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# An Overview of Three-Dimensional Culture Systems for Recapitulation Human Organs in Research: Advances and Applications

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## ABSTRACT

Cell culture is a vital method in biological and biomedical research. The global cell culture market, valued at around USD 26.54 billion in 2023, is projected to surpass USD 63.60 billion by 2032. While two-dimensional cell culture has led to significant advancements in biology, its simplicity does not accurately reflect the complex *in vivo* environment. This can result in misleading data with limited predictive value for *in vivo* applications, prompting increased interest in three-dimensional (3D) cultivation methods. The 3D cell culture mimics the behavior and organization of cells *in vivo* by emulating the extracellular matrix (ECM), providing better insights into 3D interactions among cells and between cells and the matrix, thus reconstructing their natural microenvironment. In this review we will outline the various types of 3D models (include spheroids, organoids, bio-printed structures, and tissue chips). Subsequently, we will examine the methodologies employed to develop 3D culture systems (include four category methods). Lastly, the practical applications and challenges of these 3D models will be addressed. The future research will likely concentrate on incorporating cutting-edge technologies to improve the reproducibility and applicability of 3D models in research.

**Keywords:** Cell culture, 3D models, Spheroid, Organoid, Matrix-based structures, Microfluidic system, Personalized medicine, Cancer research



## INTRODUCTION

Cell culture is a vital method in biological and biomedical research, involving the cultivation of cells in a controlled environment with regulated parameters like temperature, pH, and oxygen levels. This allows researchers to grow and manipulate cells outside their natural environment, providing a valuable model for studying key life processes such as growth, metabolism, and differentiation (1). Cultured cells are valuable models for studying diseases like cancer, genetic disorders, and infections, allowing researchers to explore cellular mechanisms. They also help assess new pharmaceuticals' effects on cellular behavior before human trials, aiding in identifying promising therapies and understanding their mechanisms. Advancements in cell culture methods have enhanced tissue engineering, allowing for the cultivation of cells to create artificial tissues for transplantation and regenerative medicine (2). The global cell culture market is expected to grow significantly due to rising awareness of cell-based vaccines, more funding for research, increasing chronic disease rates, greater use of single-use technologies, higher demand for monoclonal antibodies, a shift towards personalized medicine, and the launch of innovative cell culture products. The global cell culture market, valued at around USD 26.54 billion in 2023, is projected to surpass USD 63.60 billion by 2032,

with a compound annual growth rate (CAGR) of 10.20% from 2023 to 2032 (3).

Cell cultures can be categorized into three main types: adherent culture, suspension culture, and three-dimensional (3D) culture. Adherent cells are typically grown in two-dimensional (2D) monolayers on plastic dishes or coated substrates, known as adherent culture. Suspension culture is a method where cells grow in liquid media without attachment, often used for specific cell types like hematopoietic cells. Three-dimensional culture cultivates cells in environments that better mimic *in vivo* conditions and enable more complex interactions (4).

The 2D *in vitro* cell culture system is a traditional method that uses a flat substrate for growing cells in a monolayer, allowing for the study of various cell types and drug screening. This system has been used in research since the early 1900s, especially for co-culturing cellular heterogeneity and assessing bioactive compounds in cancer research (5). While 2D cell culture has led to significant advancements in biology, its simplicity does not accurately reflect the complex *in vivo* environment, including cell morphology, signaling, differentiation, and biochemical interactions. This can result in misleading data with limited predictive value for *in vivo* applications (6), prompting increased interest in 3D cultivation methods. The first 3D cell culture model was introduced in 1992 by Petersen and Bissell to replicate breast tissue in cancerous and non-cancerous conditions (7). The 3D cell culture mimics the behavior and organization of cells in vivo by emulating the extracellular matrix (ECM), providing better insights into 3D interactions among cells and between cells and the matrix, thus reconstructing their natural microenvironment (5, 8).

The ECM is a framework of non-cellular fibrillary proteins and structural macromolecules that provide structural and biochemical support to cells. (9). Cell-binding sites that regulate cell adhesion and migration are established by the ECM (10). The ECM consists of: ***(i)*** Interstitial ECM (stromal), which includes proteins like collagen I and polysaccharides such as fibronectin, hyaluronic acid, and proteoglycans; ***(ii)*** The basement membrane, located beneath epithelial or endothelial cells, aids in gas diffusion and signaling molecule transport while acting as a barrier between epithelial cells and connective tissue (11, 12). To achieving 3D culture models, four category methods can be done: scaffold-free methods, scaffold-based methods, 3D printing technologies, and microfluidic systems (5). Initially, this review will outline the various types of 3D models. Subsequently, we will examine the methodologies employed to develop 3D culture systems and their associated benefits. Lastly, the practical applications and challenges of these 3D models will be addressed. The content of this review article is summarized in **Table 1**.

## 3D Cell Culture Models

### Spheroids

One of the earliest 3D models, characterized by spheroids, was developed in 1970 by Sutherland and his team to replicate cancer cell behavior and study their responses to radiotherapy (13). Spheroids are cellular clusters made from one or multiple cell types, typically grown as free-floating aggregates (14). Spheroids have an outer layer of cells in contact with the environment, with additional layers inside, creating a nutrient and oxygen gradient that mimics *in vivo* conditions. As a result, cells within the sphere can display proliferative, non-proliferative, or necrotic characteristics based on their location (15). Spheroids are sophisticated models used to investigate cellular behavior, especially in the fields of cancer research and drug development (16).

### Organoids

Organoids are 3D miniatures that mimic the structure and function of human organs (17). Organoids represent a more intricate model system, typically originating from the self-organization of pluripotent stem cells (induced pluripotent stem cells or embryonic stem cells) or cells derived from tissues, including cancer cells. They serve as a robust platform for investigating human biology, the mechanisms underlying diseases, and responses to pharmacological agents. Their inherent complexity facilitates extended culture periods, a capability that is not achievable with spheroids (18).

### Matrix-based Structures

As one of the most important and widely used 3D model based on the matrix, hydrogels are adaptable materials used to develop 3D cell culture models that replicate the natural ECM and tissue microenvironment. They offer a biomimetic framework that facilitates cellular growth and interaction. Hydrogel-based models have revolutionized biomedical research, especially in tissue engineering and drug development, thanks to their hydrophilic polymer networks that retain moisture while ensuring structural integrity (19). Hydrogels may consist of natural, synthetic, or hybrid polymers, with each type providing distinct characteristics that can be customized for particular uses (20).

### Bio-printed structures

Bio-printed structures are advanced 3D constructs created using layer-by-layer 3D printing technology that deposits living cells, biomaterials, and bioactive molecules to form tissue-like architectures. This innovative technique shows great potential in regenerative medicine, drug testing, and tissue engineering, driven by the global shortage of organs needed for restoring damaged tissues. Key applications include engineered skin, cartilage, bone, cardiac tissue, and vascular grafts (21, 22).

### Microfluidic-based structures

Organ-on-a-chip technology has gained significant attention for its applications in precision medicine, drug development, and screening. This innovative platform mimics the structure and function of human organs by integrating biology and engineering, creating biomimetic systems that simulate physiological conditions. It usually includes tiny channels that are covered with living cells, enabling the replication of organ-level functions in dynamic environments. (23-25).

## METHODS TO ESTABLISH A 3D CELL CULTURE

### Scaffold-free Methods

Scaffold-free methods mean methods that do not require scaffolds depend on the natural ability of cells to aggregate without external support, using particular cell plates and physical parameters. Four primary methods exist for cells to aggregate and form scaffold-free 3D structures:

i) The **forced-floating technique** utilizes well plates coated with low-adhesion polymer and is the most straightforward method for 3D cell culture. Initially, cells are centrifuged to form a pellet, which is then suspended in a spheroid culture medium and placed in multi-well plates with a low-adhesion surface. Research has demonstrated that this method can stimulate the chondrogenic differentiation of MSCs (26). Dental pulp spheroids (27), bone marrow, and endothelial spheroids have also been successfully cultured using pellet cultures (28). This approach is classified as a static culture method for producing 3D models.

ii) The **hanging drop technique**, another form of static cell culture, involves placing a small amount of cell suspension into micro trays to form and shape spheroids in the shape of droplets (29). After preparing the cell suspension, a drop of the culture medium containing the desired number of cells is deposited on the lid of a plate, and the lid is then turned upside-down. This technique relies on surface tension and gravitational force to create a 3D cell aggregate within the droplets. It is possible to regulate the size of the spheroid (29). Hurrell et al. utilized the hanging drop technique to establish hepatic spheroids (30), and Gupta et al. generated cancer spheroids using this method (31).

iii) In the **magnetic levitation technique**, a solution of magnetic nanoparticles is combined with cells and exposed to a magnetic force. The cells are allowed to internalize the magnetic nanoparticles after an overnight incubation. Subsequently, the cells are detached and placed in low-adhesive plates. A magnet positioned on the plate lid generates a magnetic force, causing the cells to levitate in opposition to gravity. This promotes cell-cell interactions, resulting in cell aggregation (32, 33).

iv) The **agitation-based methods** use a continuously rotating bioreactor to simulate microgravity. By gradually turning isolated cells into aggregates, the cell suspension prevents them from adhering to the container wall due to constant stirring, resulting in a wide variety of non-uniform spheroids. This approach is considered a form of dynamic cell culture. One of the primary challenges of using static cell culture conditions to create 3D models is the exchange of nutrients, as cell aggregates can grow to a thickness of 1–2 mm, hindering the transfer of gases and waste products. Bioreactors enable the creation of dynamic 3D cell cultures by regulating various parameters of the

extracellular microenvironment, such as flow rate, oxygen levels, temperature, pH, nutrients, and waste products. Multiple bioreactor designs are available, including direct perfusion systems, spinner flasks, and others (15). Bioreactor systems allow for precise manipulation of environmental variables, enabling the investigation of fundamental cell functions in a 3D setting and the enhancement of engineered tissue quality. Furthermore, through the automated and standardized production of tissues in enclosed systems, bioreactors have the potential to lower manufacturing expenses, making engineered tissues more accessible (34).

### Scaffold-based Methods

Cells in scaffold-based methods are cultivated on or within a supportive matrix that imitates the ECM. These matrices can be specifically designed to have pores, which enable the exchange of nutrients and waste while encouraging cell adhesion and growth. To establish this system, cells are introduced into natural or synthetic materials, allowing for cell proliferation, aggregation, and 3D organization. The key characteristics for a biomaterial to be deemed suitable for scaffold preparation include high biocompatibility, biodegradability, reactivity to cell adhesion, elasticity, and minimal toxicity (15, 35).

Scaffold materials like silk fibroin, chitosan, collagen, and alginate contain bioactive signals necessary for cell growth, whereas synthetic scaffolds, such as polyethylene glycol (PEG), polycaprolactone (PCL), poly L-lactic acid (PLLA), and polyacrylamide (PAm), as well as metals and ceramics like calcium phosphate biomaterials and hydroxyapatite, offer adjustable mechanical properties that can simulate the rigidity of different tissues. To address the limitations of using natural or synthetic biomaterials alone for scaffold preparation, hybrid scaffolds with multiple components have been created to overcome the weaknesses of each individual material (36-38).

### Bio-printing Techniques

In tissue/organ engineering, the primary 3D bioprinting methods employed are inkjet-based, extrusion-based, laser-assisted, stereolithography (SLA)-based, and scaffold-free-based bioprinting techniques. The key distinctions among these methods lie in their print speed, resolution, cell viability, and the biological materials utilized for bioprinting (39, 40).

### Microfluidic Systems

Microfluidic devices are an advanced technique for culturing cells in 3D. These tools are highly valuable in modern science, offering innovative answers for various uses and continuously advancing with technological developments (41). Tiny wells connected by microfluidic pathways make up microfluidic setups, allowing continuous delivery of nutrients and growth stimulants. Using microfluidic systems, it is possible to culture different types of cells at the same time, as demonstrated by Sun et al., who developed a method for drug testing by growing spheroids that contain both tumor cells and fibroblasts (23, 42). These devices make use of microfluidic channels to regulate the movement of fluids and nutrients, closely imitating the dynamic conditions present in real organs. These channels enable precise control of the microenvironment, including shear stress and concentration gradients, which are crucial for preserving cell function. Various models have been effectively created using different organs, such as lung on a chip, liver on a chip, kidney on a chip, heart on a chip, intestine on a chip, and skin on a chip (25). A fundamental technique for fabricating tissue chips is soft lithography, which is widely recognized as the predominant method for producing organ chips, especially those composed of polydimethylsiloxane (PDMS). This technique entails the development of a master mold through photolithography methods, which subsequently serves as a template for casting PDMS (25, 41).

## APPLICATIONS OF 3D CELL CULTURE MODELS

### Organ physiology and Disease Modeling

3D cell cultures have been used to replicate different organs and tissues like bone, brain, heart, liver, lung, and skin. In vitro 3D models are now crucial for developing and evaluating potential treatments and approaches to enhance the regeneration process. They are valuable for studying and enhancing the incorporation of newly formed tissue with the surrounding

environment (15). In this section, we provide instances of how 3D culture models are utilized in research pertaining to various organs or tissues:

Studying bone trauma, articular cartilage degeneration, and bone diseases like osteoporosis relies heavily on 3D cell culture models (43). The testing of potential treatments, enhancement of tissue integration, and examination of bone remodeling events are all areas where 3D cell culture models provide assistance. A micron-scale bone organoid prototype, known as a human trabecular organoid, has been developed by Iordachescu and colleagues. This prototype is used to explore the impact of microgravity, degenerative events, and associated temporal occurrences on bone remodeling, which cannot be replicated using other in vitro and in vivo technologies (44). Caire and colleagues created 3D models by utilizing synoviocytes from individuals with rheumatoid arthritis (RA) and merged them with Matrigel to study the long-term inflammatory bone condition (45). Recent research indicates that human neuron characteristics, like transcriptional patterns, can be replicated by 3D neuronal cultures (46). Furthermore, a 3D brain organoid was established by Abud and colleagues, incorporating iPSC-derived human microglia-like cells that exhibit functional properties and transcriptome profile similar to human microglia (47). The use of 3D cell cultures is important for studying conditions such as non-alcoholic fatty liver disease (NAFLD) (48) and viral infections like hepatitis C (49) and SARS-CoV-2 (50). These models enable scientists to replicate disease conditions, examine the effectiveness of antifibrotic properties, investigate viral infections, and assess the impact of pathogens on particular organs such as the liver (51). In 2021, Leibel et al. demonstrated the use of 3D models in research by creating a 3D whole-lung organoid from iPSCs. This organoid is valuable for investigating the development and maturation of the lungs through branching morphogenesis. (52).

**Toxicology and Drug Screening Studies**

Three-dimensional cell culture systems are increasingly being recognized as a transformative approach in toxicology drug screening. These systems provide a more accurate representation of human physiology compared to traditional 2D cultures, which often fail to mimic the complex interactions and environments found in vivo. Engineered microtissues in 3D are effective for drug testing, particularly in the study of cardiotoxicity. They offer a way to evaluate how different drugs impact cardiac tissues, allowing for a more precise forecast of drug reactions and toxicity (53). The combination of various cell types in these models results in a more mature reaction to pharmacological substances, which helps in forecasting the inotropic impacts of medications and comprehending the role of non-myocyte cardiac cells in cardiotoxicity (54). The development of bone chip systems and bone metastasis models for pre-clinical pharmaceutical testing makes use of 3D cell cultures. These models play a crucial role in assessing the effects of substances like vitamin K2 (55) and celastrol (56) on bone microenvironments, enhancing the functions of osteoblasts and preventing bone loss.

**Precision Medicine and Regeneration Study**

3D culture systems represent a significant leap forward in regenerative medicine, offering tools that enhance our understanding of cellular behavior in a more physiologically relevant context. Three-dimensional cell cultures offer a more accurate in vivo representation, diverse nutrient concentrations, and allow cell proliferation without immortalization. They also enable gene editing in patient-derived organoids ex vivo, significantly speeding up the development of transgenic mouse models (57). In bone tissue engineering, 3D models play a crucial role by facilitating the development of bone constructs using mesenchymal stem cells (MSCs). These models assist in the design of scaffolds that imitate the natural architecture of bone, thereby improving osteogenic differentiation and promoting bone regeneration (38). In skin tissue engineering, skin models in 3D have been created for the purpose of healing wounds and transforming them into fully functional skin (58). Skin substitutes such as Dermagraft have been developed based on these models, offering long-lasting coverage for extensive wounds and enhancing the results of skin regeneration (59). Using 3D cell culture techniques, scientists have effectively created

sweat gland organoids. These organoids, which are derived from human epidermal keratinocytes in a 3D Matrigel system, demonstrate potential for regenerative treatment in individuals with extensive skin injuries. They facilitate the formation of new sweat glands and tissue replacement therapy (60).

## Cancer research

3D cell cultures have become a revolutionary approach in cancer research, offering significant advantages over traditional 2D systems. Tumor tissue chips have been utilized in 3D cell cultures for screening anticancer drugs. Testing drug-tissue interactions and characterizing drug kinetics is enabled by these models, which help in the identification of effective drugs for eliminating cancer cells (5). 3D cell cultures are utilized for investigating the connections between cancer cells and the tumor microenvironment (TME). These models replicate the intricate web of interactions among cancer cells, ECM, and stromal cells, offering valuable insights into cancer cell behavior and their reactions to treatments (61). 3D models are utilized by researchers for investigating the processes of cancer cell migration and invasion. These models provide a more realistic portrayal of cell motility and invasion in contrast to traditional 2D cultures, which helps in comprehending the mechanisms behind tumor spreading and invasion. Currently, the utilization of 3D microfluidic systems may offer an improved model for studying cancer cell migration and, consequently, tumor spreading. A new model has been suggested by Goh et al. for visualizing and quantifying migrating cells in a 3D microfluidic plate filled with Matrigel, under various extracellular stimuli, such as nutrient gradient, cytokines, and co-culture with fibroblasts (62, 63).

**Table 1.** Properties of 3D Cell Culture Models.

| 3D Cell Culture Models | Techniques to obtain | Features | Applications |
|---|---|---|---|
| Spheroid | -Hanging drop method<br>-Low-attachment plates<br>-Agitation-based methods<br>-Magnetic levitation technique | -Metabolic similarity to tissues<br>-Cellular heterogeneity<br>-Enhanced cell-cell interactions | -Cancer research<br>-Regeneration Study<br>-Drug testing |
| Organoid | -Hanging drop method<br>-Low-attachment plates<br>-Agitation-based methods<br>-Embedding in matrices | -Self-organization<br>-Organ-like structures<br>-Cellular heterogeneity<br>-Maintaining functional properties over time | -Cancer research<br>-Organ and disease modeling<br>-Drug discovery and development<br>-Precision Medicine<br>-Gene editing |
| Matrix-based structure | Seeding cells on/within scaffold | -Hydrophilic polymer network<br>-Tissue-like stiffness<br>-Biocompatibility<br>-Nutrient and waste transport<br>-Customizable composition | -Drug Development<br>-Regeneration Study<br>-Tissue engineering |
| Bio-printed structure | 3D Printing techniques | -Self-assembly<br>-Biomimicry<br>-Customizable bio-inks | -Organ and disease modeling<br>-Drug discovery and development<br>-Tissue engineering |
| Microfluidic-based structure | Soft lithography and microfluidic design | -Real-time monitoring<br>-Multi-organ systems<br>-Mimicking real organ environments | -Organ and disease modeling<br>-Personalized medicine<br>-Toxicology and Drug Screening Studies |

## CONCLUSION

3D cell culture models have the ability to accurately replicate cell behavior and organization seen in living organisms. Due to their distinctive characteristics and increasing potential applications, 3D cell models are being increasingly suggested as viable substitutes for in vivo models in studying both physiological and pathological processes, as well as in understanding pharmacological responses. When using animals for experimentation, it is important to consider various complex factors, including the high costs associated with managing and caring for laboratory animals, the ethical considerations related to animal welfare in experiments, particularly concerning potential causes of their distress and fear, and the issue of how applicable results from animal studies are to humans (64). Many countries are working towards ending animal testing by 2035 by adopting the 3Rs approach: reduce, refine, and replace (65). Although 3D cell culture structures offer benefits, they also encounter challenges. The creation methods involving complexity and variability can result in inconsistent outcomes, posing difficulties for standardization. Additionally, numerous 3D models still do not feature fully functional blood vessel networks, which may restrict the delivery of nutrients and oxygen in larger constructs. The upcoming research will likely concentrate on incorporating cutting-edge technologies like artificial intelligence and high-throughput screening to improve the reproducibility and applicability of 3D models in research.

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There is nothing to say.

## CONFLICTS OF INTEREST

The authors report no conflicts of interest.

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## REFERENCE

[1] Leland DS, and Ginocchio CC. Role of cell culture for virus detection in the age of technology. *Clinical microbiology reviews* 20: 49-78, 2007.

[2] Weiskirchen S, Schröder SK, Buhl EM, and Weiskirchen R. A Beginner's Guide to Cell Culture: Practical Advice for Preventing Needless Problems. *Cells* 12: 2023.

[3] Cell Culture Market Size, Share, and Trends 2024 to 2034. https://www.precedenceresearch.com/cell-culture-market.

[4] Justice BA, Badr NA, and Felder RA. 3D cell culture opens new dimensions in cell-based assays. *Drug Discovery Today* 14: 102-107, 2009.

[5] Habanjar O, Diab-Assaf M, Caldefie-Chezet F, and Delort L. 3D Cell Culture Systems: Tumor Application, Advantages, and Disadvantages. *Int J Mol Sci* 22: 2021.

[6] LaBarbera DV, Reid BG, and Yoo BH. The multicellular tumor spheroid model for high-throughput cancer drug discovery. *Expert opinion on drug discovery* 7: 819-830, 2012.

[7] Petersen OW, Rønnov-Jessen L, Howlett AR, and Bissell MJ. Interaction with basement membrane serves to rapidly distinguish growth and differentiation pattern of normal and malignant human breast epithelial cells. *Proceedings of the National Academy of Sciences* 89: 9064-9068, 1992.

[8] Redmond J, McCarthy H, Buchanan P, Levingstone TJ, and Dunne NJ. Advances in biofabrication techniques for collagen-based 3D in vitro culture models for breast cancer research. *Materials Science and Engineering: C* 122: 111944, 2021.

[9] Geiger B, and Yamada KM. Molecular architecture and function of matrix adhesions. *Cold Spring Harbor perspectives in biology* 3: a005033, 2011.

[10] Brown NH. Extracellular matrix in development: insights from mechanisms conserved between invertebrates and vertebrates. *Cold Spring Harbor perspectives in biology* 3: a005082, 2011.

[11] Frantz C, Stewart KM, and Weaver VM. The extracellular matrix at a glance. *Journal of cell science* 123: 4195-4200, 2010.

[12] Lu P, Takai K, Weaver VM, and Werb Z. Extracellular matrix degradation and remodeling in development and disease. *Cold Spring Harbor perspectives in biology* 3: a005058, 2011.

[13] Sutherland RM, Inch WR, McCredie JA, and Kruuv J. A Multi-component Radiation Survival Curve Using an in Vitro Tumour Model. *International Journal of Radiation Biology and Related Studies in Physics, Chemistry and Medicine* 18: 491-495, 1970.

[14] Gunti S, Hoke AT, Vu KP, and London Jr NR. Organoid and spheroid tumor models: techniques and applications. *Cancers* 13: 874, 2021.

[15] Urzì O, Gasparro R, Costanzo E, De Luca A, Giavaresi G, Fontana S, and Alessandro R. Three-Dimensional Cell Cultures: The Bridge between In Vitro and In Vivo Models. *International Journal of Molecular Sciences* 24: 12046, 2023.

[16] Białkowska K, Komorowski P, Bryszewska M, and Miłowska K. Spheroids as a Type of Three-Dimensional Cell Cultures-Examples of Methods of Preparation and the Most Important Application. *Int J Mol Sci* 21: 2020.

[17] Hofer M, and Lutolf MP. Engineering organoids. *Nature Reviews Materials* 6: 402-420, 2021.

[18] Yi SA, Zhang Y, Rathnam C, Pongkulapa T, and Lee K-B. Bioengineering Approaches for the Advanced Organoid Research. *Advanced Materials* 33: 2007949, 2021.

[19] Maji S, and Lee H. Engineering Hydrogels for the Development of Three-Dimensional In Vitro Models. *Int J Mol Sci* 23: 2022.

[20] Zhao Z, Vizetto-Duarte C, Moay ZK, Setyawati MI, Rakshit M, Kathawala MH, and Ng KW. Composite Hydrogels in Three-Dimensional in vitro Models. *Frontiers in Bioengineering and Biotechnology* 8: 2020.

[21] Deo KA, Singh KA, Peak CW, Alge DL, and Gaharwar AK. Bioprinting 101: Design, Fabrication, and Evaluation of Cell-Laden 3D Bioprinted Scaffolds. *Tissue Eng Part A* 26: 318-338, 2020.

[22] Papaioannou TG, Manolesou D, Dimakakos E, Tsoucalas G, Vavuranakis M, and Tousoulis D. 3D Bioprinting Methods and Techniques: Applications on Artificial Blood Vessel Fabrication. *Acta Cardiol Sin* 35: 284-289, 2019.

[23] Ortiz-Cárdenas JE, Zatorski JM, Arneja A, Montalbine AN, Munson JM, Luckey CJ, and Pompano RR. Towards spatially-organized organs-on-chip: Photopatterning cell-laden thiol-ene and methacryloyl hydrogels in a microfluidic device. *Organs-on-a-Chip* 4: 100018, 2022.

[24] Leung CM, de Haan P, Ronaldson-Bouchard K, Kim G-A, Ko J, Rho HS, Chen Z, Habibovic P, Jeon NL, Takayama S, Shuler ML, Vunjak-Novakovic G, Frey O, Verpoorte E, and Toh Y-C. A guide to the organ-on-a-chip. *Nature Reviews Methods Primers* 2: 33, 2022.

[25] Danku AE, Dulf E-H, Braicu C, Jurj A, and Berindan-Neagoe I. Organ-On-A-Chip: A Survey of Technical Results and Problems. *Frontiers in Bioengineering and Biotechnology* 10: 2022.

[26] Grigull NP, Redeker JI, Schmitt B, Saller MM, Schönitzer V, and Mayer-Wagner S. Chondrogenic Potential of Pellet Culture Compared to High-Density Culture on a Bacterial Cellulose Hydrogel. *International Journal of Molecular Sciences* 21: 2785, 2020.

[27] Zhang S, Buttler-Buecher P, Denecke B, Arana-Chavez VE, and Apel C. A comprehensive analysis of human dental pulp cell spheroids in a three-dimensional pellet culture system. *Archives of Oral Biology* 91: 1-8, 2018.

[28] Inglis S, Kanczler JM, and Oreffo ROC. 3D human bone marrow stromal and endothelial cell spheres promote bone healing in an osteogenic niche. *The FASEB Journal* 33: 3279-3290, 2019.

[29] Bartosh TJ, Ylöstalo JH, Mohammadipoor A, Bazhanov N, Coble K, Claypool K, Lee RH, Choi H, and Prockop DJ. Aggregation of human mesenchymal stromal cells (MSCs) into 3D spheroids enhances their antiinflammatory properties. *Proc Natl Acad Sci U S A* 107: 13724-13729, 2010.

[30] Hurrell T, Ellero AA, Masso ZF, and Cromarty AD. Characterization and reproducibility of HepG2 hanging drop spheroids toxicology in vitro. *Toxicol In Vitro* 50: 86-94, 2018.

[31] Gupta P, Kar S, Kumar A, Tseng FG, Pradhan S, Mahapatra PS, and Santra TS. Pulsed laser assisted high-throughput intracellular delivery in hanging drop based three dimensional cancer spheroids. *Analyst* 146: 4756-4766, 2021.

[32] Lewis NS, Lewis EEL, Mullin M, Wheadon H, Dalby MJ, and Berry CC. Magnetically

levitated mesenchymal stem cell spheroids cultured with a collagen gel maintain phenotype and quiescence. *Journal of Tissue Engineering* 8: 2041731417704428, 2017.

[33] Miyamoto Y, Koshidaka Y, Noguchi H, Oishi K, Saito H, Yukawa H, Kaji N, Ikeya T, Suzuki S, Iwata H, Baba Y, Murase K, and Hayashi S. Observation of Positively Charged Magnetic Nanoparticles inside HepG2 Spheroids Using Electron Microscopy. *Cell Medicine* 5: 89-96, 2013.

[34] Martin I, Wendt D, and Heberer M. The role of bioreactors in tissue engineering. *Trends in Biotechnology* 22: 80-86, 2004.

[35] Moradi N, Kaviani S, Soufizomorrod M, Hosseinzadeh S, and Soleimani M. Preparation of poly (acrylic acid)/tricalcium phosphate nanoparticles scaffold: Characterization and releasing UC-MSCs derived exosomes for bone differentiation. *BioImpacts: BI* 13: 425, 2023.

[36] Chaudhary S, and Chakraborty E. Hydrogel based tissue engineering and its future applications in personalized disease modeling and regenerative therapy. *Beni-Suef University Journal of Basic and Applied Sciences* 11: 3, 2022.

[37] Edmondson R, Broglie JJ, Adcock AF, and Yang L. Three-Dimensional Cell Culture Systems and Their Applications in Drug Discovery and Cell-Based Biosensors. *ASSAY and Drug Development Technologies* 12: 207-218, 2014.

[38] Moradi N, Soufi-Zomorrod M, Hosseinzadeh S, and Soleimani M. Poly (acrylic acid)/tricalcium phosphate nanoparticles scaffold enriched with exosomes for cell-free therapy in bone tissue engineering: An in vivo evaluation. *Bioimpacts* 14: 27510-27510, 2024.

[39] Wu CA, Zhu Y, and Woo YJ. Advances in 3D bioprinting: Techniques, applications, and future directions for cardiac tissue engineering. *Bioengineering* 10: 842, 2023.

[40] 40. Lu T-Y, Xiang Y, Tang M, and Chen S. 3D printing approaches to engineer cardiac tissue. *Current cardiology reports* 25: 505-514, 2023.

[41] Niculescu AG, Chircov C, Bîrcă AC, and Grumezescu AM. Fabrication and Applications of Microfluidic Devices: A Review. *Int J Mol Sci* 22: 2021.

[42] Sun Q, Tan SH, Chen Q, Ran R, Hui Y, Chen D, and Zhao C-X. Microfluidic Formation of Coculture Tumor Spheroids with Stromal Cells As a Novel 3D Tumor Model for Drug Testing. *ACS Biomaterials Science & Engineering* 4: 4425-4433, 2018.

[43] Liu X, Li T, Wang F, Sun F, Hu J, Ye X, Wang D, and Yang X. Controlling sustained statins release in multi-layered composite scaffolds for healing of osteoporotic bone defects. *Biomaterials Advances* 137: 212838, 2022.

[44] Iordachescu A, Hughes EAB, Joseph S, Hill EJ, Grover LM, and Metcalfe AD. Trabecular bone organoids: a micron-scale 'humanised' prototype designed to study the effects of microgravity and degeneration. *npj Microgravity* 7: 17, 2021.

[45] Caire R, Audoux E, Courbon G, Michaud E, Petit C, Dalix E, Chafchafi M, Thomas M, Vanden-Bossche A, Navarro L, Linossier M-T, Peyroche S, Guignandon A, Vico L, Paul S, and Marotte H. YAP/TAZ: Key Players for Rheumatoid Arthritis Severity by Driving Fibroblast Like Synoviocytes Phenotype and Fibro-Inflammatory Response. *Frontiers in Immunology* 12: 2021.

[46] Tekin H, Simmons S, Cummings B, Gao L, Adiconis X, Hession CC, Ghoshal A, Dionne D, Choudhury SR, Yesilyurt V, Sanjana NE, Shi X, Lu C, Heidenreich M, Pan JQ, Levin JZ, and Zhang F. Effects of 3D culturing conditions on the transcriptomic profile of stem-cell-derived neurons. *Nature Biomedical Engineering* 2: 540-554, 2018.

[47] Abud EM, Ramirez RN, Martinez ES, Healy LM, Nguyen CHH, Newman SA, Yeromin AV, Scarfone VM, Marsh SE, Fimbres C, Caraway CA, Fote GM, Madany AM, Agrawal A, Kayed R, Gylys KH, Cahalan MD, Cummings BJ, Antel JP, Mortazavi A, Carson MJ, Poon WW, and Blurton-Jones M. iPSC-Derived Human Microglia-like Cells to Study Neurological Diseases. *Neuron* 94: 278-293.e279, 2017.

[48] Romualdo GR, Da Silva TC, de Albuquerque Landi MF, Morais JÁ, Barbisan LF, Vinken M, Oliveira CP, and Cogliati B. Sorafenib reduces steatosis-induced fibrogenesis in a human 3D co-culture model of non-alcoholic fatty liver disease. *Environmental Toxicology* 36: 168-176, 2021.

[49] Ananthanarayanan A, Nugraha B, Triyatni M, Hart S, Sankuratri S, and Yu H. Scalable

Spheroid Model of Human Hepatocytes for Hepatitis C Infection and Replication. *Molecular Pharmaceutics* 11: 2106-2114, 2014.

[50] Yang L, Han Y, Nilsson-Payant BE, Gupta V, Wang P, Duan X, Tang X, Zhu J, Zhao Z, Jaffré F, Zhang T, Kim TW, Harschnitz O, Redmond D, Houghton S, Liu C, Naji A, Ciceri G, Guttikonda S, Bram Y, Nguyen D-HT, Cioffi M, Chandar V, Hoagland DA, Huang Y, Xiang J, Wang H, Lyden D, Borczuk A, Chen HJ, Studer L, Pan FC, Ho DD, tenOever BR, Evans T, Schwartz RE, and Chen S. A Human Pluripotent Stem Cell-based Platform to Study SARS-CoV-2 Tropism and Model Virus Infection in Human Cells and Organoids. *Cell Stem Cell* 27: 125-136.e127, 2020.

[51] Cacciamali A, Villa R, and Dotti S. 3D cell cultures: Evolution of an ancient tool for new applications. *Frontiers in Physiology* 13: 836480, 2022.

[52] Leibel SL, McVicar RN, Winquist AM, and Snyder EY. Generation of 3D whole lung organoids from induced pluripotent stem cells for modeling lung developmental biology and disease. *J Vis Exp* 170: 62456, 2021.

[53] Ribas J, Sadeghi H, Manbachi A, Leijten J, Brinegar K, Zhang YS, Ferreira L, and Khademhosseini A. Cardiovascular Organ-on-a-Chip Platforms for Drug Discovery and Development. *Applied In Vitro Toxicology* 2: 82-96, 2016.

[54] Ravenscroft SM, Pointon A, Williams AW, Cross MJ, and Sidaway JE. Cardiac Non-myocyte Cells Show Enhanced Pharmacological Function Suggestive of Contractile Maturity in Stem Cell Derived Cardiomyocyte Microtissues. *Toxicological Sciences* 152: 99-112, 2016.

[55] Mandatori D, Penolazzi L, Pelusi L, Lambertini E, Michelucci F, Porreca A, Cerritelli P, Pipino C, Di Iorio A, Bruni D, Di Nicola M, Buda R, Piva R, and Pandolfi A. Three-Dimensional Co-Culture System of Human Osteoblasts and Osteoclast Precursors from Osteoporotic Patients as an Innovative Model to Study the Role of Nutrients: Focus on Vitamin K2. *Nutrients* 13: 2823, 2021.

[56] Ma H-P, Deng X, Chen D-Y, Zhu D, Tong J-L, Zhao T, Ma J-H, and Liu Y-Q. A microfluidic chip-based co-culture of fibroblast-like synoviocytes with osteoblasts and osteoclasts to test bone erosion and drug evaluation. *Royal Society Open Science* 5: 180528, 2018.

[57] Teriyapirom I, Batista-Rocha AS, and Koo B-K. Genetic engineering in organoids. *Journal of Molecular Medicine* 99: 555-568, 2021.

[58] Szymański Ł, Jęderka K, Cios A, Ciepelak M, Lewicka A, Stankiewicz W, and Lewicki S. A Simple Method for the Production of Human Skin Equivalent in 3D, Multi-Cell Culture. *International Journal of Molecular Sciences* 21: 4644, 2020.

[59] Marston WA, Hanft J, Norwood P, Pollak R, and Group ftDDFUS. The Efficacy and Safety of Dermagraft in Improving the Healing of Chronic Diabetic Foot Ulcers: Results of a prospective randomized trial. *Diabetes Care* 26: 1701-1705, 2003.

[60] Sun X, Xiang J, Chen R, Geng Z, Wang L, Liu Y, Ji S, Chen H, Li Y, Zhang C, Liu P, Yue T, Dong L, and Fu X. Sweat Gland Organoids Originating from Reprogrammed Epidermal Keratinocytes Functionally Recapitulated Damaged Skin. *Advanced Science* 8: 2103079, 2021.

[61] Li S, Yang K, Chen X, Zhu X, Zhou H, Li P, Chen Y, Jiang Y, Li T, Qin X, Yang H, Wu C, Ji B, You F, and Liu Y. Simultaneous 2D and 3D cell culture array for multicellular geometry, drug discovery and tumor microenvironment reconstruction. *Biofabrication* 13: 045013, 2021.

[62] Goh A, Yeh C-C, and Lei KF. Visualization and Quantification of 3D Tumor Cell Migration under Extracellular Stimulation. *ACS Applied Bio Materials* 3: 1506-1513, 2020.

[63] Polacheck WJ, Charest JL, and Kamm RD. Interstitial flow influences direction of tumor cell migration through competing mechanisms. *Proceedings of the National Academy of Sciences* 108: 11115-11120, 2011.

[64] Langhans SA. Three-Dimensional in Vitro Cell Culture Models in Drug Discovery and Drug Repositioning. *Front Pharmacol* 9: 6, 2018.

[65] Tannenbaum J, and Bennett BT. Russell and Burch's 3Rs then and now: the need for clarity in definition and purpose. *J Am Assoc Lab Anim Sci* 54: 120-132, 2015.