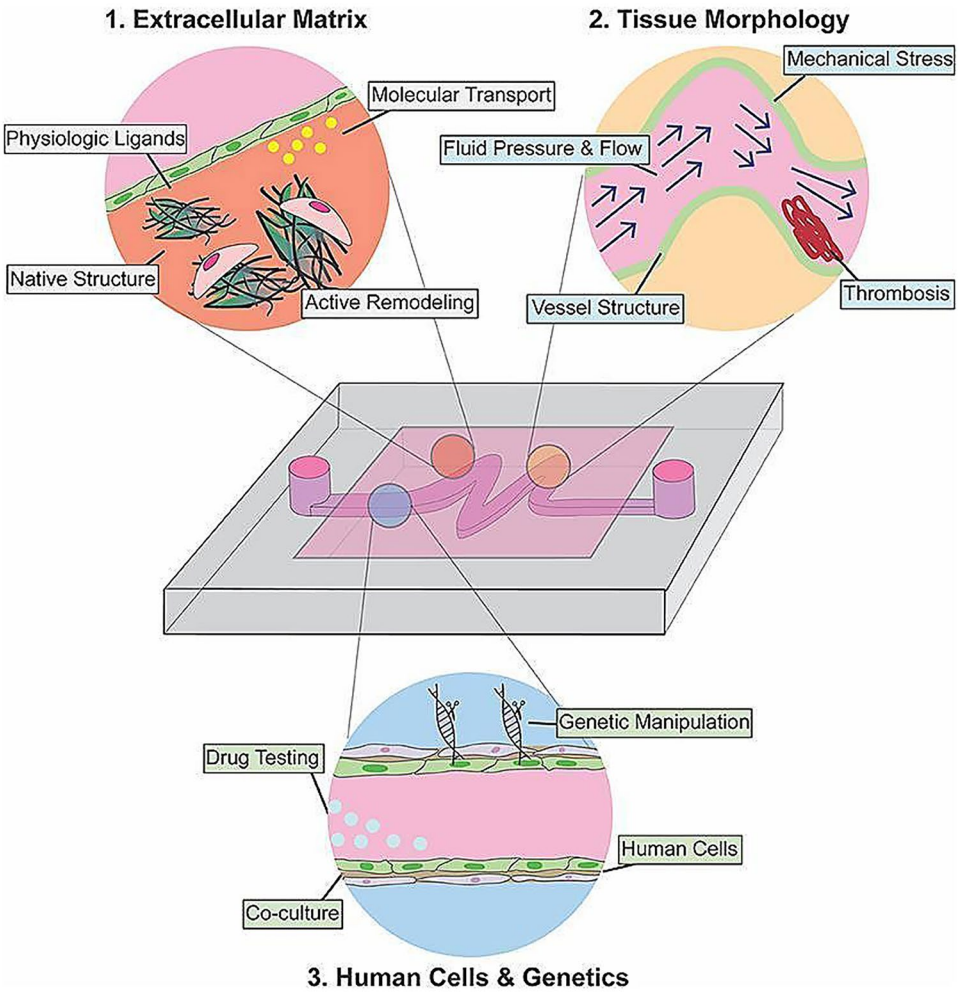

Fig. 5 Use of microfluidic devices for modeling cardiovascular disease pathophysiology (adopted from Doherty et al. [58])

encompasses four key elements, including (i) microfluidics, (ii) living cell tissues, (iii) stimulation or drug delivery, and (iv) sensing. The chip is made with accuracy using techniques such as lithography and contact printing. Biocompatible materials such as hydrogels can prevent mechanical damage and can retain their 3D structure [56]. In 2019, Kane et al. developed a technique to monitor cells in a 3D microfluidic arrangement that can produce a time-lapse image by microscopy to evaluate cellular electrical activity [57]. An organ-on-chip cell culture/model cannot be made and retrieved without microsensor-mediated reading of its metabolic state and characteristic points in the system.

Three-dimensional bioprinting

Three-dimensional bioprinting has attracted interest in the field of tissue engineering and regenerative medicine in recent times, and this technology is employed in various industries for the fabrication of complex structures and materials. The basic principle of bioprinting is the layer-by-layer biological constitution with precision by spatial control. It relies on three fundamental principles: (i) biomimetics, (ii) self-assembly, and (iii) tissue building blocks. The process of 3D printing begins with the imaging of the organ of interest using MRI or CT, followed by segmentation which is the creation of 3D geometry of the area of interest. After segmentation, the 3D shape is transformed into a file ready for printing, i.e., from DICOM to STL. After the selection of an appropriate 3D bioprinter and the materials for 3D fabrication, the 3D object is printed which is then tested for its surface properties and such. However, the limitations of bioprinting remain to be unsolved [59]. The types of bioprinting techniques can be classified into four categories:

(i) Droplet-based: this type of bioprinter is also referred to as inkjet-based bioprinters where the cells are deposited in the biomaterials as droplets by using heating reservoirs or piezoelectric actuators to produce cytosine which was developed in the year 1998 by Klebe [60]. It can be used for the fabrication of tissues in a determined 3D structure. Two methods

are commonly employed in the generation of droplets in droplet-based microfluidics: (i) active and (ii) passive methods. In the active method, droplets are generated such as an electric, magnetic, or centrifugal field. In the passive method, droplets are generated by using properties such as surface and channel properties [61]. In a study, this method is employed by printing such droplets using lipid monolayers and allowing the mammalian cells to be loaded in the droplets by using hydrogel bioink [62], although this technique is not preferred as it is not as efficient as using photolithography for the fabrication process.

(ii) Extrusion-based: it is also referred to as the fusion deposition modeling 3D printing technique. This technique used plastic or metal-based filaments that were extruded through a heated nozzle and printed structures in 3D form layer by layer. Using a computer-aided drug design, this method of 3D printing can be employed for precision printing of the biomaterial. Recently, researchers are focusing on developing patient-customized 3D scaffolds by obtaining the CT/MRI images, importing them into CAD software, and developing a 3D model. Based on the biomaterials used, a wide range of bioinks are developed; e.g., extrusion-based bioprinting can be done to produce a blood vessel–like architecture that can perfuse under high pressure using bioinks such as carbohydrate glass, PEG, fibrin, Matrigel, alginate, and agarose. Endothelial monolayers can be developed using GelMA and agarose. A cellularized tubular structure for cell attachment and proliferation is constructed using gelatin and hyaluronic acid bioinks [63]. The major challenge is vascularization in in vivo conditions.

(iii) Laser-assisted: it is a technique that uses a laser as the energy source and deposits biomaterials onto a substrate. The crucial components in this technique are a pulsed laser source, a ribbon coated with liquid biological materials, and receiving substrate made out of biopolymers [64]. Researchers have used this technique to develop 3D models such as fibroblast, myoblast, and neural stem cells [65]. The non-contact orienting of 3D models with high resolution and activity and the precise delivery are some of the unique advantages of using laser-assisted method when compared to other 3D printing techniques [66].

(iv) Stereolithography: it is also known as a solid-free form (SFF) technique for printing 3D models. It is the most powerful and versatile, with great precision in the fabrication of biomaterials. This method also makes use of the images obtained from CT/MRI and computer-aided drug design [67]. It uses a liquid, photosensitive polymer that is solidified upon illumination. Microarrays are used to control the light intensity for the polymerization of light-sensitive polymer materials [68]. Despite its immense advantage to fabricate biomaterials, it has various limitations such as biocompatibility, biodegradability, and residual toxins which can evoke an immune response. The UV light source is also harmful to the cells as it can damage the DNA. To overcome this, in 2015, Wang et al. designed a protocol to use visible lights instead of UV and demonstrated it using 3T3 cells which resulted in high resolution and cell viability for a minimum of 5 days [69]. Previous research on 3D bioprinting explained the computer-aided production of tissues and organs using alginate hydrogel bioink (Fig. 6) [70].

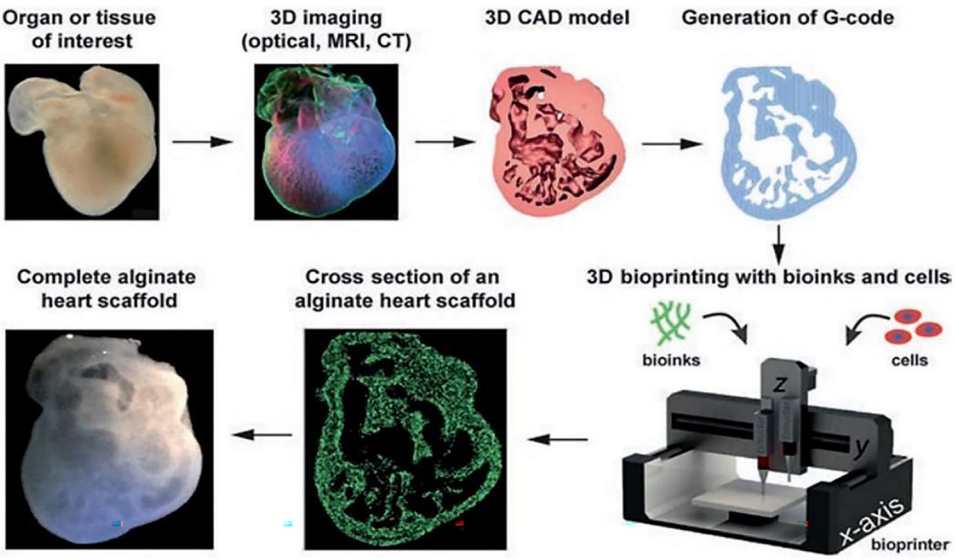

Fig. 6 3D bioprinting based on an alginate hydrogel bioink, using a computer-aided design for the production of tissues and whole organs (adopted from Ramiah et al. [70])

Origin and nature of cells used in 3D cell culture models

While the traditional 2D cultures are grown as monolayers on a flat surface such as glass or plastic, 3D cultures are generally grown as 3D aggregates or spheroids by using a scaffold or matrix or sometimes in a scaffold-free manner [4]. In 3D culture, the scaffold/matrix technique uses an acellular 3D matrix where the cells are seeded or it can also be done by dispersing the cells in a liquid matrix which is then followed by either solidification or polymerization [4]. The materials used in this technique can be biological materials or synthetic materials. Biologically derived materials include alginate, chitosan, and hyaluronic acid. Some examples of synthetic-based materials include polyethylene glycol (PEG), polyvinyl alcohol (PVA), polylactide-co-glycolide (PLG), and polycaprolactone (PLA). The scaffold-free system is generated by using techniques such as hanging drop templates, magnetic levitation, and magnetic 3D bioprinting [71]. In the above techniques, the cells grow in their natural 3D environment which also enables them to communicate with the ECM as well as their microenvironment which directly influences a variety of cellular functions, such as cell proliferation, differentiation, morphology, gene expression, protein expression, and cellular responses to external stimuli [4].

Many cells have a natural tendency to form aggregates by establishing a contact and specific microenvironment allowing them to express a tissue-like phenotype. For example, spheroid cultures are a simple 3D model that can be produced from an extensive range of cells and has the tendency to self-aggregate [72]. This is specifically used in cancer research as it enables rapid detection of morphological changes in the transformed cells. These cells are embedded in the ECM and left to proliferate and polarize conferring to its primary organ of origin. This will result in the establishment of a perfect sphere shape only if the cells are normal, or it will appear as a distorted structure if the cells are malignant. ECM thus aids the cells to move freely within their spheroid that are corresponding to the way in which cells would move in live tissue inside the body. The spheroid cultures are thus an enhanced model to study the cells for their migration, differentiation, survival, and growth [72].

In a scaffold-free spheroid culture, cells are grown in suspended media. This can be achieved either by continuing spinning or by using low-adherence plates. No adherence signal is given to these cells and the culture is mainly reliant on its cell-to-cell contact. Spheroids are mainly used in modeling solid tumor growth and metastasis studies. These cells can be collected and analyzed using quantitative techniques such as colorimetric, fluorescence, and luminescence assays measured with a plate reader and also qualitatively by confocal microscopy [72].

Advantages of 3D cell culture models over 2D models for drug testing

For decades, the 2D cell culture technique has been used in the drug discovery process as a very crucial part of drug testing. They form a two-dimensional monolayer of cells on a flat plastic surface. Over the past decade, it has provided abundant information about the vital biological process and the cell's microenvironment. Although this technique possesses various advantages and is well established and most understood with lots of comparative literature available and still in use, it has its own limitations. The first and foremost is the lack of real microenvironment expression of the cells. It is difficult to understand the functions of the human body; the cell lines are prone to changes in the morphology and its genotype which might not resemble the parent cells, issues related to the growth media used and the growth of the cells, the lack of productivity, and increased cost and failure rate [73]. Moreover, this technique is now superseded with a three-dimensional cell culture model. The foremost advantage of using such 3D culture is the exact mimicking of the physiological environment of the human cells which exist in the 3D structure. It has shown better cellular responses to drug treatments which resemble that of the in vivo condition. This model has also shown resistance to the anti-cancer drug when compared to the 2D model indicating that the genotypic and phenotypic changes in the cell culture models influence the drug testing process [74].

This model has a higher degree of structural complexity and retains a steady state. Overall, this technique provides a much better simulation of cells in a more realistic way to grow and understand the physiological process and the response of a cell. Although this technique outweighs the limitations of a 2D cell culture, it has its own limitations.

Limitation of 3D cell culture models

As discussed above, in spite of their contribution to many areas of research, each of the 3D cell culture types has its own advantages and disadvantages. The biggest challenge of the 3D culture technique is the unfamiliar and complex workflow of culturing cells in 3D and analyzing them. Even the most skilled scientist will have practical difficulty in achieving reproducibility and the uniformity of spheroids in cell culture. Compared to the scaffold 3D model, the scaffold-free 3D model is less complex and easily adaptable to the cell culture environment. Poor reproducibility with biomimetic scaffolds has been reported, disturbing the consistency of spheroid formation, and the non-human origin of some of these scaffolds may limit their effectiveness in their applications [75].

Table 1 Application of different types of 3D cell culture models

3D cell culture model	Applications
Spheroids	Drug testing, nanoparticle examination, models to study neurodegenerative disease, Parkinson's disease, hepatocyte models to study liver functions and disorders, etc. [10]
Organoids	Human developmental biology, human disease modeling, tissue engineering, regenerative medicine, personalized medicine, preclinical disease modeling, and drug screening [22]
Scaffold	Tissue reconstruction and regeneration, tissue engineering, biomedical sensing, etc
Organ-on-a-chip	Drug screening, toxicity studies, understanding disease and metabolic disorders, drug discovery and development, identifying cancer biomarkers, etc
3D Bioprinting	Tissue engineering, regenerative medicine, organ and drug printing, toxicology studies, clinical transplantation, high-throughput screening, and cancer research [81]

The major disadvantage of spheroid cell culture includes the development and maintenance of spheroids in its uniform structure, the forming the spheroids from a small seed number of cells, accuracy in controlling the specific ratios of different cell types in spheroid structure when they are co-cultured, and other factors such as lack of reliability, simplicity, standardization, and high-throughput compatible assays for drug screening using spheroids [76].

In organoids, the major limitation is that not all the cells will mimic the structure and function of the actual organ [77]. Above all, it lacks vasculature which is the most vital for the transport of nutrients and waste materials to move in and out of the cell. Some of them may imitate only the early stages of organ development; for example, in recent research, it was shown that retinal organoids failed to fully mature to become light sensitive, whereas cerebral organoids failed to fully develop as cortical plate layers. Other technical challenges still persist when harvesting such organoids with in vivo–like complexity, to increase the rate of maturity, and screening-compatible reproducibility [76].

In scaffold/matrix-based material, the disadvantages include the lot-to-lot variability and complex nature of their composition makes it difficult to identify exactly which signals are promoting a particular cell function. There are other natural gels such as fibrin, hyaluronic acid, chitosan, and alginate that have also been used for the 3D cell culture technique; however, these natural gels are less preferred than synthetic scaffolds because they have less versatility to promote the 3D culture. Moreover, porous scaffolds have issues arising in their inadequate diffusion properties, which is a challenge to fabricating more complex tissues such as the heart and liver [76].

Most organs-on-chips are commonly made only for the apprehension of the important features of an organ or a particular disease but due to its practical difficulties. To date, a wide range of organs-on-chips is being investigated for their properties, including the skin, lung, vasculature, heart, muscle, liver, and intestine. However, most organs-on-chips lack vasculature and also are difficult to adapt to high-throughput screening procedures [76].

3D bioprinting when compared with other 3D cell culture models has additional disadvantages such as maintaining the tissue maturity and functionality [78], lack of vasculature, challenges with the cells and materials used, and difficulty in adapting to high-throughput screening.

Application of 3D cell culture models in drug testing and research

For decades, cell-based drug discovery highlighted the screening of well-characterized cell monolayers, regularly in cancer drug discovery. However, in recent years, the 3D cell culture technique has been emphasized yielding results with higher precision for clinical outcomes and has become more prominent in the field of drug discovery. This technique has also avoided the ethical issues raised by using animal models for drug testing. It can be used to screen a wide range of drugs which does not limit to one particular cell or tumor type. It models the natural physiological conditions that can promote a specific cell behavior. Researchers focus on the development of models that enhance the motility of cells, initiation of cell dormancy, promoting the differentiation of cells such as epithelial cells and neurons, or an anticipated microenvironment like that of a metastatic function [79, 80]. It has opened ventures for preclinical testing of a tailored set of drug candidates to advance the outcomes and ultimately reduce the side effects of cancer therapy, although much research still needs to be done in developing systems that can precisely epitomize in vivo conditions and also the disease pathology [5]. 3D systems have been in use for a long time in cancer research for examining the safety and efficacy and to do other fundamental research. Monitoring tumor relapse is made possible by 3D culture's ability to survive for longer periods. Table 1 summarizes the applications of various 3D cell culture models.

Future trends and conclusion

The 3D cell culture technique was founded decades back but researchers are now focusing on standardizing this technique to become more organized. This technique has already empowered a ground-breaking understanding of the precision of the native physiological environment of the cell. 3D systems have been used for a long time in cancer research for their safety and efficacy in testing and research. Monitoring tumor recurrence has been facilitated by 3D cultures' capability to thrive for a longer period of time. Growing organoid models on electronic chips can help us to keep track of the electrical activity of cardiac cell cultures in real time. Personalized medicine would become a lot more achievable when growing an extensive variety of cell types from the harvested cells using this technique. Testing any substantial new drug on cells in vitro first, before exposing it to the body, can become a standard practice if the effect on patient outcomes is significant enough. Therefore, the 3D cell culture technique has grown exponentially in the past decade, with a broad application in the field of drug discovery and development and toxicity screening of the drug for various diseases. Considering all its advantages, it is necessary for research to be done for developing systems that can accurately represent in vivo microenvironment and disease pathology.

Acknowledgements The authors are grateful to Saveetha Dental College and Hospitals, SIMATs, Chennai, India.

Author contribution Conceptualization, A.P.F; writing original draft preparation, T.S.B.; writing, review, and editing, V.V.P. and A.P.F.; project administration, A.P.F. All authors have read and agreed to the published version of the review manuscript.

Funding T.S.B and A.P.F. acknowledge the financial support for the Start-up Research Grant (SRG/2022/001432), by the Science and Engineering Research Board, Department of Science and Technology, Government of India, New Delhi, India.

Data availability Not applicable.

Declarations

Ethics approval and consent to participate Not applicable.

Consent for publication Not applicable.

Competing interests The authors declare no competing interests.

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