

ADVANCES IN MAMMALIAN CELL CULTURE TECHNIQUES FOR CANCER THERAPEUTIC STUDIES

Abstract

The emergence of cell culture as a pivotal technique in understanding cellular processes involves isolating cells from tissues and cultivating them in a controlled environment. This method aids in studying various aspects like pharmacology, mutations, cancer development, and cell behavior. Cells can be cultured directly from tissues using enzymatic or mechanical methods or by utilizing established cell lines. Cultured cells require specific nutrients, growth factors, hormones, and gases for optimal growth. Initially, cell culture was carried out in two dimensions (2D) on flat surfaces, but this method has limitations in replicating the complex microenvironments found in the human body. Research comparing 2D and 3D cell culture models for drug testing, particularly in cancer cells, demonstrated that cells cultured in 3D environments displayed increased resistance to common chemotherapy drugs. This emphasizes the significance of considering cellular microenvironments in evaluating drug responses and highlights the drawbacks of 2D models in predicting drug efficacy in real tumor contexts. Despite its simplicity and cost-effectiveness, 2D cell culture remains widely used among researchers. To address the limitations of 2D culture, 3D cell culture techniques have been employed. This method provides more comprehensive insights into cell-cell and cell-matrix interactions and offers a more clinically relevant response to therapeutic drugs.

Keywords: Cell lines, 2D cell culture, 3D Cell culture

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I. INTRODUCTION

To better understand the basic biophysical science and cellular processes of cells, formation of tissues and organs, their function, and the impact of disease on these tissues, cell culture has emerged as a crucial technique. Cell culture entails separating cells from tissue before they develop in a synthetic environment. This can be done either directly by enzymatic/mechanical means or using an existing cell line. It offers model systems for researching various applications such as pharmacological effects, mutagenesis, carcinogenesis, and cell physiology(Correia, 2017). Consequently, Cell culture systems have been widely used in biology to advance research, pave the way for the development of new drugs, and ensure the evolution of medicine. Essential nutrients, growth factors, hormones, and gases all contribute to the environment where cells develop. The majority of cells can be cultured either floating in the culture media or attached to a solid or semi-solid substrate since they are anchorage-dependent(Abe-Fukasawa et al., 2018).

In its inception, cells were reproduced in two dimensions (2D) and adhered to flat adherent surfaces or polystyrene objects as shown in figure 1. 2D *in-vitro* culture models may not accurately represent the results of a research investigation. This is due to the lack of intricate microenvironment that cells in the human body experience. A study comparing 2D and 3D-culture models for drug testing in breast cancer found that cells cultured in 3D environments exhibited heightened resistance to paclitaxel and doxorubicin (two commonly used chemotherapeutic agents). This highlights the importance of considering the cellular microenvironment when evaluating drug responses in breast cancer research, and highlights the limitations of 2D-culture models in predicting real-world drug efficacy within the complex context of tumors(Imamura et al., 2015).

The extracellular matrix (ECM) is one of the many supporting structures that surround cells in the body. Although due to its effectiveness, simplicity, and affordability, 2D cell culture is still the method of choice for the majority of researchers.To overcome the limitations of the conventional 2D *in-vitro* cell culture, 3D cell culture technique was used as it offers more insightful data on 3D cell-cell and cell-matrix interactions, and displays more clinically accurate response to therapeutic drugs(Alghuwainem et al., 2019; Jensen & Teng, 2020). Table 1 summarizes the properties of 2D and 3D cell culture model.

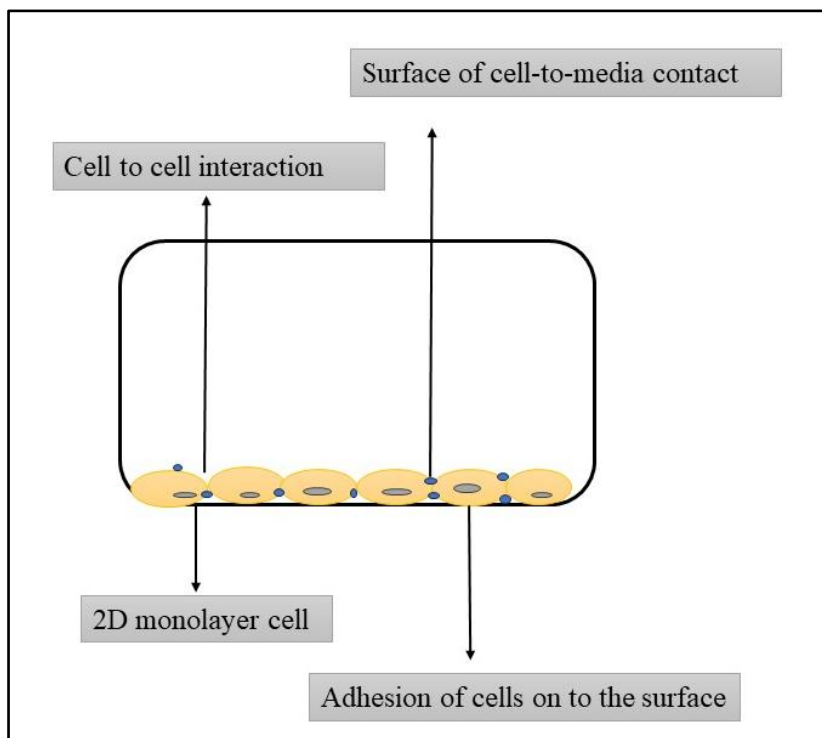


Figure 1: Illustration of Cells in 2D Cell Culture

Table 1: Properties of 2D and 3D Cell Culture Model

Properties	2D	3D	Reference
Duration of culture	It simply takes a few minutes to a few hours to develop a culture.	Typically, it lasts a few hours to a few days to develop a culture.	(Chen et al., 2012)
In-vivo like	Avoids replicating the natural architecture of the tissue or tumor mass.	It resembles the <i>in-vivo</i> structure and behavior of tumor cells.	(Griffith & Swartz, 2006)
Cell-cell interaction	No “niches” are produced and lack interaction between cells-cell and with the extracellular environment	Biological "niches" are produced by appropriate cell-cell and cell-extracellular environment interactions.	(Lv et al., 2016)
Availability of nutrients	A greater percentage of cells in the culture plate are synchronized in the same phase of the cell cycle as a result of uniform distribution of nutrients and growth	Although nutrients can be dispersed unevenly throughout cells as required, Since they receive less oxygen and growth substance from the core cells, they	(Senkowski et al., 2016)

	substances to all cells.	frequently remain dormant.	
Cell Morphology and polarity	Altered morphology and means of division leads to decline in polarity and diversity of phenotype.	Maintained morphology and unaltered division mechanisms ensure the retention of diverse phenotypes and cellular polarity.	(Langhans, 2018; Soetje et al., 2020)
Cell Division	The rate of cell division is abnormally fast.	The rate of cell division might range from extreme to low, based upon the technology used and the particular cell types being studied.	(Munir, 2019)
Drug sensitivity	Drugs fails to metabolize well.	Accurately represents the effects of the medicine	(Senkowski et al., 2016)
Molecular expression	Modifications in the topology, biochemistry, mRNA splicing, and gene expression of cells.	Cellular structure, gene expression, splicing patterns, and metabolic traits closely mimic those seen in vivo.	(Park et al., 2021)
Cell viability	The actions of cytotoxins can affect viability.	High survivability and less reactivity to environmental factors	(L. Chen et al., 2021)
Cost of maintenance	The use of inexpensive commercially available assays and growing media keeps the cost of maintaining a culture to a minimum.	Tends to be more expensive, time-consuming, and dependent on the availability of few commercially available tests.	(Athukorala et al., 2022)

II. 3D CELL CULTURE

Intricate cell-cell and cell-matrix interactions, complex nutrient transport dynamics, and three-dimensional groupings of cells constitute an organism. In the *in-vivo* condition, the elimination of waste products by the circulatory system ensures that the cells are kept in a chemo-static environment with a steady new supply of nutrients. The spatial arrangement of the cell must be taken into consideration by the culture environment. In 1992, Petersen and Bissell made the first 3D cell culture model. They described the 3D organotypic structures

resembled breast tissues in both malignant and non-cancerous conditions (Petersen et al., 1992). The primary purpose of 3D culture is to mimic the structure of the tissue's ECM. Non-cellular fibrillary proteins, several accessory proteins, and sticky molecules make up the extracellular matrix (ECM), which supports cells structurally and biochemically, creates cell-binding sites that regulate cell adhesion and motility, and is crucial to many fundamental functions. Structurally ECM is composed of Stroma and a basement membrane. Stroma contains major types of macromolecules such as proteins, glycoproteins, and proteoglycans. Several protein molecules, namely collagen I and III, as well as self-arranged polysaccharides in glycosaminoglycan fiber networks, including hyaluronic acid, fibronectin, and proteoglycan, make up the majority of this structure. The basement membrane serves as a structural barrier between epithelial cells and the connective tissue of the organ, which is located on the basal side of epithelial or endothelial cells in healthy tissues. It is composed of laminins, nidogen, perlecan, collagen IV, VIII, and X, as well as integrin receptors. It keeps these processes constant while facilitating gas diffusion and the transport of signaling chemicals. In three-dimensional cultures, immortalized cell lines (such as A549, HeLa, HEK 293 cells etc.), stem cells, or explants are arranged inside hydrogel matrices that strongly resemble the conditions associated with living cells (Erdogan & Webb, 2017; Kyriakopoulou et al., 2023). In mammals, the ECM is made up of around 300 proteins, 36 of which are proteoglycans (PG), 200 of which are complex proteins, and 43 of which are collagen subunits. Galectins make up the rest. The gel in the middle of the intercellular space is made up of PGs called PG glycosaminoglycans. Fibrous proteins like fibronectin, laminin, collagen, and elastin are the key components of the ECM (Hynes & Naba, 2012). The cells are initially suspended in a hydrogel precursor solution and are subsequently trapped by a gel initiation reaction that yields molecules with covalent or non-covalent interactions. The ability to analyze the morphology and cellular organization influenced by ECM interactions, which are changed during oncogenic transformation, is thus made possible by 3D culture models. As a result, 3D tumor models created *in vitro* are crucial tools for understanding the mechanisms behind cancer growth and spread. The 3D systems are more physiologically relevant, and hence, 3D cell culture is anticipated to produce cellular responses with a higher level of biological relevance. 3D cultures can be achieved using two methods: scaffold-free or scaffold-based culture models. The scaffold-free method does not depend on rigid structures, whereas in scaffold-based culture models, Cells are seeded on a scaffold (a porous matrix, such as collagen), where they interact and colonize to create 3-D cell culture (Lv et al., 2016). Spheroids are an example of a scaffold-free structure, whereas hydrogel-based supports and hard polymer material-based supports are examples of scaffold-based structures (Duval et al., 2017; Jensen & Teng, 2020).

III. SCAFFOLD-FREE STRUCTURE

Scaffold-free techniques includes pellet culture, magnetic levitation, spheroid microplates, spinner culture, hanging drop, Cultivation of Molded Lozenges and Liquid Overlay (Chaicharoenaudomrung et al., 2019). These techniques are considered to be the simplest due to the lack of a substrate or scaffolds that facilitates the self-assembly of cell colonies (figure 2 (a)). The aggregation of non-adhered 3D microtissue is known as spheroids. A necrotic nucleus and a peripheral layer combine to form a hard spherical structure with a well-balanced shape that range from 50 to 150 μm in size (Vu et al., 2021). In order to create models of various types of cancer *in vitro*, such as breast cancer, spheroids have been widely used several applications that enables the production of more spheroids (Djomehri et al.,

2018). However, this culture demands attentive effort and poses the potential of the development of spheroids with consistent dimensions and shape. Further, the distribution of oxygen, paracrine factors and nutrients are affected by the degree of diffusion in 3D structures, while their long-term durability and behavior are greatly influenced by the rate of self-disassembly.

- 1. Pellet Culture:** Centrifugal force (500g for 5 min) is used in the technique to concentrate cells to the bottom of the tube, remove supernatants, and then resuscitate them in a medium for spheroid development. The resulting suspension can be centrifuged immediately after an hour on an agitator to reduce cell damage. This approach is particularly effective at encouraging mesenchymal cell differentiation, which supports procedures like chondrogenesis and bone creation. This technique promotes strong cell connections, which fosters the growth of specific kinds of cells and tissue-like structures. It's crucial to consider a potential downside, though. Shear stress may result through the use of centrifugation to improve cell adhesion and aggregation, which could harm cultured cells (Achilli et al., 2012). To overcome this drawback and guarantee success in this potential culture technique, careful consideration and improvement of centrifugation factors are required.
- 2. Magnetic Levitation:** In this method, magnetic nanoparticles are introduced into the cells and exposed to an external magnetic field, resulting in the formation of a spheroid. This results in a dense cellular environment conducive to the synthesis of ECM (Anil-Inevi et al., 2021). In addition, this design is also easier to analyze the contents of the spheroid using techniques like western blotting and other biochemical experiments. In general, Magnetic levitation is a flexible method that enables scientists to carefully control cell spatial arrangement, encouraging the creation of complex multicellular structures in both fundamental and advanced technology conditions (Jensen & Teng, 2020).
- 3. Spheroid Microplates:** This technique is employed to grow multicellular cultures as well as for studying tumor cells (Imamura et al., 2015). According to studies, multicellular spheres formed from two NSCLC cells lines have highly distinct growth traits from 2D cell cultures. The cells revealed stem-cell-like characteristics, multidrug resistance, and enhanced cell motility (Achilli et al., 2012; Imamura et al., 2015).
- 4. Spinner Flask Culture:** A technique for growing cells in a liquid medium while providing agitation to keep the cells suspended is known as spinner flask culture or suspension culture. This method is frequently used in bioprocessing and research to culture cells in a controlled environment, especially those that are sensitive to shear stresses. Cells are placed in a culture vessel (spinning flask) that contains a liquid growth media to create a spinner culture. A stirring device, such as a magnetic stir bar or impeller, is included in the flask to mix the medium and keep cells from sinking to the bottom (Ryu et al., 2019).
- 5. Hanging Drop Technique:** Johannes Holtfreter developed the hanging drop method for fabricating three-dimensional spheroids in 1944 to cultivate embryonic stem cells (Chaicharoenadomrung et al., 2019). The hanging drop technique is a method used in spheroid culture to create precise spheroids of precise sizes. These droplets self-disassemble under gravity, allowing single cells to aggregate and form spheroids within

them. By controlling factors like drop volume and cell suspension density, the size of the spheroids can be regulated (Duval et al., 2017). The technique involves preparing a cell suspension within mini-plateau wells, placing it on a tray, and inverting the setup. The droplets remain suspended due to surface tension. The hanging drop approach is preferred for its ability to yield well-defined and controlled spheroids using simple and cost-effective equipment. It is particularly useful for generating large numbers of spheroids and producing spheroids with a narrow size distribution, even when different cell types are combined (Foty, 2011; Nguyen, 2017).

- 6. Static Suspension Culture:** Utilizing this cultivation method, spheroids are produced by disrupting cell adhesion on non-adherent culture plates or gels with non-adherent properties. This involves using substances like agarose with micro-well gel or pHEMA to overlay cellular suspensions (Metzger et al., 2011). These methods offer a straightforward means of observing spheroid formation and growth. Due to the prevention of cell binding to the support, cells naturally aggregate to form spheroids. Agitation, with or without centrifugation, further promotes their aggregation. However, while agarose boasts excellent non-adherent properties, it does have limitations, such as yielding spheroids of varying sizes and shapes, thus introducing heterogeneity (Ryu et al., 2019).

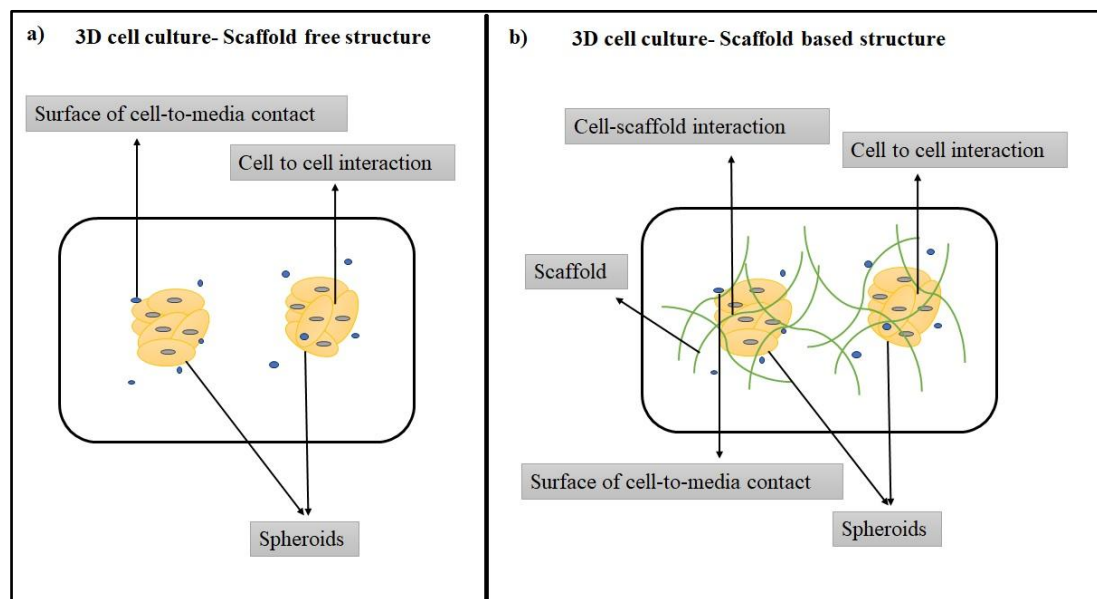


Figure 2: Illustration of Cells in a) 3D Cell Culture- Scaffold Free Structure b) 3D Cell Culture- Scaffold based Structure

- 7. Scaffold-Based Structures:** This method involves growing cells on a scaffold or supportive structure that is made of a hydrogel or a rigid polymer substance (figure 2 (b)). The scaffold can be synthesized from natural polymers, synthetic polymers, or hybrid materials that combine both natural and synthetic components. The material is selected based on the expected advantages of biocompatibility or certain physico-chemical qualities (Jensen & Teng, 2020).

- **Hydrogel Based Scaffold:** Cross-linked networks of hydrophilic polymers bonded by physical, ionic, or covalent bonds are known as hydrogels. They form soft and elastic textures that closely mimic living tissues after swelling. A porous hydrogel allows cells to recapitulate cytokines and growth factors (via diffusion) when they are deeply sown into a hydrogel (Godugu et al., 2013). Alginate, chitosan, hyaluronan, dextran, collagen, and fibrin are examples of natural polysaccharide and proteins that can be used to create hydrogels (Zheng & Xiao, 2023). Alginate is derived from brown seaweed and can also be produced by *pseudomonas* and *azotobacter* bacterial strain (Hay et al., 2013). Using materials originating from animals in clinical settings may increase the risk of immunogenicity and infections, Therefore, employing such material for such applications may be challenging. Non-animal origin polysaccharide-based hydrogels are greatly desired due to their biocompatibility and gel formation, yet they encounter difficulties with material composition, control kinetics, and mechanical qualities. For example, covalently cross-linked alginate hydrogels can be synthesized by conjugating methacrylate groups to the alginate backbone and exposing them to UV light. If cells are suspended in the alginate solution before the photo-crosslinking, they will be equally dispersed throughout the hydrogel. A hydrogel that has been covalently bonded is chemically stable and offers a variety of ways to relieve tension. The degree of methacrylation of alginate can be changed to alter the mechanical characteristics and biodegradation rates of a hydrogel (Andersen et al., 2015; Li et al., 2022).

Collagen, a primary element of ECM, is crucial to the growth and proliferation of cancer. Collagen fibrils arrange themselves in a wide range of forms, depending on the tissue type, to serve the distinct roles and characteristics of tissues. By manipulating the concentrations and preparation conditions, the mechanical characteristics, architecture, and biodegradability of the hydrogel can be precisely controlled (Short et al., 2017). Research on prostate cancer pathogenesis and therapeutic approaches has been largely focused on cell lines like PC3 and LNCaP. Researchers have used collagen-based scaffolds to simulate bone metastases, a key aspect of advanced disease progression. These scaffolds provide a more accurate platform for studying cancer behavior and therapeutic responses. When cultured in a 3D environment, these cell lines showed increased resistance to docetaxel, a common chemotherapy agent. This highlights the importance of using three-dimensional culture models to predict drug efficacy and address bone metastasis challenges, leading to more effective treatment strategies for advanced prostate cancer (Fitzgerald et al., 2015).

The fundamental benefit of hydrogels is that they can accurately mimic the biochemical and mechanical characteristics of the real native ECM due to their adjustable physico-chemical properties. However, the chance for cellular heterogeneity is more in hydrogels those made of collagen, despite their high cost, lack of replicability, lengthy handling, and need for specialized equipment (Lv et al., 2016; Short et al., 2017).

- **Rigid Polymer-Based Scaffold:** Synthetic materials including polyethylene glycol (PEG), polyvinyl alcohol, polyglycolic acid (PGA), poly-hydroxyethyl methacrylate

(poly-HEMA), polycaprolactone (PCL), and poly-lactide-co-glycolide (PLGA) is used for the development of scaffolds. Due to their active chemical groups (amine, acid, or alcohol activities) that they naturally carry and their ability to form a 3D framework, synthetic polymers can create an ECM model with specific characteristics. These hydrogels are made by polymerizing synthetic polymers, and as a result, they have a variety of biophysical, mechanical, and biological properties. In 3D breast cell cultures, synthetic hydrogels are crucial as they offer a controlled setting for studies on the complex interactions between malignant tumors and their *in-vitro* microenvironments. Their unique composition enables specific features that are essential for simulating the complexity of breast tissue and stimulating illuminating research into the development of malignancies in this dynamic environment.

In order to build a 3D artificial microenvironment for human embryonic stem cell development, Levenberg et al., (2003) utilized PLGA and PLA to form porous scaffolding. Even while they had some success with this, they also showed how challenging it is to encourage cells to invade the entire structure. The biodegradation of scaffolding made of poly-glycolic acid, poly-lactic acid, and their copolymer PLGA can also result in the production of by-products like lactic acid. When it applies to standard 3D cell culture, considerations for concerns including durability, preservation, and consistency of the product must be made, biodegradable materials are therefore not practicable. In some situations, using synthetic hydrogels poses difficulties that should be carefully explored. These hydrogels might not be as useful physiologically since they might expose the cells to dangerous breakdown products. Their suitability for *in vitro* tumor engineering is thus constrained. Furthermore, because of the active chemical groups in their makeup, they may be subject to unfavorable chemical reactions. Their applicability is further complicated by the probable loss of cell signaling patterns and sensitivity to pH, as is the case with polyethylene glycol (PEG). Biophysical factors including mechanical qualities, permeability, and stiffness must be taken into consideration while using synthetic hydrogels to make sure they as closely resemble natural tissue conditions as possible (Fan et al., 2019).

3D cell culture includes drawbacks such as immune rejection and circulatory system cell loss. The Cell Sheet Method is being developed to overcome these restrictions. The extracellular matrix (ECM) is preserved by this innovative method, which also enables direct implantation into tissue beds and stacking to produce 3-D tissue-like structures (Nair & Kumary, 2016).

IV. CONCLUSION

The complexity and functionality of 3D cell culture make them essential for research on tumor and stem cells. A crucial tool for better comprehending the alterations, interactions, and biomolecular signals that occur during malignant transformation and metastasis is the 3D culture. Numerous 3D cell culture technologies have been created by the pharmaceutical sector, with prospective uses in tissue replacement materials, illness simulation, drug development, and 3D disease models. Currently, a variety of natural and synthetic polymer hydrogels have been created into 3D biomaterial scaffolding. Experiments on lab animals should be replaced by the validation of preclinical results by 3D cell systems. The therapeutic

application of these cultures, however, necessitates extensive research into their functional, safe, and transplantable indices.

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