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Two-Dimensional and Three-Dimensional Cell Culture and Their Applications

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Abstract

Cell culture is one of the most important and commonly used *in vitro* tools to comprehend various aspects of cells or tissues of a living body such as cell biology, tissue morphology, mechanism of diseases, cell signaling, drug action, cancer research and also finds its great importance in preclinical trials of various drugs. There are two major types of cell cultures that are most commonly used- two-dimensional (2D) and three-dimensional culture (3D). The former has been used since the 1900s, owing to its simplicity and low-cost maintenance as it forms a monolayer, while the latter being the advanced version and currently most worked upon. This chapter intends to provide the true meaning and significance to both cultures. It starts by making a clear distinction between the two and proceeds further to discuss their different applications *in vitro*. The significance of 2D culture is projected through different assays and therapeutic treatment to understand cell motility and treatment of diseases, whereas 3D culture includes different models and spheroid structures consisting of multiple layers of cells, and puts a light on its use in drug discovery and development. The chapter is concluded with a detailed account of the production of therapeutic proteins by the use of cells.

Keywords: Cell culture, 2D culture, 3D culture, drug action, therapeutics

1. Introduction

The growth of cells in a controlled artificial environment isolated from their natural habitat is referred to as cell culture [1]. It is a significant tool used widely to study cell and molecular biology, screening drugs and toxicity analysis, the role of a particular gene in a disease, and cancer research. Due to their unique properties, they also have been tuned for screening and developing biopharmaceutical compounds such as vaccines and recombinant proteins. One of the major advantages of using cell culture is the homogenous and reproducible data generated [2].

Drug discovery is a lengthy and time-consuming process that undergoes several stages of testing and optimization. This encompasses identification of the target, lead discovery, pre-clinical validation, and clinical trials [3]. Therefore, it is very pertinent to obtain information about the biological activity, biochemical mechanisms, toxicity, and off-target interactions of drug molecules leading to the early stages of drug discovery. *In vitro*, cell-based assays prove futile to understand the

effects of drugs on the cells at an early stage of drug discovery which attributes an increased chance of development of drugs with good efficacy and safety [4].

Two-dimensional (2D) cell culture was introduced many decades ago that has been the major type of cell culture technique in numerous fields. This traditional approach has been extensively used for drug screening due to its relatively inexpensive feature and convenience to use. However, the issue of mimicking the *in vivo* environment restricts its use [5]. The 2D cell cultures grow as a monolayer in controlled flat environments, such as a glass or polystyrene flask that comprises live proliferating cells because of the detachment of dead cells from the surface (**Figure 1**). As a result, this leads to uniformity in nutrients and growth factors present in the medium to which the cells get access and proliferate at a faster rate than they would *in vivo* [6]. Thus, the morphology of the cells is completely changed as they appear flattered and stretched as compared to the *in vivo* environment. Besides this, the cell–cell interactions and cell-extracellular interactions become different in comparison to the tumor [7].

Recently there has been an upsurge of interest towards three-dimensional (3D) cell culture in biomedical research and drug development processes due to its high-throughput accuracy and refined *in vitro* models [8]. They have been broadly used in understanding the cell shape, cell–cell interaction, and the cellular environment that efficiently mimics the *in vivo* environment. 3D cultures grow as clusters or aggregates called spheroids either with a matrix or without a matrix [9] (**Figure 1**). There is a gradient of nutrients across these spheroids due to which the cells at the surface of spheroids proliferate more as compared to the cells that are present in the interior [10]. As a result of the difference in the proliferation rate, the cells in the spheroids are usually in different stages of the cell cycle such as proliferating, quiescent, hypoxic, and necrotic cells. In the 3D culture, the cells have uniform access to nutrients as in the case of a tumor. Also, the shape of the cells, cell–cell interactions, and cell-environment interactions are well defined in 3D culture [7]. It has also been observed that 2D cell cultures are more sensitive to drugs as compared to 3D cells [11]. This chapter intends to provide the true meaning and significance of both cultures. It starts by making a clear distinction between the two and proceeds further to discuss their
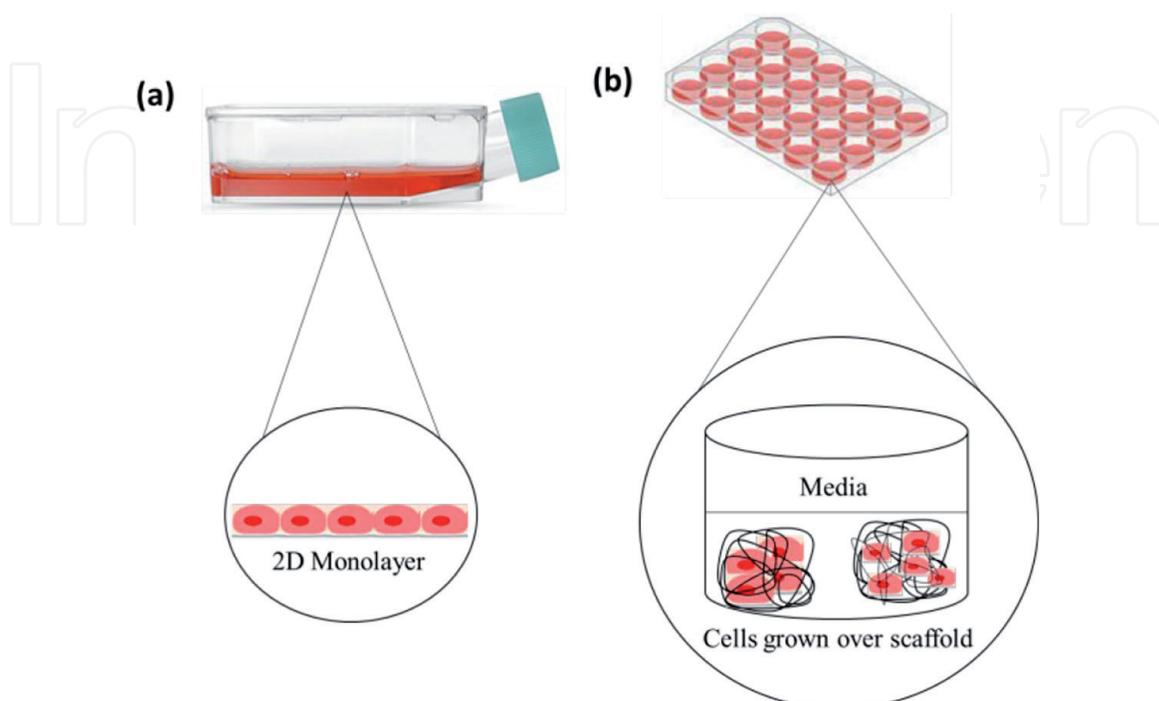

Figure 1.
Simplified sketch of 2D cell culture (a) and 3D cell culture (b).

different applications *in vitro*. The significance of 2D culture is projected through different assays and therapeutic treatment to understand cell motility and treatment of diseases, while 3D culture includes different models and spheroid structures consisting of multiple layers of cells, and puts a light on its use in drug discovery and development. The chapter is concluded with a detailed account of the production of therapeutic proteins by the use of cells.

2. *In vitro* applications of 2D culture

2.1 In vitro **cytotoxicity assays and tissue-engineered tissue models**

Cytotoxicity assays are commonly used for *in vitro* toxicology and pharmacology studies for the screening the effect of chemicals and drugs on the cultured cells. There are different assays available for measuring cytotoxicity namely- the colony-forming assay and dye inclusion or exclusion such as neutral red and trypan blue assay is the most significantly used. Cytotoxicity assays can be broadly divided into the following categories– (i) Assays based on metabolism, (ii) Adenosine triphosphate (ATP) Bioluminescence Assay and (iii) Assays based on the release of enzymes.

Assays based on metabolism generally include the 3-(4,5-dimethythiazol2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay and its alternatives such as 2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2H-Tetrazolium-5-Carboxanilide (XTT),3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium(MTS) and sulforhodamine B (SRB) assay. Due to rapid, quantitative, versatile, and highly reproducibility of MTT, it is widely used in large-scale, anti-tumor drug-screening program. MTT is a quantitative colorimetric assay that quantifies the reduction of yellow tetrazolium dye by mitochondrial succinate dehydrogenase to purple insoluble formazan crystals by the NADPH dependent cellular oxidoreductase enzymes [12]. The crystals are dissolved in an appropriate solvent. The absorbance is then recorded using a spectrophotometer to analyze the cell viability wherein the crystals get accumulated in the viable cells due to their impermeability to the cell membrane.

ATP Bioluminescence Assay is used to measure the ATP level that is well regulated in the metabolically active live eukaryotic cells as compared to the dead cells wherein the ATP level falls due to the activity of ATPases. This assay includes a luciferase enzyme that utilizes energy from ATP that converts luciferin into oxyluciferin and thus produces luminescence. Therefore, luminescence could be used to measure the ATP level. Assays based on the release of enzymes are more significant as they measure the products released by the dead cells [13].

Assays based on the release of enzymes include Lactate dehydrogenase(LDH) leakage assay involving the formation of pyruvate from lactate in the presence of LDH with simultaneous reduction of NAD to NADH that alters the absorbance at 340 nm [14]. Research in cancer and cell biology is greatly dependent on *in vitro* assays and models. This help in understanding the various responses of the cultured cells when exposed to different conditions. Tissue-engineered *in vitro* tissue models serve as an alternative to *in vivo* animal studies to study the physiology of various diseases. Example of tissue-engineered *in vitro* models includes Skeletal Muscle Models [15], blood–brain barrier model [16], aneurysm models [17] and the Pre-vascularized Human Vaginal Mucosa model [18].

2.2 Cell migration assay

Cell migration is well known for its significant role in embryonic morphogenesis, cancer invasion and metastasis, immune responses, tissue formation, and

angiogenesis [19]. Mainly, cell migration is of two types; single-cell migration and collective cell migration. Single-cell migration is regulated by cytoskeletal activity without cell-to-cell interactions with neighboring cells. This type of migration is important for embryonic development, immune response, and in the early stages of metastasis. On the other hand in the collective cell migration, the group of cells retains their cell to cell interactions as well as collective polarity. Wound healing assay or scratch assay is a 2D *in vitro* technique used to study collective cell migration. In this assay, a scratch is made on the confluent cell monolayer resulting in the formation of a gap or wound which is monitored by taking pictures of the migrating cells at regular intervals of time. These pictures are then used to measure the speed of wound closure and thus quantify migration. Live-cell imaging using Time-lapse microscopy can be used for a more detailed study of cell migration behavior [20]. In order to reduce the effect of cell proliferation on gap filling, the readings are taken for a time period of 24 hours but this may vary depending on the cell line.

Another assay involving the response of single cells to various chemo-attractants is the transwell assay or the Boyden Chamber assay. This assay can be used for both adherent and non-adherent cells wherein the cells are placed in a serum-free medium on one side of a porous membrane and analyzed on the basis of the cell's ability to migrate through the pores to the other side. Cell migration can be quantified by counting the cells that have traversed through the membrane towards the higher concentration of chemoattractant [21]. A drawback of this assay is visualizing the cells and their morphology while migrating through pores due to the transitive state of cells [22].

2.3 In vitro **tumorigenicity assay**

Cancer is one of the most frightful diseases in both developing and developed countries and imparts a major health burden to the society. Tumorigenicity is the tendency of the cultured cells to form tumors. The two common *in vitro* tumorigenicity assays are - Colony-forming assay and Tumorsphere assay. The colony-forming assay is also referred to as clonogenic assay that analyzes the potentiality of a single cell to undergo a clonal expansion to form a colony composed of a minimum of 50 cells [23]. This assay is usually used to distinguish there productive viability of untreated cells from the cells that are treated with ionizing radiation or cytotoxic agents. It is also used to study the stemness and the clonogenicity of stem cells [24].

Colony forming assay is performed using the soft agar method. The basic steps involved in this assay are treating the cell monolayer in the flask, seeding the required number of cells on the agar and incubate for 1–3 weeks, fixing and staining the colonies and finally observing the colonies under the stereomicroscope [23]. Another *in vitro* tumorigenicity assay is tumorsphere assay which analyzes the potential of cancer stem cells (CSCs), a major cause of tumor initiation, progression, and recurrence after treatment. This assay is carried out under non-adherent conditions and serum-free medium supplied with growth factors of choice leading to the proliferation of CSCs and formation of spheres whereas the non-CSCs undergo apoptosis due to loss of adherence and abundant nutrients [25].

2.4 Cell invasion assay

Cell migration is an important process in biology where the cells changes and reaches their destination within a proper environment, in order to execute their respective function. It is a normal physiological process that takes place in nearly all forms of organisms. However, changes or deregulation of any kind in the pattern of cell migration or invasion are an indication of pathological conditions

including inflammatory diseases and cancer metastasis, with the latter being the most explored one [21]. There are various biological methods that are commonly employed in the scientific community to study the above-mentioned events in depth namely, the cell culture wound-healing assay, the transwell migration, and invasion assay, individual cell-tracking assay, and spreading assay. These assays aim to provide relevant information pertaining to the pattern of cell migration or its response to chemoattractant(s).

2.4.1 The cell culture wound-closure assay

It is the simplest of all methods in determining the migration of whole-cell masses altogether. Going further in detail, it can be used to interpret individual cell's morphological characteristics and phenotypes during migration. Measuring the closed distance compared to the control over regular intervals of time shows specific migration changes or phenotype that was unknown in the past [26].

2.4.2 The transwell migration and invasion assay

The transwell migration and invasion assay are used to determine the capability of single cells to respond to various chemoattractant(s) including chemokines, growth factors, lipids, or nucleotides. It also contributes to assessing differential cell migration due to the over-expression of a receptor. It also identifies and characterizes the key regulators participating in cell migration [26].

2.4.3 Individual cell-tracking assay or single-cell tracking assay

Conducting single-cell tracking and its live imaging under appropriate conditions adds to the overall advantage of cell migration assay. The software includes a time-lapse video-microscopy protocol comprising of post-processing tracks of the cell populations with single-cell resolution. It greatly helps to understand the cell biology and lineage progression of distinct cell populations [27].

2.4.4 Cell spreading assay

In this type of assay, the spreading process of individual cells is seen and recorded with the help of Differential Interference Contrast microscopy (DIC). The spreading state is recorded every 5 seconds with a Charge-Coupled Device (CCD) of the camera, producing high-quality grayscale images. The process of taking images could extend to several hours [28].

2.5 Hybridoma technology and monoclonal antibodies

Antibodies, one of the major elements of the immune system are the glyco-proteins produced by the immunoglobulins; B-cells provide protection against invading pathogens. The antibodies are highly specific and selective, thus have been used as an extraordinary tool in bioengineering and biomedical research for many years. The antibodies are majorly classified into two categories, Monoclonal Antibodies (mAbs) and Polyclonal Antibodies (pAbs) are based on their origin from the lymphocytes. mAbs are produced by only B lymphocyte or B cells and are monospecific. Due to this property, they possess high specificity and affinity towards a single epitope of an antigen whereas pAbs are produced by different B-cells and possess different affinities for multiple epitopes of a specific antigen. Since mAbs are highly specific, they are produced on a large scale through culturing

of antibodies-producing cells widely known as 'Hybridomas', which are commonly derived from mice, and the method is known as 'Hybridoma Technology [29].

Hybridoma technology was discovered and developed by two eminent scientists, Georges Kohler and Cesar Milstein in 1975 and is considered to be one of the biggest breakthroughs. It has proved to be a robust, effective, and successful methodology employed in the field of biotechnology and biomedical research that solely deals with mAb isolation. The B cells go through the antibody maturation process in the germinal centers of secondary lymphoid tissues (for example, lymph nodes, spleen, tonsils, and Peyer's patches). Upon proliferation, certain mutations are experienced by the B cells, specifically in the genes encoding the variable region of the antibodies that helps in the selection for high-affinity tight binding to the corresponding antigen. The overall resulting antibodies by B cells consist of a natural pairing of the light chain and variable heavy chain genes with constant region genes. This region contains Class Switch Recombination (CSR) differentiates from the hybridoma technology in which CSR is absent [29].

Following are the steps employed for the production of monoclonal antibody by hybridoma technology.

2.5.1 Isolation of antibody-producing B lymphocyte

The mouse/mice is/are immunized every 2–3 weeks with red blood cells taken from sheep in order to produce the B cells. These antibodies are isolated from the spleen cells of mice.

2.5.2 Screening of mouse for production of antibody

After the process of immunization, the blood samples are taken from the mouse to determine the serum antibody titer. When the titer reaches the optimal level, the mouse is boosted by injecting antigen 3 days prior to fusion with myeloma cells [30].

2.5.3 Fusion of B cells with myeloma cells

Fusion of isolated spleen cells (limited life span) with tumor lymphocytes (immortal) with the help of PEG (Polyethylene Glycol) leads to the development of hybridomas with an unlimited life span.

2.5.4 Culturing of hybridomas

Hybridomas are grown in a selective medium containing Hypoxanthine, Aminopterin and Thymidine (HAT). Aminopterin present in the media blocks pathway for nucleotide synthesis, making the cells dependent on the alternative pathway which is not evident in myeloma cells.

2.5.5 Screening and selection of the desired colony

The cells are screened and chosen or selected for production of antibodies with the desired specificity.

2.5.6 Culturing of the selected hybridoma cells on large scale

The cells are cultured and used for the production of large quantities of antibodies [31].

2.5.7 Storage for future use

The cells are frozen and stored for future use in therapeutics.

2.6 Gene therapy

Gene, the fundamental biological unit of heredity that constitutes an ordered sequence of nucleotides present in chromosomes. The functional aspect of a gene is to encode a protein or RNA molecule inherited from parents such as texture and color of the hair and eyes. Any kind of alterations/mutations in a gene sequence can lead to abnormal functionality of the genes. Gene therapy is a modern type of experimental technique in the medical field which involves rectifying the non-functional or malfunctioning of genes by replacing them with healthy and functional genes. Several approaches have been implemented by researchers in terms of correcting a mutated gene with a healthy copy of the gene or by inactivating the mutated gene causing disease. It has been widely studied for various diseases such as immune deficiency, blood disorders, eye problems, metabolic disorders, regeneration of nerve cells, and cancer [32]. The first case of gene therapy was discovered in the 1990s whereby a functional Adenosine Deaminase (ADA) gene was incorporated in the white blood cells of the patient, replacing the non-functional ADA [33]. This application led to interesting results with the immune systems and hence, was considered the most reliable technique.

There are two main methods for gene therapy such as- *Ex-vivo* gene therapy *and In-vivo* gene therapy. The former is the transfer of genes into patient cells outside the body and the latter one is the transfer of genes directly to cells inside the body. To carry this, several techniques are used like- direct or liposome-mediated injection of DNA, calcium phosphate transfection, electroporation, dendrimers, hybrid methods, retrovirus, and other viral vectors. Clinical conditions on which gene therapy has been applied are as follows:

2.6.1 Parkinson's disease (PD)

The strategy of gene therapy has been applied to this disease in order to improve the advanced symptoms of PD. Gene therapy was applied to transfer 'Glutamic Acid Decarboxylase (GAD), a chemical produced by a gene into the basal ganglia. GAD showed an increased amount of a neurotransmitter called as Gamma-Aminobutyric Acid (GABA), responsible for inhibiting brain signals and decreasing activity in the nervous system Decreased GABA activity leads to certain brain-related disorders [34].

2.6.2 Alzheimer's disease (AD)

AD and other frontotemporal dementias (FTDs) are caused by the accumulation of amyloid-β peptide (Aβ) and protein tau in the brain. It is characterized by having memory loss, difficulty in learning and communicating along with the inability to organize things. The use of recombinant Adeno-Associated Viruses (rAAVs) has provided new ways for studying AD and other related neurological disorders [35]. Such strategies or approaches have added novel dimensions to medical treatments.

2.6.3 Cystic fibrosis

Cystic fibrosis is a disease known to affect the lungs primarily. Its symptoms include inflammation, airway obstruction leading to respiratory tract infection and deformity.

Insertion of the Cystic Fibrosis Transmembrane Regulator (CFTR) gene directly into the epithelium cells of the respiratory tract bear the capability to lessen the symptoms but not totally cure the disease in patients suffering from cystic fibrosis [36].

2.7 Cell therapy

Cell-based therapy is one of the most important and well-known forms of all treatments in the fields of modern science & medicine. It is not only a curative option for treating deadly or threatening diseases but is also making 'Regenerative Medicine' the most vital technique in health care with the specific goal of replacing diseased cells, tissues or organs and thereby restoring their normal function(s) [4]. Over the years, there has been a gush of interests and work done in understanding the potential of stem cells. They are the cells found naturally in the living bodies, characterized by two defining properties of eternal self-renewal and the propensity to differentiate into an adult cell type. There are three main types of stem cells: Totipotent (a cell developing into a healthy organism independent of the permissive environment), Pluripotent (a cell developing into any type of adult cell) and Multipotent (a cell developing into a limited type of cell) [37].

Following is the account of different stem cells used for the treatment of various diseases:-

2.7.1 Pluripotent stem cells

Reportedly, pluripotent cells have been used successfully to treat animals per se. Animals diagnosed with diabetes are incorporated with cells containing insulin responsive to glucose levels. Additionally, the treatment of the animals suffering from acute spinal cord injury and visual impairment is performed with myelinated neurons and retinal epithelial cells, respectively. Researchers are still conducting studies with the use of pluripotent stem cells to cure several disorders such as Parkinson's disease, muscular dystrophy and heart failure.

2.7.2 Induced pluripotent stem cells

The stem cells created artificially from normal adult somatic cells through co-expression of genes and factors are known as Induced Pluripotent Stem Cells (iPSCs). These are important for maintaining the characteristic properties of Embryonic Stem (ES) cells. Some reports have stated the successful use of iPSCs in conditions like Parkinson's disease, spinal muscular atrophy, cardiac diseases, blood disorders, diabetes, amyotrophic lateral sclerosis, Huntington's disease, and familial dysautonomia.

2.7.3 Multipotent stem cells

The multipotent stem cells derived from bone marrow (Hematopoietic stem cells) have been used in the 1960s to treat cancer conditions like leukemia, myeloma and lymphoma. Mesenchymal stem cells with the capability of forming whole joints in mouse models have been used regenerating bone and cartilages form. Curing heart ailments are still under clinical trial.

3. Applications of 3D cultures *in vitro*

Spurred by the recent advent in cell culture technologies, three-dimensional (3D) cell culture is paving the way in promoting tissue organization and cell

differentiation by triggering tissue-based diseased microenvironment. An ideal 3D cell culture system generally composed of tightly bound tissues that involve cell–cell fluent interaction almost mimicking the extracellular matrix (ECM) that is highly dynamic and includes scaffolds of cells in a fluid that enhances them to differentiate (**Table 1**). The key parameter of a 3D culture environment is the ability to organize the spatial arrangement of cells with other surrounding cells along with physical constraints [8]. This significant approach has gardened great focus on understanding complex cellular biology and their responses by validating mammalian tissue studies via linking the gap between *in vitro* and *in vivo* environments. The two-dimensional (2D) cell cultures lack several features that 3D cultures impart such as tissue-specific architecture and complex cellular interactions that make them poor models for complex diseases. Based on the process of preparation, 3D techniques are categorized into (i) scaffold-based, (ii) scaffold-free culture systems. Scaffold-based technique efficiently is more responsive towards cell-to-ECM connection because of their potentiality of mimicking ECM whilst scaffold-free technique persuades physiological and cellular gradients. Scaffold or matrix is designed according to the tissue of interest, higher is the complexity of the scaffold, and the higher is the difficulty to extract the cells for analysis [9]. Majorly, the

Approach	Merits	Demerits
3D Spheroids	• High reproducibility	• Expensive to prepare
	• Therapeutic inhibition to drug exposure can be easily evaluated through image analysis	• Optimization protocols for each cell line is required
	• Constant perfusion	• Difficult light matter interactions for large spheroids
	• Lower consumption of reagents	• Differences in spheroids' diameters,
	• Control of shear stress and pressure on cells	• Low-throughput
	• Capable of imbibing large amount of water or biological fluid	• Labour intensity
Hydrogels	• Ease of maintenance	• Low mechanical strength
	• Amenable of controlling the micro-tissue size and large amount of micro-tissues per plate	• Difficult to handle
		• Expensive
Organoids	• Amenable to high-throughput screening	• Absence of microenvironment
	• Long lived organoid production from single cells	• Optimization protocols are not globally standardized.
		• Organoid cultures rapidly die due to contamination
Cancer co-culture Models	• Easily evaluate cell–cell interactions of cancer microenvironment	• Microbial contamination
	• Provide fluid flow	• Static condition
	• Easy to handle and quantify	
	• Relevant mechanical cues	
Organ-on-a-chip	• Enable stable co-culture of living human cells	• Architectural complexity of developing human tissues and organs
	• Good control over microenvironment	• Difficulty in standardization and scale-up

Table 1.
Merits and demerits of different 3D cell culture techniques.

scaffolds manufactured are polymeric hard material-based support, microfluidic-based assembly, hydrophilic glass fiber, and organoids. In contrast, scaffold-free systems form cellular aggregates called spheroids that have evolved with improvement in their techniques such as magnetic levitation, liquid overlay (low adhesion plates), hanging drop microplates, and spheroid microplates yet the demand for 3D scaffolds preferentially increased due to their immense property of modulating the behavior of cultured cells according to the matrix in which they are cultured.

3.1 3D Spheroids

3Dspheroids or multi-cellular aggregates are spherical micro-sized cellular constructs that are produced from numerous gamuts of cell types, originally from scaffold-free systems. The most characteristic features of 3D spheroids are the ability to recapitulate a cell's typical physiological behavior, cellular heterogeneity, gene expression, cell–cell signaling, and structural architecture with respect to cell–cell contact [10]. Various types of 3Dspheroids include embryonic bodies, tumor spheroids (spheres of different tumorcells), hepatospheres (spheres of hepatic cells), neurospheres (spheres of different cell types of the central nervous system (CNS)) and mammospheres (spheres of mammary glands) [38]. An ideal 3D spheroid constitute ECM components such as proteoglycans, laminin, collagen, fibronectin, tenascin, and glycosaminoglycans [39] which tightens the spheroid density with close ECM-cell and cell–cell anchors eventually increase interstitial fluid pressure (IFP). Depending on the primary amount of cells seeded, the size of spheroid increases with an elevation in cell number, oxygen, and nutrient gradients equivalent to the tissue of interest [5]. Alongside, the different techniques enabling spheroid cultures are illustrated further.

Hanging drop technique is a non-scaffold method wherein a drop of media containing cells are suspended inversely on the lid of the culture dish (bottom-less and open) such that there is no surface provided for the cells and tend to hang. This attempt forms a cluster called spheroid at the tip of the droplet when cultured for a longer period [40]. Spheroids formed through hanging drop cultures have fetched considerable stance in cell culture technology with 100% reproducibility owing to ubiquitous applications in cancer research [41], toxicity testing in hepatocytes [42], and constructing cardiac spheroids [43]. Another method involves the use of a liquid overlay that eases the formation of aggregates and commercially produced as low adhesion plates. These spheroid microplates contain either hydrophilic or hydrophobic coating with V-shaped bottom and allow mild attachment to the surface such that the cells tend to self-aggregate and form spheroid. Unlike the hanging drop technique, low adhesion plates generate one spheroid per plate that signifies its importance for multicellular culture. This ensures a medium-throughput screening that requires no modification in spheroid formation [44]. Spheroids can also be cultured with the use of magnetic nanoparticles with the application of the magnetic field. The process is called magnetic cell levitation that is highly applied to produce spheroids of mesenchymal stem cells and tissue engineering [45, 46]. An *in vivo* study showed that human glioblastoma cells levitated by a magnetic approach closely mimicked the protein expression of human glioblastoma tumor xenografts [47].

3.2 Organoids

Organoids refer to the primary cultures derived from cell aggregates through *in vitro* process that is grown in 3D gels containing ECM to produce organ-like buds with the application of either physical support (cell adherence) or biochemical

cues (signaling pathway modulation). Various types of cells such as embryonic, adult, primary, and stem cells are utilized for the development of organoids. Based on organ-like structures formed, organoids are classified into tissue and stem cell organoids. The application of organoids has helped in producing numerous *in vitro* organoids such as rectal [48], gastric [49], lung [50], liver [51], pancreas [52], retina [53], thyroid [54], kidney [55] and intestine [56] that had successfully recapitulated the structural and functional motif of real organs. 3D organoids are extensively used by researchers to decipher the toxicity analysis, examine the genetic pathologies and investigate the local immune responses to infections. In addition to this, current reports have suggested the promiscuous application of organoids in platforms like transcriptomics and proteomics technologies. One such example illustrates the interaction study between Zika virus and Toll-like receptor 3 is performed by the generation of cerebral organoids from embryonic stem cells [57]. They have been also used as models for distinct genetic diseases. For instance, a study applied the rectal organoid model of cystic fibrosis for the investigation of the potency of transmembrane regulator-modulating compounds [48]. Besides, the tubular organoids model of polycystic kidney disease was also used to unravel the cause for cyst formation [55]. Apart from this, organoids have been an excellent source of models to understand the depth of neurodegenerative diseases viz.; Alzheimer, Parkinson's, HIV, diabetes, or cancer.

3.3 Cancer co-culture models

Cancer cell lines have emerged as an eminent tool for comprehending complex physiology of cancers. The cell cultures have eased the outlook in preclinical research to understand the process of disease, morphological changes occurring in tissue, gene function, cell biology and tissue engineering [58]. They have evolved with immense features of offering homogenous samples without any sort of modification and variations. However, a big leap was noted when monolayer cell cultures (2D) obtained from solid tumors were incapable of mimicking the structural elements of tumor microenvironment. Thusly, 3D cancer cell culture models have placed an enduring platform recently whereby ECM in 3D construct is same as that of original cell culture and imparted knowledge of predicting tumor response to treatment [59]. The application of 3D cell culture models of tumors have ought to manifest typical properties of tumor microenvironment such as gene and protein expressions, morphology, angiogenesis, malignancy and invasiveness. From this standpoint, 3D tumor cell culture models scintillate anticancer therapeutics and cancer drug discovery. To date, a vast content of literature owes the significance of these 3D co-cultures models in varying applications. In a study, tumor-associated macrophages (TAM) or cancer-associated fibroblasts (CAF) and gelatin hydrogel microspheres (GM) have been applied to produce cancer co-culture models from different cancer cells including HepG2 (liver), MCF-7 (breast) and WA-hT (lung) in order to inspect sustained release of drugs. They induced metastatic proteins involved in epithelial-mesenchymal transition (EMT) with transforming growth factor-β1 (TGF-β1) and reported elevation in N-cadherin and Vimentin proteins with deceleration in E-cadherin protein [58]. Recently, cancer co-culture models evinced interest in numerous approaches such as 3D breast cancer co-culture models obtained from MCF-7, MRC-5 and MDA-MB-231 tumor cells were used in investigating radiation-induced fibrosis [59], tumor-associated fibroblast differentiation [60] and development of immunotherapies [61], 3D lung cancer co-culture models derived lung squamous carcinoma and Non-Small Cell Lung Cancer Cells (NSCLC)fromTUM622, A549 and Colo699 tumor cells were utilized to explore tumor-stroma interactions [62, 63], 3D renal cancer co-culture models formed from

Caki 1 (skin metastasis derived) and ACHN (pleural effusion derived) were sought for determining the efficacy of produced 3D models in stem cell physiology research and drug toxicity screening [64]. 3D colon cancer co-culture models acquired from LS 174 T, HCT 116, Colo205, MCF7, SW480, SW620, CCD-18Co, Caco-2, HT-29, and H446 have also been used to explore tumor-stroma interactions [65].

3.4 Tissue co-culture models

In vitro tissue models with the use of co-culture cells have emphasized greater applications to represent varying mechanisms of human body which is a daunting task. These models have served a vital role over several animal models that once were used to examine human physiology and pathophysiology. The major limitation of animal models was the failure of mimicking true human facets and their ethical constraints. This led to fetch insights into development of tissue models as a research tool from co-cultures such that the created models would possibly recapitulate the natural microenvironment of cells and examine the pathophysiological bases of diseases. Distinct *in vitro* 3D tissue models have been achieved with the approach of tissue engineering comprising human characteristics with increased complexity as compared to the 2D monolayers. Some of the examples of 3D models constructed from tissue co-cultures include kidney [66], neuro-glia [67], lung [68], liver [69], ovary [70] and intestine [71] that have potentially predicted and represented physiological responses of the original culture of cells. Most probably, primary cells are used as they possess feasible *in vivo* features of not being immortal, incapable of getting transformed and consist of limited survival time in culture. Pertaining to these advantages, they have been applied to develop models that would combat various disease and physiological studies. Reportedly, tissue models have been revolutionized in terms of investigating multiple changes in real-time processes. A pulmonary endothelium model was constructed to investigate massive inflammation in patients with acute respiratory disease syndrome (ARDS). The authors performed this study using lavage samples of the patients for determining the etiology of ARDS that took place during the process of disease [72]. In addition, endothelial cells have also played a key role in constructing 3D tissue models with their fascinating physiological roles. For instance, *in vitro* gastrointestinal epithelial cell cultures derived from adult murine colon allowed the authors to analyze epithelial cell–cell interactions, microbiological infections and cellular signaling [73]. Another instance showed the potential of hepatocyte tissue cultures in maintaining the cancer cell hierarchy in human hepatocellular carcinoma [74].

3.5 Organ-on-a-chip

Organ-on-a-chip is a biomimetic system that uses fabrication of computerized microchips and microfluids consisting of living cells, mimicking the natural environment of organs from which it is been created. There are several factors that made organ chips be listed in "Top Ten Emerging Technologies" in the World Economic Forum [75] such as shear force, tissue-boundaries, concentration gradients, tissue–organ interactions and cell patterning. Organ chips have intensified in the field of drug therapeutics for their ability of high throughput screening. **Table 2** summarizes the recent researches carried out using various organ chips. These organ chips use microtechnology that provides nutrients to the cells for their better growth and proliferation. Microfluids are one such component that has been used in various studies for efficient treatment in drug sensitivity testing [86]. Talking of this notion, a microfluidic chip was produced in order to monitor and document real-time impedimetric biosensor changes. Other organ-on-a-chip models such as blood–brain barrier chips

Organ type	Incorporated cell types	Organ-specific properties	Ref.
Lung	Primary lung alveolar epithelial cells	• Breathing movements	[76, 77]
	Primary lung endothelial cells	• In vivo functionality array of tiny alveoli	
		• Human lung parenchyma (lung alveoli and ultrathin air–blood barrier)	
		• Recreates the native viscoelastic microenvironment of the cells	
	Human vascular endothelial cells	• Alveolar capillary barrier in the human lung	[78]
	Human alveolar epithelial cells		
Skin	Peripheral perfusion fluid (PPF)	• Franz diffusion cell system	[79]
		• Drug absorption across the dermal barrier	
		• Microfluidic Diffusion Chamber (MDC)	
Liver	Hepatic cell lines	• Hepatoprotective effect assessment	[80]
		• Hepatic activity (cell viability, albumin synthesis, urea secretion, and cytochrome P450 enzyme activities)	
		• Drug Screening and Toxicity Testing	
Kidney	Human podocytes Glomerular endothelial cells	• Human glomerular filtration barrier	[81]
		• Functions and structure of the glomerulus	
	Human-derived renal proximal tubule epithelial cells	• Cell Polarization-Dependent Cisplatin Toxicity	[82]
		• Proximal tubule	
Heart	Cardiomyocytes	• Contractile behavior (contraction force, frequency, and synchronization) of a 3D cardiac tissue construct	[83, 84]
		• Three-dimensional beating tissue from human cardiomyocytes	
Pancreas	Human pancreatic beta-cell line	• Human pseudo-islets in biomechanical flow conditions	[85]

Table 2.
Summary of recent organ-on-a-chip models.

have been developed to represent the *in vivo* architecture of brain involving micro blood vessels by using type 1 collagen hydrogel. Another model entails the significance of human-on-a-chip that depicts the normal human physiology in combination with single organ chip within a microfluidic system that ultimately forms multi-organ chip [87]. One study dealt with an *in vitro* 3D-tumor-on-a-chip device that illustrated its importance in quorum sensing phenomenon in tumor cells activated by salmonella [88]. Hence, organ-on-a-chip has been diversified in many scientific platforms due to their efficient physiological bio-mimicry.

3.6 Patient-derived cells

Animal models used in laboratories have been greatly avoided due to the fact that they are costly and require a large number of laborers. This approach was replaced by the use of *in vitro* models wherein despite having several advantages; still, the application is constrained due to poor cell-to-cell and cell-to-matrix interactions. *In vitro* culture of cells has been observed to acquire multiple genetic and epigenetic variations that eventually make the cells lose their originality. The above-listed models comprise their own merits and demerits in respect of cellular response, cellular composition, and structural features. The above-mentioned models consist of a few advantages and disadvantages with respect to cellular composition, mimicking the *in vivo* physiology of original tumor architecture, tumor microenvironment (TME) and the response to different exogenous stimuli. Therefore, patient-derived cells have come into the picture which is generally a co-culture-based technology that is grown in a culture medium supplemented with all sorts of nutrients [89]. Different studies have exemplified the use of patient-derived cells as a preclinical model in drug discovery (screening and responses) in several types of cells. Patient-derived xenograft (PDX)models are made of minute pieces of tumor tissue of the surgical patients, utilized for implantation into an immune-deficient mouse. In a study, Fong et al. used PDX models from prostate cancer to investigate tumor-stromal interactions via the use of a 3D hydrogel system [90]. Likewise, in another study, Liu et al. demonstrated the establishment of patient-derived cell cultures from colorectal cancer cells of biopsies of cancerous and non-cancerous tissue that could grow in *in vitro* culture indefinitely by recapitulating exactly the same phenotypic and genotypic features of the original tissue [91]. Some of the researchers have also elaborated patient-derived 3D culture using a scaffold-based organoid culture that is prepared to preserve the genomic features of the original tissue [92].

4. Three-dimensional cell culture in drug discovery and development

Drug discovery is a lengthy and time-consuming process that undergoes several stages of testing and optimization. This encompasses identification of the target, lead discovery, pre-clinical validation, and clinical trials [3]. Due to the constant failure of drugs in Phase II and Phase III clinical trials, there has been constant pressure on the pharmaceutical industry to seek more novel drugs with lower side effects and cost-effectiveness. 3D cell culture has emerged as a significant high-throughput system that has uplifted the standards of cell culture [93]. Specifically, spheroids are considered the most reliable model for testing drugs in various diseases because of their capability of resembling the natural environment of original tissue [93]. The spatial organization of spheroids in different layers of cells leads to cellular death by forming reactive oxygen species [94]. In the case of investigating the effect in 3D spheroids, fluorescence microscopy plays a key role in determining pharmaceutical dispersion within spheroids (eg-doxorubicin and epirubicin) [95]. The capital importance of any drug testing involves cell-based assays that are efficient enough and easily reproducible compared to expensive animal models. Cell-based assays have shaped the physiological relevance of 2D cultures [96]. While the reaction may vary from technique to technique such as cell viability, proliferation, signaling and migration and drug to drug for achieving better sensitivity. It is now broadly accepted that compared to 2D cultures, 3D models serve the resemblance of the natural environment of original tissue efficiently and differently in 3D environments. Research has nested stance on novel 3D culture technologies that impart functional basis of tissues such as spheroids and organoids [97]. A study

used 3D hydrogel-based model for the determination of drug sensitivity in HepG2 cell lines by comparing cytotoxicity effect with cytotoxicity (CT_{50}) and lethal dose (LD_{50}) values [98]. Organoid 3D models also aid as a resourceful tool for modeling neurodevelopmental disorders [98]. Microfluidic chips have also been utilized in drug sensitivity testing whereby a study elaborated its efficacy in lung cancer which was in combination with stromal cell lines [98]. Evaluation of absorption, distribution, metabolism, excretion, and toxicity (ADMET) of the drug is primarily examined in *in vitro* cell culture experiments. 3D cell cultures have fostered drug pharmacokinetics in several studies with the implementation of various types of cell culture models.

5. Cell based manufacturing of therapeutic proteins

Therapeutic proteins production using human cell lines has greatly influenced different medical areas including biopharmaceutical research and vaccine production. Mammalian cell lines prove futile in protein production due to their likelihood of possessing post-translational modifications (PTMs) achieved from recombinant proteins that are in accordance with the endogenous human proteins. These cell lines show exquisite specificity to produce similar proteins to those in humans naturally synthesized, an advantage over mammalian expression systems [99]. One of the most routinely and high yields of proteins production is performed by using cell-based expression systems such as Chinese hamster ovary (CHO) a cell line that constitutes major advances such as accomplishment of gene amplification, specific productivity, better selection strategies, and devising greater expression units and advanced hosts. CHO cells have established their safety profile for 20 years from the production of its first recombinant biotherapeutic protein in 1986 [100]. Other human cell lines such as BHK-21 cells are used for the generation of few coagulation factors such as factor VIII [101]. There are two vital human cell lines namely, HEK293 and HT-1080 that are used to manufacture licensed products of human PTMs. The advancement in protein-based drug development and technologies has driven more towards the therapeutic proteins market that comprises of sales of these therapeutic proteins. The methods that are involved in the production of these proteins are pegylation, glycoengineering, albumin fusion, Fc-fusion, product purity, targeting, and functionality of therapeutic protein drugs. Few examples of therapeutic protein drugs which has been produced using protein engineering technologies and approved by the Food and Drug Administration (FDA) from the past five years are imiglucerase, Belimumab, alfa, coagulation factor IX recombinant human and albiglutide [102]. A French pharmaceutical company named Sanofi accomplished a great achievement of strengthening its R&D strategy with the best proprietary therapeutic proteins production pharmaceutical company, Ablynx, for a nanobody technology platform.

6. Conclusion

In particular, a plethora of research studies have shed light on the fact that in spite of the availability of advanced organ-on-chip technologies and bioengineered 3D models, the application is limited by drug companies due to their relatively novel approach which is more likely requires to undergo further validation and characterization. Moreover, 3D cell culture models with high-throughput screening in combination with high-content leads to the identification of clinically relevant compounds. However, still many difficulties are being faced as 3D cell cultures do not meet certain criteria in the drug discovery process with regard to size, morphology, complexity, and protocol

for assaying. It requires ample standardization and optimization to extract successful specific phenotypes for drug screening. Thus, there are few 3D models that are constrained for their restricted access due to limited permeability. Following the advances in protein therapeutics, more improvements in generating sophisticated therapeutic protein products will be developed for better futuristic research.

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Conflict of interest

There are no conflicts of interest.

Abbreviations

2D	Two-dimensional
3D	Three-dimensional culture
Aβ	Amyloid-β peptide
ADMET	Absorption, distribution, metabolism, excretion and toxicity
ADA	Adenosine Deaminase
AD	Alzheimer's disease
ARDS	Acute respiratory disease syndrome
CAF	Cancer-associated fibroblasts
CCD	Charge-Coupled Device
CFTR	Cystic fibrosis transmembrane regulator
CHO	Chinese hamster ovary
CNS	Central nervous system
CSCs	Cancer stem cells
CT50	Cytotoxicity 50 percent
CSR	Class Switch Recombination
DIC	Differential Interference Contrast microscopy
ECM	Extracellular matrix
ES	Embryonic Stem
EMT	Epithelial-mesenchymal transition
FTDs	Frontotemporal dementias
GABA	Gamma-Aminobutyric Acid
GAD	Glutamic Acid Decarboxylase
GM	Gelatin hydrogel microspheres
HAT	Hypoxanthine, Aminopterin, Thymidine
IFP	Interstitial fluid pressure
iPSCs	Induced pluripotent stem cells
LDH	Lactate dehydrogenase
LD50	Lethal dose 50 percent
MTT	3-(4,5-dimethythiazol2-yl)-2,5-diphenyl tetrazolium bromide

MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
MDC	Microfluidic Diffusion Chamber
mAbs	Monoclonal Antibodies
NAD	Nicotinamide adenine dinucleotide
NADH	Reduced nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NSCLC	Non-Small Cell Lung Cancer Cells
pAbs	Polyclonal Antibodies
PDX	Patient-derived xenograft
PD	Parkinson's Disease
PPF	Peripheral perfusion fluid
PTMs	Post-translational modifications
rAAVs	Adeno-Associated Viruses
SRB	Sulforhodamine B
TAM	Tumor-associated macrophages
TGF-β1	Transforming growth factor-β1
TME	Tumor microenvironment
XTT	2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2H-Tetrazolium-5-Carboxanilide.

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