Chapter 14
3D Cell Culture Techniques

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14.1 Introduction

The detailed studies that are conducted on the formation, function and pathogenesis of tissues and organs using cell culture systems and animal models are beneficial for managing any pathological condition like cancer and neurodegenerative diseases (Kapałczyńska et al., 2018; Koledova, 2017). Traditional two-dimensional (2D) cell culture models or animal model systems are widely used for better understanding the formation, function of tissue/organ under normal and diseased conditions. It has been inferred by various studies that the 2D cell culture models have helped in understanding the fundamental concepts of biological processes. The cells produced on a flat 2D surface (such as polystyrene substrates) are obserevd to be dramatically different from cells grown in 3D cell culture systems in terms of their shape, cell-cell interactions, cell-matrix interactions, and cellular differentiation (Freshney, 2015; Koledova, 2017). Experimental studies on animal models offer relevant information about specific molecules and biological processes. However, regular disparities have also been observed in results that were gathered from studies of desired gene and protein expression profiles. Besides this, animal model systems also could not reflect the appropriate features of human tumours, therapeutic drug responses, stem cell differentiation and autoimmune diseases (Yamada & Cukierman, 2007;

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S. Mani et al., *Animal Cell Culture: Principles and Practice*, Techniques in Life Science and Biomedicine for the Non-Expert,
https://doi.org/10.1007/978-3-031-19485-6_14

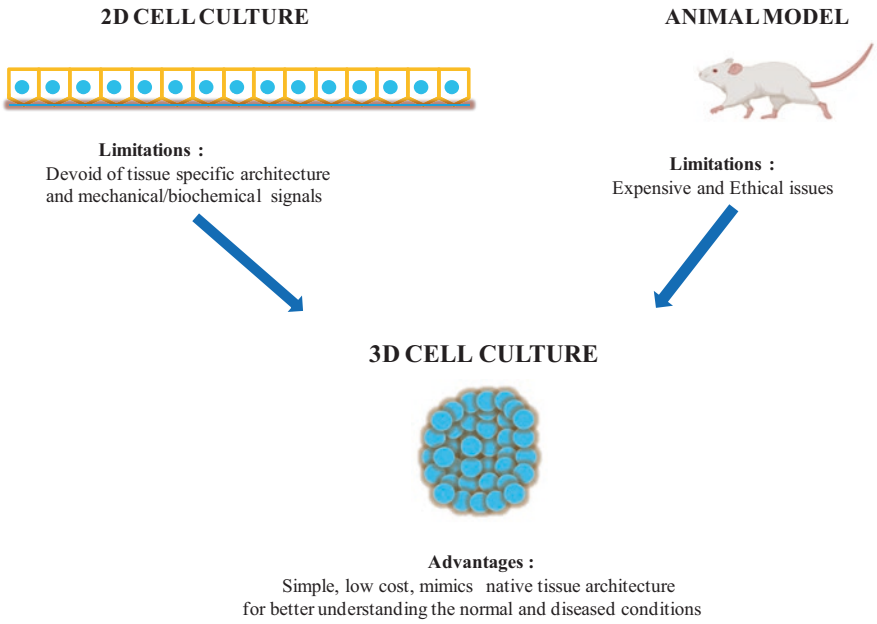

Fig. 14.1 A comparison between two-dimensional cell culture, animal model system and 3D cell culture methods. On the one hand, the 2D cell culture model lacks tissue architecture as well as biochemical/mechanical signals, whereas in vivo models require high costs for maintenance and are also associated with ethical concerns. Therefore, 3D cell culture systems are recognised as a link between 2D cell culture and animal model systems. It also mimics native tissue architecture, which helps in better understanding the physiology of normal and diseased conditions

Justice et al., 2009). Henceforth, in vitro 3D cell culture models are recognised as a third approach that works efficiently over the pitfalls of traditional cell culture techniques and animal model systems (Fig. 14.1).

3D cell culture models have made a revolutionary path towards gathering information about biological processes in in vivo molecular mechanisms. Further, these models have also enabled advancement in cellular and molecular biological studies for the cellular growth, cell proliferation and profile expression studies of genes and proteins. The popularity of 3D models is due to their capability to mimic specific environments and tissues, which facilitated their use in applications like advances in tissue engineering, development and screening of new therapeutics against pathological conditions (Edmondson et al., 2014).

The rationale behind this book chapter is to discuss the general principles, ideas and cautions towards the use of in vitro 3D cell culture systems for enhancing knowledge in morphogenesis of tissues and pathological conditions, mainly carcinogenesis. In the present chapter, we deliberate the different types of 3D cell culture methods that help in gaining information about cell growth, cell proliferation, growth conditions and expression profiles of genes and proteins. We will also intend to compare the characteristics of the 3D cell culture and two-dimensional (2D) monolayer culture in order to perceive the advancement of 3D culture over the traditional cell culture. Finally, we will also connote the applications of 3D cell culture models and challenges faced by researchers and medical professionals for their use in personalised medicine.

14.2 3D Cell Culture Versus 2D Cell Culture Systems

Earlier experimental studies conducted on 2D cell cultures in vitro for disease model conditions, including cancer, inferred that the traditional cell cultures have many limitations like changes in morphology, polarity, a flaw in interactions between cellular and extracellular environments and a division method. These limitations make their limited use in regular experimental research in the area of drug screening, drug discovery etc. Therefore, 3D cell culture systems show up-gradation in the context of real reflection of cellular organ system and pathological conditions. These modern systems have also facilitated the study of biomarkers and targeted therapies against diseases significantly. In Table 14.1, we have compiled the comparison of properties of 3D versus 2D culture. The diagrammatical comparison of 3D versus 2D cell culture is also shown in Fig. 14.1.

Table 14.1 Shows the comparative analysis of 3D cell culture systems and the traditional 2D cell culture model

Type of culture	The 3D cell culture system	2D cell culture model
Time taken for culture formation	From a few hours to few days	From minutes to few hours
In vivo initiation	In vivo tissues and organs are in 3D form.	Not reflect the native structure of tissue or tumour mass.
Quality of culture	Lacks significant performance and reproducibility. So, it is difficult to interpret and also culturing is not easy.	Exhibits significant performance and reproducibility, long-term culture in maintenance. So, it is easy to interpret due to their simplicity.
Cell interactions	Exhibits significant interactions of cell-cell and cell-extracellular environment, environmental niches	Lack of inter-cellular and intra-cellular interactions and also in vivo-like microenvironment and niches
Cellular characteristics	Native morphology and way of division, diverse phenotype and polarity	Altered morphology and way of division; loss of diverse phenotype and polarity
Accessibility towards crucial compounds	Approachable contents such as oxygen, nutrients, metabolites and signalling molecules are inconsistent as in vivo.	Unlimited approachability towards the oxygen, nutrients, metabolites and signalling molecules (in contrast to in vivo)
Molecular mechanisms	Possesses expression of genes, mRNA splicing, topology and biochemistry of cells as in vivo	Exhibits alterations in gene expression, mRNA splicing, topology and biochemistry of cells (in contrast to in vivo)
Cost of Maintenance a culture	Expensive, time-consuming, Fewer commercially available tests	Cheap, commercially available tests and the media

Adapted and modified Kapałczyńska et al. (2018)

14.3 Overview of 3D Cell Culture Techniques

Various approaches were recognised like whole animals, organotypic explants culture, cell spheroids, tissue-engineered models and micro-carrier cultures for the use of 3D cell culture in many applications (Carletti et al., 2011). These models require both scaffolds for culturing and scaffold-free cultures. Organotypic explants are mainly used in those studies where complete information is required in whole animals such as *Drosophila melanogaster* and zebrafish. These models provide crucial data wherein the cells are physically located in their native niche. The growth conditions can be varied in cell culture studies on animal models like *Drosophila melanogaster* and zebrafish. However, those involving mouse embryos require highly controlled conditions such as the pH, temperature and oxygen levels should be highly precise (Corrò et al., 2020).

Organ explantation is mainly done for brain and neural tissues. The explanted tissue is grown on gels or semi-permeable membranes in the presence of a growth medium or isotonic solutions. These systems can stably maintain the tissue architecture; but time availability is an essential factor. Differentiated cells are also present in these culture systems. Although, deep imaging of these issues presents a significant challenge.

One of the most common ways of 3D cell culture is the spherical cellular aggregation model known as spheroids. This model does not use any scaffold model and is easily visualised by using imaging techniques such as light or fluorescence microscopy. Henceforth, this model has been found to be very crucial for experimental research on cancer and therapeutics studies. Some spheroids such as multicellular tumour spheroids (MCTS), mammospheres, neurospheres, hepatospheres and embryoid bodies are recognised for studying the different pathological conditions. These models have been ideally used for the study of solid tumour models. The best characterised is the MCTS. These tumour spheroid models have helped a lot in better understanding cancer mechanism and in the development of a highly sensitive cancer drug testing platform. These models are highly beneficial as they mimic the in vivo tumours very well in terms of their morphology, cell proliferation, aeration, nutrient uptake and drug intake.

14.4 Methods of 3D Cell Culture Techniques

Currently, different types of 3D cell culture techniques are available at the global level. Broadly these can be categorised into two main types; scaffold-based techniques and scaffold-free techniques. These techniques are discussed in the below section:

14.4.1 Scaffold-Based Techniques

As the size and complexity of 3D culture increase, it demands a need for scaffolds or matrices. In scaffold-based 3D culturing, the cells are grown on substrates that represent the extracellular matrices making this system a lot closer to the native environment. The porosity of scaffolds promotes the transportation of oxygen, nutrients and waste, enabling cells for their easy proliferation and migration within the scaffold and eventually adherence to it. There are two scaffold categories – in vitro 3D scaffolds used for cell culturing and experimental applications such as drug testing. The second is biomedical engineering scaffolds that are exploited as supports for tissue regeneration applications. Scaffolds can be in the form of *hydrogels*, membranes and *3D matrices*. Based upon their sources, they can be divided as natural and synthetic scaffolds too (Table 14.2).

Hydrogels Scaffolds are basically polymers of hydrophilic molecules that possess a large amount of water without dissolution. Hydrogels offer several benefits over solid scaffolds, such as these provide a hydrated environment that is favourable for cell colonisation and infiltration and also high resemblance with the tissues (Rodrigues et al., 2015). The biomaterial used for designing such scaffolds plays a crucial role as these provide a 3D template for cell adhesion, proliferation and differentiation. The scaffolds could be made of different biomaterials either of natural origin (obtained from plant, animal or human tissues) such as fibrin, fibroin, glycosaminoglycans (GAGs), alginate, gelatin, chitosan, hyaluronic acid and collagen or could be synthetic such as polyethylene glycol (PEG), poly –L- lactic acid (PLLA), polycaprolactone (PCL) and polylactic acid-co–caprolactone (Afewerki et al., 2019). The cell type and the nature of the study are essential when choosing the type of scaffold.

The most commonly used matrix in 3D cultures is *collagen*, as they require low-cost maintenance, high biocompatibility and biodegradability and a very low antigenicity. The structure of collagen matrices (such as the pore size, stiffness) is also easily manipulated. This could be achieved by altering its concentration or introducing new cross-linking compounds such as glutaraldehyde and formaldehyde or physical treatment like ultraviolet or gamma irradiation (Lee et al., 2001; Ravi et al., 2015).

Table 14.2 Biomaterials used for scaffolding

Natural polymers	Synthetic polymers
Fibrin	Polyethylene glycol (PEG)
Fibroin	Poly –L- lactic acid (PLLA)
Glycosaminoglycans (GAGS)	Polycaprolactone (PCL)
Alginate	Polylactic acid- co–caprolactone
Gelatin	
Chitosan	
Hyaluronic acid	
Collagen	

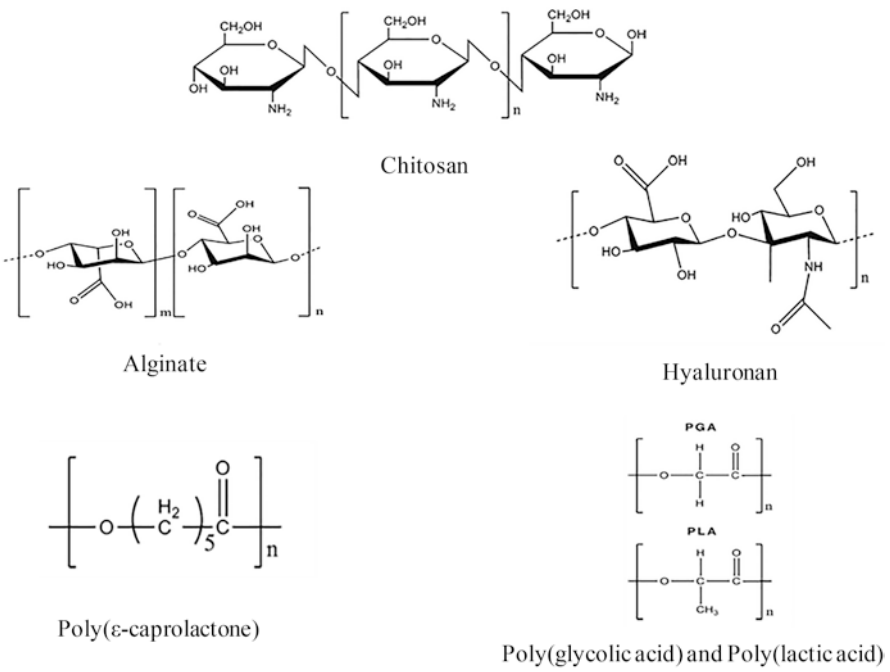

Fig. 14.2 It depicts the chemical formulas of various natural and synthetic polymers that are used for the hydrogel scaffolds

A standard method for making collagen scaffolds is freeze-drying (Carletti et al., 2011). Collagen scaffolds are used extensively for culturing osteoblast and chondrocytes and have major biomedical applications (Lee et al., 2001; Zhou et al., 2006; Negri et al., 2007) (Fig. 14.2).

Chitosanisan N-deacetylated derivative of chitin, which is found in the exoskeleton of crustaceans and insects. Chitosan is sensitive to enzymes such as chitonase and lysozyme, and the degree of degradation depends on the acetyl content. The mechanical properties of chitosan are affected by the molecular weight and degree of deacetylation (Carletti et al., 2011). Chitosan hydrogels can be prepared by ionic bonding or covalent cross-linking. Chitosan-based scaffolds have been mainly used for culturing chondrocytes for cartilage regeneration (Rogina et al., 2021). Chitosan-chondroitin sulfate scaffolds have been used to study the tumour microenvironment in prostate cancer (Xu et al., 2020). The application of chitosan-based scaffolds has also been used in improving the bioavailability of molecules. Epigallocatechin-3-gallate (EGCG) is used to differentiate mesenchymal stem cells to osteoblast, but as it is metabolised during cell culture, its bioavailability is reduced. Therefore, CS (Chitosan)/Alg (alginate)-ECN (EGCG-Chitosan nanoparticles) scaffolds have been designed for improving their bioavailability. In this type of scaffold, EGCG is loaded onto chitosan nanoparticles which are then entrapped into a chitosan alginate scaffold (Wang et al., 2021).

Glycosaminoglycans such as hyaluronic acid have also been used as a scaffold for the culture of chondrocytes, bone and skin (Carletti et al., 2011). Hyaluronic acid hydrogels have been used for delivering growth factors that are osteoinductive and angiogenic for bone tissue engineering (Rodrigues et al., 2015).

Gelatin is also used as a biomaterial for scaffolds as it shows chemical similarity to the extracellular matrix in native tissues, low cost-effectiveness, bioavailability and compatibility and low antigenicity. However, it has low solubility in concentrated aqueous media, is highly susceptible to enzymatic digestion and has a high viscosity and poor mechanical properties. Therefore, it has limited use for 3D cell culture. However, to overcome this limitation, we can synergistically use it with a wide range of polysaccharides resulting in gelatin-composite hydrogels (Afewerki et al., 2019).

Apart from the natural polymers, many synthetic polymers have also been used as scaffolds due to their high reproducibility and versatility. The processing of synthetic polymer is relatively easy in contrast to a natural polymer. The functional group of synthetic polymers can be designed due to which their structure and properties can be easily modified. For example, their degradation rate can be easily modified depending upon the molecular weight and chemical composition. This is advantageous as they can be designed according to specific applications (Donnaloja et al., 2020). However, their biocompatibility and bioactivity are less than natural polymers. So far, the most extensively used synthetic polymers for scaffolds are polyglycolic acid (PGA), polylactic acid (PLA) and polycaprolactone (PCL) (Fig. 14.2).

Among all the synthetic polymers, PCL is non-toxic in nature and a biodegradable aliphatic polyester. Its degradation rate is slow; however, it has hydrophobic properties, which create problems in cell adhesion and penetration (Donnaloja et al., 2020). To overcome these hurdles, several co-polymerisation techniques such as PCL/alginate composite scaffolds have been developed (Fig. 14.2).

14.4.2 Scaffold-Free Techniques

This category includes those techniques where cells are grown without any solid support. It includes the *forced floating method*, the *hanging drop method* and the *agitation-based method*s which are discussed below:

In the *forced floating method*, the vessel's surface is modified in a way such that the cells are not able to attach to it, which results in their forced floatation. This is a straightforward method for the formation of 3D spheroids as the floating of the cells increases the cell to cell contact promoting the sphere formation. It has been mainly used for the development of 3D spheroids in 96-well plates. In this approach, plates were coated with 0.5% poly-2-hydroxyethyl methacrylate (poly-HEMA) and dried for 3 days. This type of coating prevents the attachment of cells to the plate surface.

After this, cells were added to the plates and centrifuged, which results in adherence of cells with each other and further leads to the formation of spheroids. This has been used for the generation of spheroids of many cancerous as well as non-cancerous cells (Breslin & O'Driscoll, 2013; Kelm et al., 2003). This method is very simple and is highly reproducible. The spheroids generated in this method are highly consistent as equal numbers of cells are added to each well. This method is highly accessible for experimental studies, and the size of the spheroids can easily be modified as per the required number of cells for seeding. This has been extensively used for drug testing. Apart from the HEMA method, 1.5% agarose is also used. This also results in the formation of a thin layer on the surface of the plate, which prevents the attachment of cells. This method is also relatively simple and can be used for the long-term culture of cells. The only disadvantage of this method is that an extra step is added before culturing of cells which increases the work and time required for coating the plates. However, there are commercially pre-coated plates available, but they increase the overall cost.

In the *hanging drop method*, a small aliquot of single-cell suspension is added in 60-well microwell mini trays. After the addition of cells, the plate is kept inverted, and the cell aliquots become hanging drops and remain in place due to the surface tension. Cells are accumulated at the tip of the drop, and they continue to proliferate (Kelm et al., 2003; Timmins & Nielsen, 2007). Here, also similar to the forced floating method, the size of the spheroid can be adjusted depending upon the cells seeded. Spheroids produced by this method are tightly packed. This method is very simple for use and also highly reproducible. The only possible limitation with this method is that the volume of liquid used to generate spheroids is very low. This is because the surface tension that holds the drop does not support larger volumes (Kelm et al., 2003).

In the *agitation-based method*, cell suspensions are kept in a container that remains in continuous motion. This can be achieved by either a continuous stirring of the cells or the container is kept in rotation. Due to the continuous motion, cells tend to form cell to cell communication and adhere to each other and not to the surface of the container (Kelm et al., 2003). This method is generally of two types: spinner flask bioreactors and rotational culture systems. *Spinner flask bioreactors* possess a container for holding the cell suspension, which has a stirring element. This ensures the continuous stirring of the cell suspension. The size of the spheroid correlates with the volume of the container and can be varied. The advantage of using spinners is that these allow the long-term culturing of spheroids as the culture medium can be changed frequently. The continuous motion of the fluids also aids in the transport of nutrients to and wastes from the spheroids. The drawback of such a system is that the force experienced by the cells as a result of continuous stirring might affect cellular physiology. Rotating culture systems function similarly to the spinners except that there is no stirring rod, but the container itself rotates. In 1992, NASA developed a rotating wall vessel bioreactor for maintaining low shear force on cells in culture. This vessel consists of a chamber that is utilised for culturing of cells. The chamber is attached to a rotator which slowly rotates about a horizontal axis (Breslin & O'Driscoll, 2013; Barrila et al., 2010).

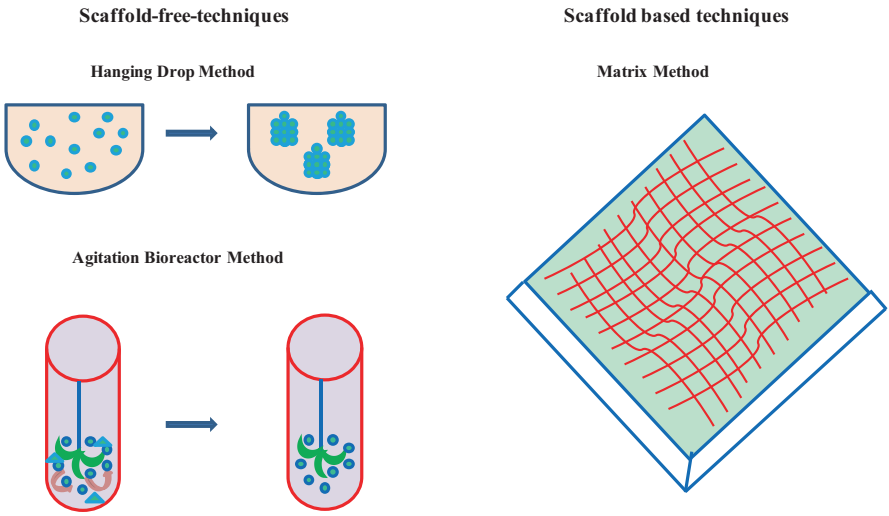

Fig. 14.3 Scaffold-based and scaffold-free techniques – 3D matrix method, hanging drop method and agitation bioreactor method

Stem cells in 3D cell culture have also been recognised in application of various biological therapeutic approaches like cell-based therapy, screening of drugs, regenerative medicine and high-throughput pharmacology. The unique properties of stem cells such as ability of self-renewal, multi-potency and clonality enable them to special use in many research areas. Human mesenchymal stem cells are mainly used due to their sectary nature for generation of spheroids culture to understand the gene expression profiles of normal physiological and diseased conditions.

All the scaffold-free and scaffold-based techniques are summarised in Fig. 14.3.

14.5 Stem Cells in 3D Spheroids and Organoids

The use of stem cells in 2D cell culture for clinical application is ineffective due to the inability of 2D culture to reflect the actual microenvironment of stem cells. Therefore, the decrease in the replicative ability of MSCs has been observed in 2D culture overtime invalidating the crucial studies. When stem cells are cultured in spheroids 3D cell culture, MSCs show a different phenotype than 2D-cultured MSCs. MSCs also have different gene expression patterns in spheroids than in 2D; e.g. MSCs show upregulation of various genes related to angiogenesis, stress response, hypoxia, inflammation, redox signalling etc. Henceforth, MSC in spheroids leads to upgradation in the development of therapeutics. Anti-inflammatory properties, reparative effect and better post-transplant survival of MSCs have also

been observed in spheroid cultures. The effectiveness of MSC spheroids over MSCs in 2D culture has been observed if MSC spheroids were administrated into the kidney of mole rats with ischemia reperfusion-induced acute kidney injury. The post-administration of MSCs spheroids showed more effectiveness in guarding the kidney to curb tissue damage, bolstering vascularisation and apoptosis and enhancing renal function in contrast to MSCs of 2D cultures (Jensen & Teng, 2020; Sakalem et al., 2021).

Pluripotent stem cells are used to generally grow as organoids for their future application in analogous tissue for transplantation in humans. According to a recent report, pluripotent stem cells from renal organoids are transplanted beneath the kidney capsules of adult mice. It has been observed that these organoids are acted as the same structures like that of a kidney in vivo. Therefore, under transplantation, glomeruli are vascularised swiftly, indicating a significant path towards developing an alternative kidney replacement therapy. It was found that the ability of organoids to represent different regions of the body makes them a crucial aspect for the study the genetic diseases for better understanding. Similarly, kidney organoid for cystic fibrosis disease model has been used to study the influences of trans-membrane conductance regulator-modulating entities and more tubular organoids used for kidney disease model where microenvironment played a crucial role in cyst formation. Organoids are also recognised for their study in difficult diseased model neurodegenerative diseases like Alzheimer and Parkinson disease. The study revealed that the brain organoids prepared from the pluripotent stem cells taken from the Alzheimer patients show significant improvement when treated with β- and γ-secretase inhibitors, which displayed significant therapeutic influences (Jensen & Teng, 2020; Sakalem et al., 2021; Chaicharoenaudomrung et al., 2019).

The medical patients who are diagnosed with a chance of organ failure require a critical care and organ transplantation treatment. The critical examination of artificial tissues and organs is very necessary for successful transplantation. Therefore, 3D bioprinting emerges as a crucial technique for the fabrication of artificial tissues and organs in three-dimensional mode before transplantation for their correct manufacturing pattern of living cells in a tissue-specific manner in which cells arrange layer by layer. This advanced medical technology has made a huge impact and saves the lives of many patients who receive successful organ transplantation.

3D Bioprinting It is the most recently developed technique of 3D cell culture in which, under the computational approach, customised 3D structures are constructed, and materials will be printed out, solidified and connected together. It has wide applications like 3D art and design, prototype, industrial manufacturing and architecture, but mainly known for tissue engineering and regenerative medicine (Mazzocchi et al., 2019; Ryan et al., 2016).

14.6 Applications of 3D Cell Culture Techniques

3D Cell culture mimics the tissues and tumour structure, thus aiding in simulating the patho-physiological microenvironment of disease or outgrowth. The structural organisation like cell-cell interaction, cell-extracellular matrix (ECM) interaction, differentiation and cumulative response to drug therapy provides valuable insight into 3D cell culture models (Ravi et al., 2015; Ryan et al., 2016) (Fig. 14.4; Table 14.3). In 2D culture, the true ECM is absent. Thus, the interaction with the cells is poorly studied. ECM plays a crucial role in the adhesive properties of cells, mechano-transduction and exposure of levels of toxic compounds and soluble factors present in media. The crucial interaction of normal cells and neighbouring cells with tumour cell physiology can be studied deeply by this model. The rate of division of cells in 2D models is higher as compared to in vivo models, which show more relevance to 3Dcell culture models (Chitcholtan et al., 2013). Peela et al. have prepared a 3D tumour model by integrating the gelatin methacrylate (GeIMA) hydrogel with a two-step photolithography technique (Peela et al., 2016). They proposed that this model shows more resemblance to tumour stiffness and architecture, thus being utilised in discovering biomarkers and targeted chemotherapy. The advancement in 3D cell culture in future may lead to superior models depicting kidney, liver etc., thus enhancing the effectiveness and rapid screening of toxic compounds in an in vitro setting. The characterisation of drugs and probable drug-drug interaction and ADMET (Adsorption, distribution, metabolism, elimination, toxicity) studies in 3D cell culture might help in a vast number of successful compounds as potential drugs (Godoy et al., 2013). Jingyun Ma et al. also suggested a 3D model for glioblastoma. They found that different drugs like temozolomide and resveratrol

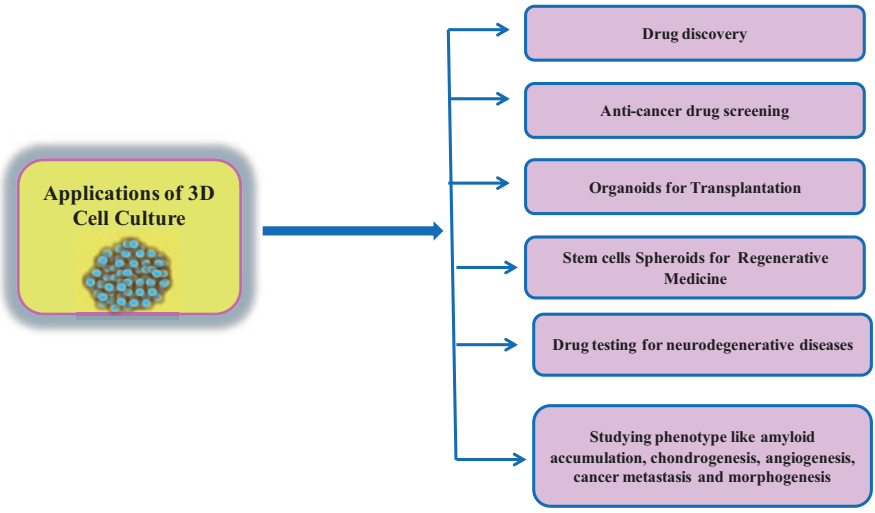

Fig. 14.4 Applications of a 3D cell culture system

Table 14.3 Application of 3D cell culture models in the study of human cancers and other pathological conditions

3D cell culture models	Applications in study of human cancers	References
3D-bioprinted agarose-alginate scaffold based, Hanging drop	Non-small cell carcinoma model	Ziółkowska-Suchanek (2021) and Amann et al. (2014)
Fibrous scaffold based	Head and neck cancer model	Young et al. (2018)
Nested hydrogel based	Osteosarcoma model	Pavlou et al. (2019)
Collagen matrix based	Glioblastoma model	Ma et al. (2018)
Fibrous scaffold based	Breast cancer model	Young et al. (2018)
Matrigel based	Lung cancer model	Pavlou et al. (2019)
Tumoroids	Colorectal cancer	Nörz et al. (2021)
3D spheroid formation with methylcellulose	Pancreatic ductal adenocarcinoma (PDAC) model	Longati et al. (2013)
3D Hanging drop	Malignant serous effusion (MSE) model	Wu et al. (2020)
Microfluidic 3D cell culture model	Vasculature study	van Duinen et al. (2015)
Silicate fibre based	Colorectal cancer	Yamaguchi et al. (2013)
3D extracellular matrix based	Antiviral drug screening	Koban et al. (2018)

in the detachable microfluidic device act similarly as in in vivo models as they inhibit the viability, proliferation and invasiveness (Ma et al., 2018). In the 2D model, the hypoxic conditions do not usually resemble the in vivo condition, but in 3D cell culture, the heterogeneous spheroidal structure allows hypoxic conditions. Recent studies on 3D models that focused on angiogenic response in hypoxic conditions observed the remarkable dysregulation in morphogenic changes and microRNA expression patterns in the model (Klimkiewicz et al., 2017). A recent report analyzed the 3D culture of drug sensitivity against AKT and mTOR inhibitor on colorectal cancer (CRC) drug screening (Nörz et al., 2021). An FDA-approved drug, gefitinib, a specific inhibitor of epidermal growth factor receptor (EGFR), was studied by a 3D cell culture system. It showed significant efficacy against the cowpox virus in primary human keratinocytes (Koban et al., 2018).

14.7 Challenges in 3D Culture Techniques

Despite having success in monolayer culture in drug discovery, it is evident that only a handful of drugs pass through the clinical phase II & III trials. Although there are many reasons, the main reason is its response and metabolism in in vivo conditions. The primary phase of drug screening is based on the monolayer culture of cells and their response. Recent advances in culture techniques enabled us a new 3D

model of cell culture, which can yield valuable insight into drug screening and its metabolism. The cost of maintaining 3D cell culture is higher as compared to the monolayer cell culture system. The various 3D cell culture techniques models allow for the transfer of experimental information to fix issues that arose in their handling and upkeep. Although the importance of the 3D cell culture model is well recognised so far, it poses many challenges that make it difficult to adapt as routine culture by researchers and medical professionals. One of the major drawbacks of a 3D cell culture system is its scalability. Current formats of 3D cell culture do not provide a solution to scale up in 384- and 1536-well plates. The techniques require automated pipettes to reduce the speed of media aspiration and dispensing of media in culture plates. This step is critical as this will impact the loss of 3D cell mass and the unwanted movement of cellular mass. The user should be attentive during pipetting as viscous liquids such as collagen and Matrigel should not be pipetted out. This technique requires special training and is labour-intensive, as well as the cost of culturing is more compared to monolayer cell culture. The biggest challenges are its disease relevance and compatibility to various detection instruments.

The visualisation of 3D structure in optical microscopy is less informational as the differential light absorption and light penetration delays the image acquisition time and complex geometrical shapes. The fluorescence microscopy also depicts the structural flaw, as the structure must obtain z-stacking to process the image output. Flow cytometry also faces challenges in 3D structures, as it was principled to single-cell movement on detectors. The techniques that can sort microorganisms, different cell populations and biomarkers are not valuable for 3D structures. If the user wants to use 3D structure in FACS, then the structure should be dissociated into single-cell suspension, and the 3D structure integrity will be lost. The dissociation of a 3D structure requires trypsinization, collagenase and mechanical disruptions. This process is time-consuming and also it limits the viability of cells even after sorting.

14.8 Conclusion

Three-dimensional cell culture emerges as a link between 2D cell culture in vitro system and in vivo animal models. Among scientific workers, the 3D cell culture techniques are increasingly becoming famous due to the ease of handling cells in vitro while obtaining data that mimic in vivo conditions and prevent concern of ethical clearance in case of animal usage. The 3D cell culture techniques are increasingly becoming crucial for in vitro modelling of basic developmental processes and human diseases. 3D culture enhances the knowledge about understanding mammalian organogenesis and carcinogenesis. Their application might also be helpful for drug testing and personalised medicine regularly. Several obstacles remain to be unravelling in the use of 3D cell culture systems for physiological studies like the inclusion of all different cell types of complex tissue, realistic modelling of regional differences in ECM composition, rigidness and arrangements of the sturdy vascular

system that facilitates the production of larger tissue constructs. This chapter provides information on 3D culture versus 2D culture, types of 3D cell culture techniques and their uses in various applications.

14.9 Future Prospects

We hope this detailed information on protocols of 3D cell culture will be helpful for researchers and graduate students in their cell culture studies. This chapter might be an inspiration and a starting point in developing new protocols and experimental designs as per the requirement for the specific scientific needs for advanced research in 3D cell culture.

References

Afewerki, S., Sheikhi, A., Kannan, S., Ahadian, S., & Khademhosseini, A. (2019). Gelatin-polysaccharide composite scaffolds for 3D cell culture and tissue engineering: Towards natural therapeutics. *Bioengineering & Translational Medicine, 4*(1), 96–115.

Amann, A., Zwierzina, M., Gamerith, G., Bitsche, M., Huber, J. M., Vogel, G. F., Blumer, M., Koeck, S., Pechriggl, E. J., Kelm, J. M., Hilbe, W., & Zwierzina, H. (2014). Development of an innovative 3D cell culture system to study tumour--stroma interactions in non-small cell lung cancer cells. *PloS one, 9*(3), e92511.

Barrila, J., Radtke, A. L., Crabbé, A., Sarker, S. F., Herbst-Kralovetz, M. M., Ott, C. M., & Nickerson, C. A. (2010). Organotypic 3D cell culture models: Using the rotating wall vessel to study host-pathogen interactions. *Nature Reviews Microbiology, 8*(11), 791–801.

Breslin, S., & O'Driscoll, L. (2013). Three-dimensional cell culture: The missing link in drug discovery. *Drug Discovery Today, 18*(5–6), 240–249.

Carletti, E., Motta, A., & Migliaresi, C. (2011). Scaffolds for tissue engineering and 3D cell culture. *Methods in Molecular Biology (Clifton, N.J.), 695*, 17–39.

Chaicharoenaudomrung, N., Kunhorm, P., & Noisa, P. (2019). Three-dimensional cell culture systems as an in vitro platform for cancer and stem cell modeling. *World Journal of Stem Cells, 11*(12), 1065–1083.

Chitcholtan, K., Asselin, E., Parent, S., Sykes, P. H., & Evans, J. J. (2013). Differences in growth properties of endometrial cancer in three dimensional (3D) culture and 2D cell monolayer. *Experimental Cell Research, 319*(1), 75–87.

Corrò, C., Novellasdemunt, L., & Li, V. (2020). A brief history of organoids. *American Journal of Physiology – Cell Physiology, 319*(1), C151–C165.

Donnaloja, F., Jacchetti, E., Soncini, M., & Raimondi, M. T. (2020). Natural and synthetic polymers for bone scaffolds optimization. *Polymers, 12*(4), 905.

Edmondson, R., Broglie, J. J., Adcock, A. F., & Yang, L. (2014). Three-dimensional cell culture systems and their applications in drug discovery and cell-based biosensors. *Assay and Drug Development Technologies, 12*(4), 207–218.

Freshney, I. R. (2015). *Culture of animal cells* (A manual of basic technique and specialized applications) (7th ed.). Wiley-Blackwell. ISBN: 9781118873373.

Godoy, P., Hewitt, N. J., Albrecht, U., Andersen, M. E., Ansari, N., Bhattacharya, S., Bode, J. G., Bolleyn, J., Borner, C., Böttger, J., Braeuning, A., Budinsky, R. A., Burkhardt, B., Cameron, N. R., Camussi, G., Cho, C. S., Choi, Y. J., Craig Rowlands, J., Dahmen, U., Damm, G., &

Hengstler, J. G. (2013). Recent advances in 2D and 3D in vitro systems using primary hepatocytes, alternative hepatocyte sources and non-parenchymal liver cells and their use in investigating mechanisms of hepatotoxicity, cell signaling and ADME. *Archives of Toxicology, 87*(8), 1315–1530.

Jensen, C., & Teng, Y. (2020). Is it time to start transitioning from 2D to 3D cell culture? *Frontiers in Molecular Biosciences, 7*, 33.

Justice, B. A., Badr, N. A., & Felder, R. A. (2009). 3D cell culture opens new dimensions in cell-based assays. *Drug Discovery Today, 14*(1–2), 102–107.

Kapałczyńska, M., Kolenda, T., Przybyła, W., Zajączkowska, M., Teresiak, A., Filas, V., Ibbs, M., Bliźniak, R., Łuczewski, Ł., & Lamperska, K. (2018). 2D and 3D cell cultures – A comparison of different types of cancer cell cultures. *Archives of Medical Science: AMS, 14*(4), 910–919.

Kelm, J. M., Timmins, N. E., Brown, C. J., Fussenegger, M., & Nielsen, L. K. (2003). Method for generation of homogeneous multicellular tumor spheroids applicable to a wide variety of cell types. *Biotechnology and Bioengineering, 83*, 173–180.

Klimkiewicz, K., Weglarczyk, K., Collet, G., Paprocka, M., Guichard, A., Sarna, M., Jozkowicz, A., Dulak, J., Sarna, T., Grillon, C., & Kieda, C. (2017). A 3D model of tumour angiogenic microenvironment to monitor hypoxia effects on cell interactions and cancer stem cell selection. *Cancer Letters, 396*, 10–20.

Koban, R., Neumann, M., Daugs, A., Bloch, O., Nitsche, A., Langhammer, S., & Ellerbrok, H. (2018). A novel three-dimensional cell culture method enhances antiviral drug screening in primary human cells. *Antiviral Research, 150*, 20–29.

Koledova, Z. (2017). 3D cell culture: An introduction. *Methods in Molecular Biology (Clifton, N.J.), 1612*, 1–11. https://doi.org/10.1007/978-1-4939-7021-6_1

Lee, C. H., Singla, A., & Lee, Y. (2001). Biomedical applications of collagen. *International Journal of Pharmaceutics, 221*(1–2), 1–22.

Longati, P., Jia, X., Eimer, J., Wagman, A., Witt, M. R., Rehnmark, S., Verbeke, C., Toftgård, R., Löhr, M., & Heuchel, R. L. (2013). 3D pancreatic carcinoma spheroids induce a matrix-rich, chemoresistant phenotype offering a better model for drug testing. *BMC Cancer, 13*, 95.

Ma, J., Li, N., Wang, Y., Wang, L., Wei, W., Shen, L., Sun, Y., Jiao, Y., Chen, W., & Liu, J. (2018). Engineered 3D tumour model for study of glioblastoma aggressiveness and drug evaluation on a detachably assembled microfluidic device. *Biomedical Microdevices, 20*(3), 80.

Mazzocchi, A., Soker, S., & Skardal, A. (2019). 3D bioprinting for high-throughput screening: Drug screening, disease modeling, and precision medicine applications. *Applied Physics Reviews, 6*(1), 011302.

Negri, S., Fila, C., Farinato, S., Bellomi, A., & Pagliaro, P. P. (2007). Tissue engineering: Chondrocyte culture on type 1 collagen support. Cytohistological and immunohistochemical study. *Journal of Tissue Engineering and Regenerative Medicine, 1*(2), 158–159.

Nörz, D., Mullins, C. S., Smit, D. J., Linnebacher, M., Hagel, G., Mirdogan, A., Siekiera, J., Ehm, P., Izbicki, J. R., Block, A., Thastrup, O., & Jücker, M. (2021). Combined targeting of AKT and mTOR synergistically inhibits formation of primary colorectal carcinoma tumouroids in vitro: A 3D tumour model for pre-therapeutic drug screening. *Anticancer Research, 41*(5), 2257–2275.

Pavlou, M., Shah, M., Gikas, P., Briggs, T., Roberts, S. J., & Cheema, U. (2019). Osteomimetic matrix components alter cell migration and drug response in a 3D tumour-engineered osteosarcoma model. *Acta Biomaterialia, 96*, 247–257.

Peela, N., Sam, F. S., Christenson, W., Truong, D., Watson, A. W., Mouneimne, G., Ros, R., & Nikkhah, M. (2016). A three dimensional micropatterned tumor model for breast cancer cell migration studies. *Biomaterials, 81*, 72–83.

Ravi, M., Paramesh, V., Kaviya, S. R., Anuradha, E., & Paul Solomon, F. D. (2015). 3D cell culture systems: Advantages and applications. *Journal of Cellular Physiology, 230*(1), 16–26.

Rodrigues, M. T., Carvalho, P. P., Gomes, M. E., & Reis, R. L. (2015). Biomaterials in preclinical approaches for engineering skeletal tissues. In *Translational Regenerative Medicine*. Elsevier Inc.

Rogina, A., Pušić, M., Štefan, L., Ivković, A., Urlić, I., Ivanković, M., & Ivanković, H. (2021). Characterization of chitosan-based scaffolds seeded with sheep nasal chondrocytes for cartilage tissue engineering. *Annals of Biomedical Engineering, 49*(6), 1572–1586.

Ryan, S. L., Baird, A. M., Vaz, G., Urquhart, A. J., Senge, M., Richard, D. J., O'Byrne, K. J., & Davies, A. M. (2016). Drug discovery approaches utilizing three-dimensional cell culture. *Assay and Drug Development Technologies, 14*(1), 19–28.

Sakalem, M. E., De Sibio, M. T., da Costa, F., & de Oliveira, M. (2021). Historical evolution of spheroids and organoids, and possibilities of use in life sciences and medicine. *Biotechnology Journal, 16*(5), e2000463.

Timmins, N. E., & Nielsen, L. K. (2007). Generation of multicellular tumor spheroids by the hanging-drop method. *Methods in Molecular Medicine, 140*, 141–151.

van Duinen, V., Trietsch, S. J., Joore, J., Vulto, P., & Hankemeier, T. (2015). Microfluidic 3D cell culture: From tools to tissue models. *Current Opinion in Biotechnology, 35*, 118–126.

Wang, J., Sun, Q., Wei, Y., Hao, M., Tan, W. S., & Cai, H. (2021). Sustained release of epigallocatechin-3-gallate from chitosan-based scaffolds to promote osteogenesis of mesenchymal stem cells. *International Journal of Biological Macromolecules, 176*, 96–105.

Wu, C. G., Chiovaro, F., Curioni-Fontecedro, A., Casanova, R., & Soltermann, A. (2020). In vitro cell culture of patient derived malignant pleural and peritoneal effusions for personalised drug screening. *Journal of Translational Medicine, 18*(1), 163.

Xu, K., Wang, Z., Copland, J. A., Chakrabarti, R., & Florczyk, S. J. (2020). 3D porous chitosan-chondroitin sulfate scaffolds promote epithelial to mesenchymal transition in prostate cancer cells. *Biomaterials, 254*, 120126.

Yamada, K. M., & Cukierman, E. (2007). Modeling tissue morphogenesis and cancer in 3D. *Cell, 130*(4), 601–610.

Yamaguchi, Y., Deng, D., Sato, Y., Hou, Y. T., Watanabe, R., Sasaki, K., Kawabe, M., Hirano, E., & Morinaga, T. (2013). Silicate fiber-based 3D cell culture system for anticancer drug screening. *Anticancer Research, 33*(12), 5301–5309.

Young, M., Rodenhizer, D., Dean, T., D'Arcangelo, E., Xu, B., Ailles, L., & McGuigan, A. P. (2018). A TRACER 3D co-culture tumour model for head and neck cancer. *Biomaterials, 164*, 54–69.

Zhou, Y., Hutmacher, D. W., Varawan, S.-L., Lim, T. M., Zhou, Y., Hutmacher, D. W., Varawan, S.-L., & Lim, T. M. (2006). Effect of collagen-I modified composites on proliferation and differentiation of human alveolar osteoblasts. *Australian Journal of Chemistry, 59*(8), 571–578.

Ziółkowska-Suchanek, I. (2021). Mimicking tumor hypoxia in non-small cell lung cancer employing three-dimensional in vitro models. *Cells, 10*(1), 141.