



A promising breakthrough in pancreatic cancer research: The potential of spheroids as 3D models

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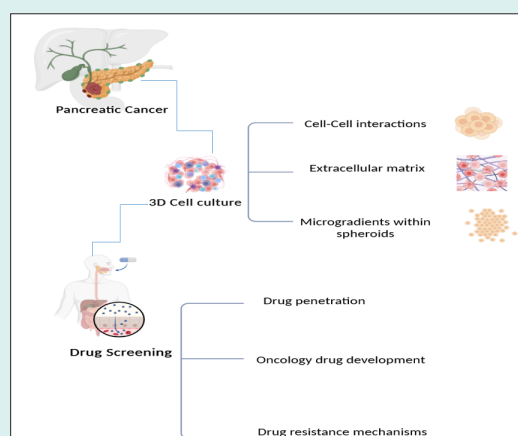
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Abstract

Pancreatic ductal adenocarcinoma (PDAC) stands as the fourth leading cause of cancer-related deaths, primarily attributable to its resistance to chemotherapy, resulting in a nearly universal fatality rate. Despite the promise exhibited by numerous drugs in preclinical studies, their subsequent failure in clinical trials underscores the inherent limitations of conventional two-dimensional cell culture models commonly employed in early drug screening endeavors. The inadequacies of two-dimensional (2D) models prompted the exploration of three-dimensional (3D) culture systems, which

more faithfully recapitulate the native tumor microenvironment. These 3D systems have distinct advantages over 2D models in morphology, proliferation, drug response, and protein expression. Among these 3D platforms, tumor organoids and spheroids, generated through different methodologies, have emerged as next-generation models that closely mirror aspects of pancreatic tumor biology. This comprehensive review scrutinizes pancreatic cancer spheroids' techniques, tissue sources, and applications, offering a nuanced analysis of their advantages and limitations. By comparing these distinct 3D culture systems, researchers gain valuable insights to inform the selection of optimal model designs aligned with their specific experimental objectives. The utilization of these advanced models holds significant promise for enhancing the clinical relevance of both *in vitro* and *in vivo* cancer research, thereby contributing to the development of improved therapeutics against pancreatic cancer.



Introduction

Pancreatic ductal adenocarcinoma (PDAC) stands out as a highly malignant form of human cancer, exhibiting an escalating incidence. The current therapeutic approach for PDAC involves a combination of surgery, chemotherapy, and radiation therapy in select cases, achieving long-term survival only for a minority of patients.¹ The dense desmoplastic stroma, distinguished by an overabundance of fibroblasts, extracellular matrix, and immune cells, is a defining feature of PDAC. This stromal milieu plays a pivotal role in disease progression and therapy response and is a distinctive PDAC feature. The complex interaction among tumor cells and diverse stromal

components along multiple signaling pathways governs the tumor microenvironment's (TME) development.² The significance of this interaction underscores the necessity for a suitable *in vitro* technique that accurately recapitulates the complexity of pancreatic TME cells, thereby advancing our understanding of pancreatic cancer development and facilitating the discovery of effective treatments. Conventional two-dimensional cell cultures, while valuable, need to catch up in capturing the intricate multicellular interactions within the TME that drive PDAC progression *in vivo*. Challenges persist despite the deployment of animal models in preclinical trials to address these limitations. For instance, the



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extended duration and engraftment issues associated with drug examination on animal models can impede timely research outcomes.³

Recently, there has been much interest in three-dimensional (3D) cell models since they can accurately mimic the characteristics of tumors in living organisms. This makes them a valuable tool that fills the gap between standard two-dimensional (2D) cell culture methods, and *in vivo* models.⁴ 3D cell cultures facilitate cell-to-cell and cell-to-matrix interactions, mirroring the physiological conditions experienced by cells *in vivo*. This approach has gained prominence as one of the most favored methods in drug development.

Moreover, employing human cells in 3D culture models can reduce reliance on alternative models, such as mouse models, which are often associated with high costs and may not precisely depict therapeutic efficacy and drug adverse effects.^{5,6} Various 3D models for cell culture have emerged, including organoids, spheroids, organ-on-a-chip, 3D scaffolds, hydrogels, 3D bioprinting, tumor microenvironment models, tumor organoids, and tumor spheres (Table 1).

To develop more effective treatments for PDAC, it is essential to use research models that accurately recreate the native tumor microenvironment and heterogeneity. This review summarizes the latest strides in utilizing 3D spheroid models derived from PDAC tissues for disease modeling, drug development, and personalized medicine applications. Our synthesis encompasses critical studies showcasing the capabilities of these models in faithfully reproducing tumor heterogeneity, microenvironment interactions, and therapeutic responses. Furthermore, we address the challenges inherent in these next-generation culture systems and outline future directions to enhance

their clinical translatability.

Spheroid

Sutherland and colleagues pioneered the concept of spheroids in the early 1970s. Since then, various models and creation methods have been developed.¹⁸ The formation of spheroids involves spontaneous cell aggregation followed by cell surface integrin binding to the extracellular matrix (ECM). As cells upregulate E-cadherin, accumulating on the cell surface, intercellular E-cadherin interactions lead to the compact structure characteristic of spheroids. Growth factors, oxygen, and nourishment are a few variables that impact this process.^{19,20}

Distinct cell lines contribute to the variability in the structure and morphology of spheroids based on their cellular sources.²¹ Additionally, the morphology of spheroids is impacted by the technique used and the primary cell origin.^{21,22} For instance, Luka et al. demonstrated that metastatic cell lines of colon cancer, when cultured in a laminin-rich extracellular matrix (IrECM), formed grape-like spheroids. In contrast, colon cancer cells derived from primary tumor tissue exhibited a round morphology.²³ In addition, 25 breast cancer cell lines were further categorized by Kenni and colleagues into four types of 3D spheroids: circular, mass, stellate, and grape-like.²⁴ Each spheroid type presents specific characteristics, such as weak cell-cell interactions and an aggressive phenotype for round, grape-like, and stellate spheroids.²⁵⁻²⁷

There are several known approaches to spheroid creation, with several of these approaches having been fine-tuned for controlled mass manufacturing.²⁸⁻³⁰ There are different sources for producing spheroids, with three

Table 1. Various 3D cell culturing approaches and applications

Model	Key Features Modeled	Preparation method	References
Spheroid	<ul style="list-style-type: none"> - Cell-cell interactions - Nutrient/oxygen gradient - Avascular tumor mimic 	<ul style="list-style-type: none"> - Hanging drop - Spinner flask - Non-adhesive surfaces 	7
Organoids	<ul style="list-style-type: none"> - Tissue complexity - Organ physiology 	<ul style="list-style-type: none"> - Matrigel - Collagen - Laminin 	8
Organ-on-chip	<ul style="list-style-type: none"> - Mimic organ physiology - TME 	<ul style="list-style-type: none"> - Microfluidic devices 	9
3D scaffolds	<ul style="list-style-type: none"> - ECM-like support 	<ul style="list-style-type: none"> - Collagen - Matrigel 	10, 11
Hydrogels	<ul style="list-style-type: none"> - Nutrient diffusion 	<ul style="list-style-type: none"> - Crosslinked hydrophilic polymers 	12, 13
3D Bioprinting	<ul style="list-style-type: none"> - 3D constructs - Spatial control 	<ul style="list-style-type: none"> - Computer-controlled deposition 	14
Tumor Microenvironment Models	<ul style="list-style-type: none"> - <i>In vivo</i> ecology - Stromal cell interactions 	<ul style="list-style-type: none"> - Co-cultures with stroma, immune cells, vasculature 	15
Tumor Organoids	<ul style="list-style-type: none"> - Intra-tumor heterogeneity - Patient-specific profiles 	<ul style="list-style-type: none"> - Patient-derived 3D models 	16
Tumor Spheres	<ul style="list-style-type: none"> - Drug screening - Cancer stem cell enrichment 	<ul style="list-style-type: none"> - Anchorage-independent spheroids 	17

ECM: extra cellular matrix.

common models:

1. Multicellular spheroids from tumor cell lines,
2. Oncospheres representing cancer stem cell (CSCs) growth,
3. Mechanical and enzymatic tumor tissue dissociation produces organotypic multicellular spheres.^{31,32}

The biological and pathologic properties of the tumor cell line are significantly influenced by the types of cell lines used, originating from pancreatic cancer, donor patients, and the region of derivation. These factors should be carefully considered when designing *in vitro* investigations. Numerous studies have explored spheroid construction with varying degrees of success and inconclusive outcomes.

Challenges and limitations

Tumor spheroids, such as multicellular tumor spheroids (MCTSs), are becoming more commonly utilized as 3D models *in vitro* for pharmacological investigations, notably in the field of cancer research. 3D cell cultures provide a more accurate depiction of the tumor environment in living organisms, as opposed to conventional 2D cell cultures. This makes them highly useful for many applications like as drug screening, drug design, drug targeting, drug toxicity assessment, and validation of drug delivery techniques.³³ Nevertheless, despite the benefits they offer, the use of tumor spheroids in pharmaceutical research is accompanied by many obstacles and restrictions.

Reproducibility and standardization

The lack of consistency in their manufacturing is one of the key obstacles to employing tumor spheroids. Both scaffold-based and scaffold-free techniques have been developed for the production of MCTSs. Cell requirements and the relevant biological inquiry often dictate the approach taken.³⁴ This heterogeneity might cause changes in the characteristics of the spheroids formed, which affects the repeatability of results across multiple experiments.

Size and testing performance

The effectiveness of tumor spheroids in testing can also be influenced by their size. An inadequate supply of nutrients and oxygen, for instance, might cause bigger spheroids to develop a necrotic core, which in turn can impact the efficacy of anti-cancer medications. Furthermore, spheroids of different sizes might affect drug penetration and distribution, which can provide misleading findings.³⁵

Complexity of TME

Tumor spheroids are more closely related to the *in vivo* tumor environment than 2D cultures, although they do not entirely recreate the complexity of the TME. For example, most spheroids are formed up of tumor cells alone and do not comprise other critical components of the tumor microenvironment, such as fibroblasts, adipocytes, and immune cells.³³ Because interactions between tumor

cells and various other cell types can greatly impact the effectiveness of medication delivery and the therapeutic value of therapy, this restriction can impact the accuracy of drug testing findings.³³

Data analysis

Data generated by research examining cancer cell metabolism and cell cycle abnormalities using tumor spheroids can be enormous, necessitating sophisticated methods for comprehensive analysis.³⁴

Spheroid of pancreatic cancer cell lines

This section discusses several studies on spheroid generation derived from pancreatic cancer cell lines. While most PDAC cell lines could form spheroids, Sipos et al noted that MiaPaCa-2 "totally failed to develop as spheroids" because it disaggregated after harvesting.³⁶ In 2013, Yeon et al. documented the creation of tumor spheroids (TS) using human pancreatic cancer cells (Aspc-1, PANC-1, Capan-2) in concave polydimethylsiloxane (PDMS) microwell plates. They evaluated their appropriateness as a model for testing the effectiveness of anticancer treatments. TS formation was observed in the three mentioned cell lines, exhibiting varying necrosis within the spheroids. PANC-1 spheroids, with a spherical shape, rough surface, and distinctive adhesion structures, were effectively formed on concave microwell plates without noticeable necrosis. Drug resistance-associated compounds, including MT1-MMP, TGF- β 1, CTGF, collagen type I, laminin, and fibronectin, were detected in PANC-1 spheroids grown in concave microwells. TGF- β 1, CTGF, and MT1-MMP are crucial molecules in pancreatic cancer associated with poor prognosis and therapy resistance.³⁷ The study also demonstrated the necessity of epidermal growth factor (EGF) for the 3D culture of Capan-2 cells. In contrast, the monolayer culture of these cells did not require EGF. This dependence may be attributed to EGF's significant role in pancreatic cancer development and its overexpression in pancreatic cancer.³⁸

In contrast to earlier findings by Sipos et al and Wen et al, another study established a 3D spheroid-based cultivation method for pancreatic cancer cell lines, specifically MiaPaCa-2 and PANC-1, to conduct pharmacological testing. Their models exhibited reproducibility and ease of manipulation, indicating that 3D cell culture has the potential to serve as an intermediary between 2D cell cultures and *in vivo* models in the medication research and evaluation method for pancreatic cancer.³⁹ Ware et al successfully generated spheroids by combining two established techniques: the hanging drop method and using methyl cellulose (MC) in the media. This method enhanced the compactness of the spheroids while preventing their separation into smaller components. Five pancreatic cancer cell lines—PANC-1, MiaPaCa-2, Capan-1, BxPC-3, and Aspc1—could be effectively

transformed into spheroids.⁴⁰

Various cell lines exhibit distinct spheroidal features, likely attributed to differences in their inherent cellular characteristics. For instance, BxPC-3 and Capan-1 displayed similar shapes and developmental patterns, generating densely packed spheroids that were one-third to one-half the size of those formed by AsPC-1, PANC-1, and MIA-PaCa-2.⁴⁰ Conversely, AsPC-1, PANC-1, MIA-PaCa-2 spheroids, and BxPC-3 and Capan-1 spheroids demonstrated a comparable phenotype.⁴¹ Despite the importance of the MIA-PaCa-2 cell line in pancreatic cancer studies, generating and maintaining homogenous and stable MIA-PaCa-2 spheroids has proven challenging. Researchers, including Cavo and colleagues, have explored various methods to overcome these challenges, such as round-bottom wells, hanging drop, and Matrigel embedding, in the presence and absence of methylcellulose in multiple mediums. Based on their results, a hydrophobic base with a methylcellulose-enriched medium may produce MIA-PaCa-2 spheroids.⁴² Genomic research also suggests that the shape and size of spheroids may reflect distinct genetic states in the cells that produce them.⁴² While studies demonstrate the feasibility of generating spheroids from different cell lines, there is no standardized procedure, and researchers are focused on refining existing methods. 3D spheroids have been the subject of much research due to their promise as a model for screening anticancer drugs. For example, Longati et al, pancreatic cancer spheroids such as AsPC-1, BxPC-3, Capan-1, and PANC-1 are superior to 2D-cultured versions of these cells when it comes to drug testing due to their chemo-resistant phenotype and matrix-rich composition.⁴³

Additional studies have explored nanoparticle

permeation in 3D multicellular spheroid models of pancreatic cancers, investigating factors such as size, surface charge, PEG decorating, and other physicochemical features.⁴⁴ Researchers have also generated spheroids from pancreatic tumor stroma cells, particularly pancreatic stellate cells, to study drug efficacy, given their role in PDAC progression and drug resistance.⁴⁵ Improved spheroid homogeneity and stability and more accurate assessment of drug responses are anticipated outcomes of future research that uses more suitable cell line models, such as primary cells with established mutation pathways.

Cultivating pancreatic cancer cell lines into spheroids, three-dimensional cell clusters suitable for diverse analyses like live cell staining and imaging, is a valuable approach. Nevertheless, creating and manipulating stable and robust spheroids from pancreatic cancer cell lines, including the challenging MIA-PaCa-2, poses a significant challenge.⁴⁶ Several methods and models have been developed to address this issue (Fig. 1).

Liquid overlay technique (LOT)

This method primarily relies on non-adhesive sheets covered by poly 2-hydroxyethyl methacrylate (poly-HEMA),^{22,47–49} agar, or agarose.^{50,51} These materials prevent cells from adhering to the plate, prompting them to adhere to each other and form spheroids.^{52,53} Plates covered with agar or agarose or low-adhesive surfaces create cell suspensions. Cells aggregate more efficiently when the plate is continuously shaken on a shaker. Liquid overlay is a straightforward and widely used method, applicable even in 96-well plates. The real-time observation of spheroid formation is a notable advantage. However, it is necessary to have more influence over the dimension and form of the produced spheroids when using the

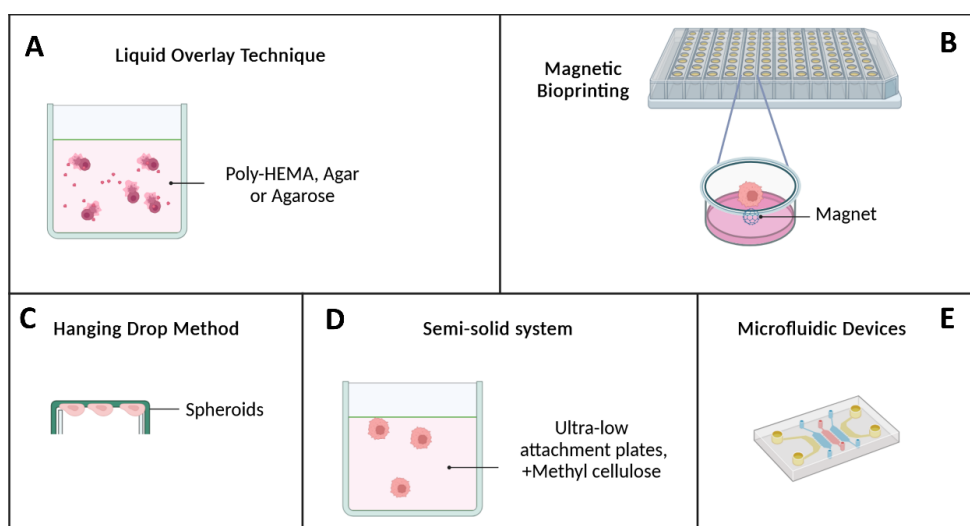


Fig. 1. Techniques for generating pancreatic cancer spheroids. **A)** Liquid Overlay Technique: Cells are seeded onto plates coated to prevent adhesion and then aggregate into spheroids. Agitation prevents spheroid fusion; **B)** Magnetic Bioprinting: Spheroids containing magnetized cells are printed using bioinks; **C)** Hanging Drop Method: Cells aggregate into spheroids by suspending drops containing cells from an inverted plate. Spheroid size can be controlled by cell number per drop; **D)** Semi-Solid System: Cells are cultured in ultra-low attachment plates with methylcellulose and growth factors to promote spheroid formation; and **E)** Microfluidic Devices: Microfluidic systems enable controlled spheroid formation through automated processing steps.

liquid overlay approach, which is a challenge.⁵⁴ Table 2 summarizes the LOT method's pros and drawbacks.

By tailoring culture substrates to individual researchers' needs, scientists have more leeway regarding experiment design and budget. Commercial low-adherence culture ware, on the other hand, provides an efficient and easy-to-use alternative. Many different kinds of cells, including cancer cells, may be effectively transformed into spheroids using these commercially accessible techniques,⁶³ dental papilla cells,⁶⁴ mesenchymal stem cells (MSCs),⁶⁵ and a mixture of heterogeneous cell types.⁶⁶ Notable products in the market include UpCell™, Nunclon™, Nunc™, Sphera™, Corning Ultra-Low Attachment surfaces, Lipidure® -COAT, CELLSTAR®, and Nanocluster.

A pancreatic cancer spheroid model was created using human pancreatic cancer cell lines MIA PaCa-2 and PANC-1 to assess the effects of chemotherapeutic treatments using the LOT approach. Cells at passage >20 were detached from the bottom of the dish using 0.05% trypsin. Using agarose-coated 96-well culture plates (50 mL 1.5% agarose per well), spheroids were started in a liquid overlay by seeding 1.2×10^3 MIA PaCa-2 cells and 1.0×10^3 PANC-1 cells per well in 200 mL of media. Except for a 72-hour drug treatment setup detailed in the drug treatment section, after an initiating period of 4 days, 50% of the supernatant was changed with the new medium. This process was repeated every 48 hours after that. Spheroid cell viability was evaluated using a modified

acid phosphatase (APH) test. The study examined the effectiveness of gemcitabine and 5-FU in MIA PaCa-2 and PANC-1 spheroid and monolayer cultures, respectively. The results indicated that the efficacy of chemotherapy drugs in spheroid culture is lower than in monolayer culture for these two cell lines. This suggests that the size and volume of the spheroid, a good simulator of the actual tumor sample in the body, may hinder the proper signaling of drugs.⁶⁷ To investigate cellular interactions at the molecular and cellular level, spheroids can be generated using soft agar. Spheroids made from cell lines MCF-7, BxPC-3, Capan-2, Panc-1, MIA PaCa-2, and Capan-1 showed that the microenvironment of 3D cultured cells is more acidic than that of 2D cells, attributed to increased phosphorylation of Tyr421 and elevated expression of cortactin. This acidity can lead to better stimulation of pro-metastasis migration of tumor cells.⁶⁸

Hanging drop method

This method is a straightforward technique for forming spheroids without requiring specialized facilities. This method leverages the surface tension of cellular suspensions to optimize spheroid formation by intensifying cell-cell interactions by including various biological factors in minimal amounts.^{69,70} The hanging drop technique involves seeding tissue culture plates with a predetermined number of cells in the shape of tiny drops. Spheroids quickly develop after a 180°

Table 2. Advantages and disadvantages of coating material used in the LOT method

Material	Advantage	Disadvantage	Ref.
Agar	<ul style="list-style-type: none"> Straightforward, low-cost, and relatively simple to handle Post-processing directly in the plates is very advantageous in high-throughput experiments. Inexpensive Dissolve in water and serum-free media. Enables optical microscopy monitoring of spheroid development by allowing single cells to spontaneously self-assemble. Preventing the loss of MCTS as a result of the MCTS being accidentally removed from the system. 	<ul style="list-style-type: none"> Used to test a specific number of cancer medicines. Dissolved in a high temperature Agar can affect cell growth and other qualities when coated plates are stored for only a few days. In a drug assay, there are no repeatable results. Heterogeneous spheroids are labor-intensive and time-consuming to mass-produce. 	53, 55-57
Agarose	<ul style="list-style-type: none"> Simple to perform and can be sterilized using an autoclave or UV light. Inexpensive Allows for accurate MCTS size adjustment and large-scale MCTS generation Allows for optical microscopy monitoring of spheroid formation. Allow for spontaneous self-assembly of single cells. Scalability The process results in the formation of irregular 3D cellular aggregates of varying sizes and forms. Preventing the loss of MCTS as a result of the medium being accidentally removed with it. 	<ul style="list-style-type: none"> Dissolve in a hot environment. Coated plates are only good for a few days and cannot be reused. They are used for short-term cultures and have a wide size distribution. Unreliable drug assay results; labor-intensive and time-consuming; difficult to mass-produce Heterogeneous spheroids Some cell lines have a hard time producing spheroids. 	58, 59
Poly (2-hydroxyethyl methacrylate) (pHEMA)	<ul style="list-style-type: none"> Performed simple and relatively easy handling very useful in high-throughput experiments direct post processing in plates Storing the coat plate and solution at 4 °C for several months to prevent the loss of MCTS due to unintended removal of the MCTS and medium. 	<ul style="list-style-type: none"> High expense; long-term cultivation problematic; 95 percent ethanol preparation Coated plates must be stored for several months and are labor-intensive and time-consuming to mass-produce. Heterogeneous spheroids Some cell lines have a hard time producing spheroids. 	60-62

rotation in a humid atmosphere when cells gather at the drop's tip—the interface between the liquid and air—caused by gravity.^{69,71} While praised for its simplicity and compatibility with high-throughput screening, tracking spheroid formation and directly assessing drug perturbations can be challenging.⁷²

One notable advantage of the hanging drop method is the concurrent culture of two or more cell lines, enabling cell-matrix interactions and cell-cell investigation. Wound healing, tumor cell interactions with stroma in aggressiveness and cancer, fetal development, and tissue engineering all rely on these interactions.⁷³ However, limitations such as a restricted suspension volume and inadequate nutrients pose challenges for long-term culture. Consequently, spheroids formed using this method often need to be transferred to other plates, affecting their integrity and time-consuming.^{74,75}

As mentioned previously, in 2016, Ware et al⁴¹ used five human PDAC cell lines—BxPC-3, PANC-1, Capan-1, AsPc-1, and MIA-PaCa-2—to create a spheroid model enriched with human pancreatic stellate cells. Adding pancreatic stellate cells enhances the simulation of the dense microenvironment characteristic of pancreatic tumors. This team used a hybrid approach, combining the hanging drop method with methyl cellulose as a medium component. The findings revealed notable differences between spheroids grown with pancreatic stellate cells and those formed with only PDAC cell lines. Spheroids cultivated with pancreatic stellate cells exhibited increased density, compactness, and more collagen than those produced using PDAC cell lines alone. Beyond similarities in collagen content, spheroids that incorporated pancreatic stellate cells closely resembled orthotopic tumors regarding the expression of KI67 and HIF-1 α .⁴¹

Magnetic bioprinting

Another innovative approach for maintaining cell cohesion until spheroid formation involves using magnetic force. Magnetic 3D bioprinting enables the magnetization of cells with biocompatible nanoparticles, which are subsequently printed onto multi-well forms. Once nearby, these magnetized cells aggregate to form a spheroid, which can be harvested for subsequent biophysical and biochemical studies. In a study by Noel et al, NanoShuttle, comprising iron oxide, poly-L-lysine, and gold nanoparticles, was employed. The Patu8902 cell line was labeled with NanoShuttle, which electrostatically binds to the plasma membrane and is spontaneously released after approximately one week. The specific membrane receptor involved in this binding has yet to be discovered. Notably, using meager magnetic forces (30 pN) ensures sufficient force for cell accumulation without compromising cell survival, metabolism, or proliferation.⁷⁶

Semi-solid system

The production of non-specific cell aggregations results from the mobility of individual cells, which is one of the constraints of spheroid formation.⁷⁷ The semi-solid culture method addresses this issue by incorporating methylcellulose into the spheroid culture medium, thereby limiting the excessive mobility of cells. In this approach, cells are seeded in ultra-low attachment plates, and the culture medium is supplemented with methylcellulose and growth factors. After 11 days, spheroids are formed. Yang et al successfully generated spheroids from the PANC-1 cell line using this method. These spheroids exhibited enhanced proliferation, differentiation, migration, and invasion properties compared to conventional 2D cultures.⁷⁸

Patterned surfaces and microfluidic devices

In 1970, microfluidics emerged as a versatile technology with applications across various industries, including cell isolation,⁷⁹ biological and diagnostic sensors,⁸⁰ pharmacological experiments,⁸¹ DNA extraction,⁸² and the formation of spheroids. Microfluidic chips consist of inlets and outlets connected by microchannels or chambers within a bulk material. The microchannel network directs, mixes, or splits liquid fluid to achieve specific applications used in biomedical and chemical settings, including microreactors, fluid mixers, cell culture, and sorting of cells and particles.⁸³

The gas permeability, cheap cost, and ease of use of PDMS make it a popular material for microfluidic system fabrication.^{84–86} PDMS's transparency enables direct fluorescent imaging of proteins and cells.^{87–92} However, challenges such as evaporation of cell culture medium over time due to gas permeability, time-consuming system development, and repeatability issues have been associated with PDMS microfluidic systems.^{93,94} Microfluidic systems have been fabricated using plastic and glass materials to address these challenges.⁹¹

Recent advancements in 3D printing technology have provided solutions for spheroid formation using microfluidics. In this technique, cells grow in layers through automated systems. Polyjet, stereolithography, and extrusion-based printing are three 3D printing techniques applicable to microfluidics.^{92,95,96} These techniques can produce systems with channels that facilitate cell-cell interaction, mimicking *in vivo* conditions.^{92,97} Additionally, chips,^{69,98–101} and biosensors⁹⁸ have been designed to produce spheroids. In these techniques, cells are trapped on a bed of ECM, including fibronectin and collagen.¹⁰² The accumulation of spheroids can be quantified by being placed between two electrodes, proving valuable in assessing the efficacy of medications.¹⁰³ The materials used for these structures typically include silicon, glass, or plastic, with transparent materials enabling fluorescent microscopy investigations.¹⁰²

Moreover, recent approaches involve using columns coated with Matrigel, poly-L-lysine, and barium chloride for spheroid culturing. This method includes suspending cells in 1% alginate, collagen, and Matrigel. The column is then introduced into the culture medium using the hanging drop method, ultimately resulting in spheroid formation at the tip of the column. This technique proves effective in assessing medication efficacy, as these columns can be placed in a medium or a 96-well plate containing the drug.^{104,105}

Integrating pancreatic stellate cells and pancreatic tumor spheroids in a 3D collagen matrix is the goal of the microchannel plate-based co-culture paradigm put out by Lee and colleagues. By recreating chemoresistance and the epithelial-mesenchymal transition (EMT), this model attempts to mimic the *in vivo* TME. Findings indicated that PANC-1 cells when co-cultured with pancreatic stellate cells, doubled the number of spheroids and generated 3D tumor spheroids after five days. By cultivating pancreatic stellate cells in close quarters with cancer cells in a 3D collagen matrix, they were able to show that the two cell types interact to enhance EMT and medication resistance in the microchannel plate. One promising approach to studying EMT and treatment resistance therapeutically relevantly is the microfluidic co-culture of pancreatic stellate cells and pancreatic tumor spheroids.¹⁰⁶ Additionally, there have been reports of spheroid models that exhibit enhanced biological complexity. These models involve the co-culturing of 3D cancer cells with one or more kinds of cells from the PDAC TME. This is a significant improvement compared to monocultures, as interactions between cells in the TME might impact the course of the illness and the effectiveness of treatment.¹⁰⁷ See Table 3 for a comparison of different methods for pancreatic-derived spheroid formation.

Patient-derived spheroid

Tumor-derived spheroids are created by isolating single-cell suspensions from tumor tissues using mechanical or enzymatic methods, followed by serum or serum-free medium cultivation. Various cancers, including brain,¹⁰⁸ breast,¹⁰⁹ lung,¹¹⁰ colon,¹¹¹ prostate,¹¹² pancreatic,¹¹³ and ovarian cancers, have been successfully used to generate tumor-derived spheroids.¹¹⁴ Enriching cancer stem cells is achieved by cultivating tumor cells with stem cell traits in a serum-free medium rich in various growth factors like progesterone, hydrocortisone, and insulin. This process promotes tumor cell proliferation while excluding non-malignant and differentiated cells. As a result, a critical feature of tumor-derived spheroids is the concentration of cancer stem cells.

Tumors are either partially physically or enzymatically dissociated into 0.3 mm pieces for use in *ex vivo* explant cultures, followed by cultivation in agar-covered plates with a serum-containing medium comparable to

organotypic multicellular tumor spheroids.¹¹⁵ Although tumor spheroids are among the most fundamental 3D cell culture models, their attractiveness lies in their ability to closely mimic solid tumors' characteristics in various ways. Importantly, they interact with other cells and the ECM. Moreover, when grown beyond 500 μm , spheroids resemble non-vascularized or minimally vascularized tumors, displaying metabolic gradients. There are three distinct layers to the structure: one with cells that are actively dividing, one with cells that are resting, and finally, one with cells that are hypoxic and necrotic.⁸⁵ Similar to human cancers, these unique characteristics of tumor spheroids make them resistant to radiation and anti-cancer therapy. Consequently, tumor spheroids are extensively utilized in drug screening investigations.¹¹⁶

3D tumor sphere models can be divided into four distinct groups based on their culture methods and sphere biology: organotypic multicellular spheres (OMS), multicellular tumor spheroids (MCTS), tissue-derived tumor spheres (TDTS), and tumorspheres (Fig. 2).¹¹⁷

1. MCTS: These spheroids comprise tumor cells co-cultured with stromal cells such as immune cells, endothelial cells, and fibroblasts, which can be mono- or heterotypic cell populations. Cell culture in non-adherent media can produce MCTS.¹¹⁸
2. Tumorspheres: Also known as cancer stem cell spheres, this method isolates and propagates CSCs from tumor tissues or cancer cell lines.¹¹⁹
3. TDTS: This model is created by partially dissolving tumor tissue using enzymes or mechanical means. This process separates cancer cells from non-tumor cells while keeping cancer cells in touch with each other. The OMS model differs from this approach, which involves cutting primary tumor tissues.¹²⁰
4. OMS: Primary tumor tissues are sliced to obtain OMS models. When compared to MCTS and tumorspheres, TDTS and OMS models do a better job of simulating tumor development and gene expression patterns. But by including stromal cells, the OMS model adds another layer of intricacy.¹²⁰ OMS has great promise for personalized medicine since it is the best 3D model for evaluating a tumor's therapeutic response to treatment.¹⁵⁰

Applications of different 3D models

As previously mentioned, many approaches are used to create three-dimensional spheroids. In this section, various 3D models have been applied to studying pancreatic cancer. According to the literature, each of these models has distinct uses. Spheroids find applications in preclinical domains, gene expression evaluation, and protein investigation. 3D tumor models generated by spherical microplates mimic the *in vivo* conditions of the microenvironment, allowing spheroids to grow as monocellular entities or in conjunction with other cells

Table 3. Different methods of pancreatic-derived spheroid formation

	Cell line	Advantage	Disadvantage	Ref.
Pellet culture	Capan-2 PDAC1, PDAC2, PDAC3, PDAC5	<ul style="list-style-type: none">• Evaluation the spatio-temporal dynamics effect of chemotherapeutics• The difference in the size of the spheroids was less than 10%.	<ul style="list-style-type: none">• Requiring large numbers of cells to produce structures of sufficient size	108-111
LOT	MIAPaCa2 PANC-1 BxPC-3 Capan-1 Capan-2 Panc-1 MCF-7	<ul style="list-style-type: none">• Easy handling• Cost-effective• The real-time formation can be followed	<ul style="list-style-type: none">• Coating the surface can be time consuming	67,68,112-116
Hanging drop	AsPc-1, PANC-1, BxPC-3 MIA-PaCa2, Capan-1,	<ul style="list-style-type: none">• Easy handling• Uniform spheroid in size and shape	<ul style="list-style-type: none">• Changing media is not possible• Low throughput	75-78,117,118
Magnetic bio printing	Patu8902	<ul style="list-style-type: none">• Rapid and simple	<ul style="list-style-type: none">• High cost• Metal nanoparticles may interfere the therapeutic method which is testing by spheroid	79
Semi-solid system	PANC-1	<ul style="list-style-type: none">• Limitation of extra mobility of cells	<ul style="list-style-type: none">• Low-throughput• Coating the surface can be time consuming	119
Patterned Surfaces and Microfluidic Devices	PANC-1	<ul style="list-style-type: none">• Easy handling• User friendly• Low-cost	<ul style="list-style-type: none">• Low-throughput	82,84,89,91,92,97,100,103,107,120

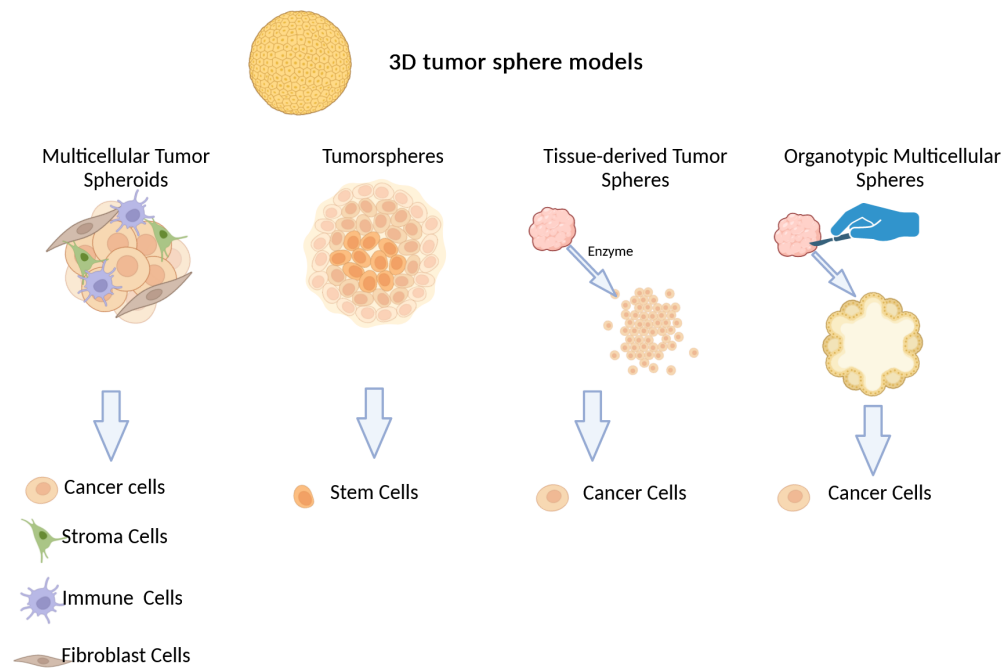


Fig. 2. *In vitro* 3D tumor sphere models for cancer research. From left to right: MCTS, spheroids of only tumor cells like cancer cell lines, useful for studying inherent behaviors like invasion, proliferation, and drug response. Tumorspheres, generated by isolating and amplifying cancer stem cells from tumor tissues or cancer cell lines. TDTS, created by partially dissociating tumor tissue enzymatically or mechanically to separate cancer cells from non-tumor cells while maintaining cancer cell connections. OMS, organotypic multicellular spheroids derived from patient tumor samples, representing a personalized model for assessing therapeutic response.

in the microenvironment. This model offers a better opportunity for predicting the effectiveness of cancer medications. The MCTS model in pancreatic cancer is employed for investigating hypoxia markers,⁴⁰ different miRNA expression,³⁷ protein levels,³⁷⁻¹²² and drug responses.^{37,123-125}

In co-culture methods, various behaviors of pancreatic cancer cells, including aggression^{126, 127}, immigration,¹²⁸ proliferation,¹²⁹ signaling pathways,¹³⁰ and drug resistance,^{129,131} can be investigated. Device-based 3D models, such as biosensors and chips, are utilized in pancreatic cancer to evaluate hypoxia,¹³²

pharmacological responses,^{106,134} and expression markers and factors.¹⁰⁶ Recreating the *in vivo* environment has led to tumor spheroids or tumoroids gaining prominence.^{135,136} Recently, novel platforms have been developed as combinations that do not require microfluidics for fabricating spheroids.¹³⁷⁻¹⁴¹ For example, microcells made by micro-molding or photolithography techniques can produce spheroids of specific sizes and compositions.^{69,137,142} Furthermore, platforms with weak attachment surfaces were designed utilizing non-adhesive materials, such as PDMS¹⁴³⁻¹⁴⁵ or agarose.^{146,147} These platforms allowed the formation of spheroids in a controlled and simple environment. A small sample size, incompatible culture media, and the inability to retrieve any samples are all potential limitations of this method. However, these models are highly effective in medical screening and are simple.^{71,141,142,148,149}

An essential part of the TME in pancreatic cancer is the presence of pancreatic stellate cells, which are involved in developing treatment resistance and the advancing the disease. Lee et al designed a culture model based on microchannels for producing spheroids with pancreatic stellate cells in a 3D collagen matrix, allowing the examination of epithelial-mesenchymal transfer and drug resistance. They designed microchannels using collagen so spheroids could grow with pancreatic stellate cells in a 3D pattern. An advantageous paradigm for therapeutically testing EMT and treatment resistance was suggested, which involves collaborating pancreatic tumor stellate cells with pancreatic tumor spheroid microfluidics.¹⁰⁶

On the other hand, the initiation of pancreatitis and pancreatic cancer follows different patterns. Acinar cells of the exocrine pancreas are positioned beneath the ductal cells and reconstruct the 3D structure of pancreatic tissue. However, the molecular mechanism of 3D structure formation still needs to be better understood. Therefore, Hakobyan et al designed a spheroid 3D model of pancreatic cancer using laser-assisted bioprinting. This model could be applied to determine the phenotypic evolution of cancer cells over time through visual analysis of phenotypic features.¹⁵⁰

Moreover, Monteiro et al devised a platform for evaluating drug resistance in 3D culture, employing classified spheroid models that mimic stromal cells. These models exhibit repeatable morphology and feature molecular biomarkers such as TGF, FGF-2, IL-1, and MMP9—essential elements secreted in the stratified microenvironment spheroid (STAMS) 3D models of human pancreatic cancer. By incorporating STAMS into an ECM-mimetic hydrogel matrix, this model reflects increased therapeutic resistance and mimics the architectural characteristics of PDAC stroma *in vitro*.¹⁵¹

Bio-fabrication of 3D spheroid models

Understanding the biological behavior of tissues,

organs, and tumors holds significant promise for disease treatment in the medical field.^{152,153} A major obstacle for tissue engineering is producing organs that mimic healthy and diseased tissues with a dense population of live cells.^{154,155} Most engineered tissues to date have been relatively thin (< 2 mm) to facilitate oxygen and nutrient transfer, and remove cell debris.¹⁵⁴

3D printing has emerged as a groundbreaking technology in tissue generation within medicine. Stents and splints are only two medical gadgets significantly benefiting from this technique.¹⁵⁶ In 3D bioprinting, the precise arrangement of layers consisting of biological, chemical-biological, and living cells is utilized to construct 3D structures by controlling the positioning of functional components. The resulting material from this technique, which incorporates cells, is called bioink. Despite their differences, all bioprinters can print cell masses, cells encased in hydrogel or other viscous fluids, and cells housed in microcarriers.^{157,158} Bioprinters encompass laser-assisted devices, extrusion devices, and printers resembling inkjet technology (Fig. 3).

In extrusion-based deposition, syringe-like instruments with polymeric or hydrogel characteristics are employed to fabricate 3D structures, typically guided by pneumatic pressure or computer-controlled mechanical pistons.¹⁵⁹ As a drop-by-drop method, inkjet printing generates the 3D structure by combining hydrogel and cell-friendly biomaterials in drops.^{160,161} The laser-induced forward transfer (LIFT) technique involves cells forming a 3D structure as drops using a laser.¹⁶²⁻¹⁶⁴ According to the recent classification by Moroni et al., bio-fabrication is categorized as 3D bioprinting.¹⁶⁵ 3D bioprinting in bio-fabrication enables the creation of 3D cancer models by printing live cells and "bio-ink" ECM together. This process allows for accurate manipulation of the position and development of primary cells. This may lead to a suitable tissue structure.¹⁶⁶⁻¹⁶⁸ In this method, bio-ink consists of cells surrounded by a matrix printed in a specific pattern. Consequently, cells self-organize, and 3D cell growth occurs. Different types of hydrogels, both natural and manufactured, including Matrigel, fibrin, collagen, PEG, alginate, and gelatin methacrylate, can be utilized in 3D bioprinting for to investigate cell function and activity.¹⁶⁹⁻¹⁷³

Recently, HeLa cells have been 3D bio-printed in a hydrogel, creating a cervical cancer model.¹⁷³ The cells within the 3D tumor bio-print demonstrate elevated growth rates, a heightened inclination to create spheroids, enhanced matrix metalloproteinase expression, and superior resistance to chemical challenges compared to cells cultured in a 2D culture. These results mirror *in vivo* responses, making this model suitable for assessing the efficacy of medications. The 3D human cancer model is a valuable tool for investigating the behavior of healthy and diseased cells, and for medication screening.^{174,175}

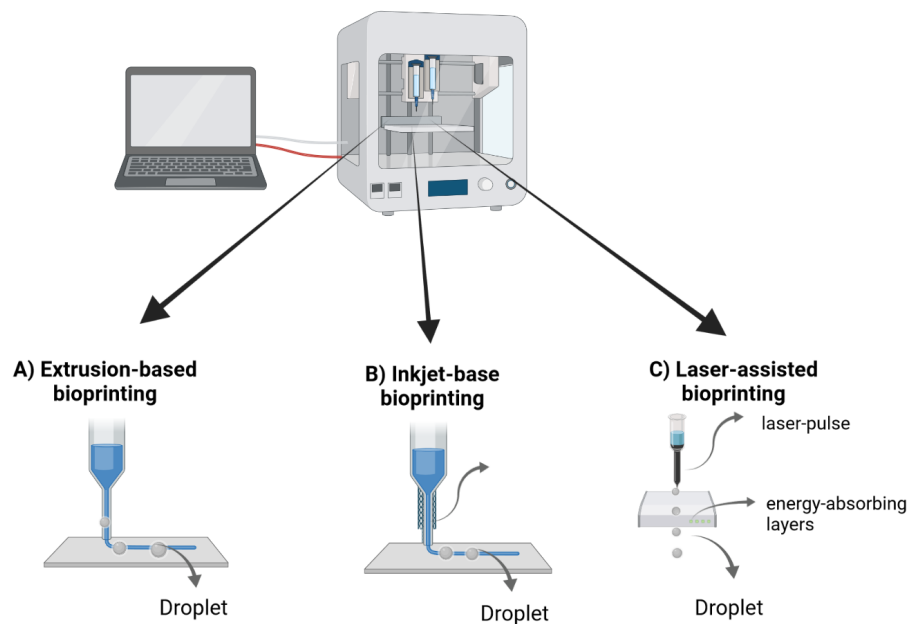


Fig. 3. The three primary bio-printing techniques: **A)** Extrusion-based bioprinting involves depositing bio-inks in layers through a controlled extrusion system. **B)** Inkjet-based bioprinting uses controlled droplet ejection to pattern biomaterials with high resolution. **C)** Laser-assisted bioprinting uses focused laser energy to position biomaterials with precision, enabling construction of intricate biological structures.

However, this technique has some limitations, such as the time-consuming nature of cellular assembly, taking weeks or days. Additionally, these models lack the capacity for the simultaneous culture of two or three cell types.^{166,172,176}

Concluding remarks

Pancreatic cancer continues to be one of the most lethal forms of cancer, often associated with a grim prognosis. Conventional 2D cell cultures have been widely employed in fundamental research and drug development; however, their ability to accurately predict the efficacy of novel treatments is constrained. More clinically relevant cell models are urgently needed to improve the success of drug development and provide deeper biological insights. Diverse *in vivo* and *in vitro* models have been established for pancreatic cancer. Unlike 2D monolayer cultures, 3D culture systems better mimic tumors' architecture and physiological activity through enhanced cell-cell and cell-matrix interactions. Current 3D approaches include multicellular spheroids, organoids, co-cultures, and microfluidic systems. Spheroid models demonstrate greater chemoresistance and expression of tumor microenvironment components compared to 2D cultures. While technical challenges of scale and reproducibility have limited spheroid generation, new technologies are emerging to enable large-scale, standardized spheroid production critical for downstream applications.

Both spheroids and organoids offer advantages over 2D models by recreating physiologically relevant tissue and tumor conditions. These 3D culture systems more faithfully reconstruct the *in vivo* microenvironment, providing higher predictive value for testing therapeutic

strategies. While organoids emphasize recapitulating native tissue organization and heterogeneity, spheroids focus on tumor cell proliferation and drug responses. The optimal 3D model depends on specific research questions and goals. In the future, interconnected spheroid and organoid models could provide insight into human tissue interactions *in vitro*. 3D culture will continue to have tremendous potential to advance pancreatic cancer research and therapy development.

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Competing Interests

The authors declare no conflict of interest.

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Review Highlights

What is the current knowledge?

- ✓ Pancreatic ductal adenocarcinoma (PDAC) is highly malignant with limited treatment options. More predictive preclinical models are needed to improve drug development.
- ✓ Conventional 2D cell cultures lack the complexity of the tumor microenvironment. 3D culture systems better recapitulate in vivo architecture and interactions.
- ✓ Spheroids focus on proliferation and drug response, while organoids emphasize tissue organization and heterogeneity.
- ✓ Emerging techniques enable large-scale, standardized spheroid production critical for drug screening.

What is new here?

- ✓ Multicellular tumor spheroids demonstrate enhanced chemoresistance and expression of microenvironment components versus 2D cultures.
- ✓ Both spheroids and organoids offer advantages over 2D models, but optimal choice depends on specific research questions.
- ✓ Continued 3D culture development has tremendous potential to advance pancreatic cancer research and therapy.
- ✓ 3D bioprinting shows promise for creating customizable models, but challenges remain regarding speed and co-culture.
- ✓ Co-culture of tumor spheroids with stromal cells adds complexity approaching in vivo conditions.
- ✓ Microfluidic models allow dynamic investigation of cell interactions within physiologically relevant microenvironments.

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