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Review Article

Why 3D *in vitro* cancer models are the future of cancer research?

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Abstract

Tumors are three-dimensional (3D) entities characterized by complex structural architecture which is necessary for adequate intercellular, intracellular and cell-to-matrix interactions among the aberrant cells in cancer. In the field of cancer research, 2D cell cultures are traditionally used for decades in the majority of experiments. The reasons for this are the vast benefits these models provide, including simplicity and cost effectiveness. However, it is now known that these models are exposed to much higher stiffness, they lose physiological extracellular matrix (ECM) on artificial plastic surfaces as well as differentiation, polarization and cell-cell communication. This leads to the loss of crucial cellular signaling pathways and changes in cell responses to stimuli when compared to in vivo conditions. Moreover, they cannot adequately mimic the complexity and dynamic interactions of the tumor microenvironment (TME) which is of great importance in anticancer drug treatments. 3D models seem more biomimetic compared to 2D cell monolayers because they offer the opportunity to model the cancer mass together with its environment which seems the key factor in promoting and directing cancer invasion. 3D cell culture with its additional dimensionality makes the difference in cellular responses because it influences the spatial and physical aspects of the cells in 3D culture. This affects the signal transduction and makes the behavior of 3D-cultured cells more physiologically relevant and reflective of in vivo cellular responses. This review focuses on major differences between 2D and 3D cell cultures, highlighting the importance of considering bioengineering humanized 3D cancer models as the future in cancer research. Additionally, it presents diverse 3D models currently used in cancer research, outlining their benefits and limitations. Precisely, this review highlights the differences between the 3D models with the focus on tumor stroma interactions, cell population and extracellular matrix composition providing methods and examples for each model from the studies done so far.

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INTRODUCTION

Tissues are three-dimensional (3D) entities, just like the tumor that arises within them *(1)*. Tumor is characterized by complex structural architecture which is necessary for adequate intercellular, intracellular and cell-to-matrix interactions of aberrant cells in cancer *(2)*. In the field of cancer research, especially anticancer drug testing, the majority of experiments are performed with 2D adherent cell cultures. However, 2D models are considered too simple and do not adequately mimic the complexity and dynamic interactions of the tumor microenvironment (TME) *(3)*.

Therefore, to adequately study the complexity of tumors, it is necessary to develop more realistic systems than the classical 2D monolayers used so far. Despite being traditionally used for decades because of the vast benefits they provide, 2D cell cultures do not realistically represent the immediate spatial, cellular, tensile and chemical environment of highly complex tumors and their stroma *(4)*. 2D models cannot effectively represent complex cellular signaling, angiogenesis, invasion and metastasis present in cancer *(5)*. Furthermore, even though 2D models represent important features of certain cancers and have been useful in unraveling biochemical pathways, and often have different genetic profiles from those of the primary cell lines derived from patients *(6,7)*. Additionally, 2D cell lines have limitations due to cross-contamination with other lines and in long-term cultures are prone to genetic drift *(1,8)*. Besides that, 2D cells are exposed to much higher stiffness which leads to exhibiting different growth conditions when compared to *in vivo* conditions. Equally important, 2D monolayers lose physiological extracellular matrix (ECM) on artificial plastic surfaces *(9,10)*. Cells grow on a flat plastic surface as a monolayer, which results in loss of crucial cellular signaling pathways and changes in cell responses to stimuli *(11)*. This has been proven troublesome in identifying preventative anti-cancer treatments. In addition, 2D cell culture properties, such as differentiation, polarization and cell-cell communication are missing as well, while wound healing, inflammatory processes, and hyper-proliferation are artificially promoted. These occurrences lead to the fact that 2D monolayer only poorly represents tumor cell biology *in vivo (12)*.

Taking into consideration that the composition of the tumor microenvironment (TME) and tumor-stromal interactions exacerbate tumor growth and metastasis, with activated stroma being a disease-defining factor leading to poor clinical outcomes *(13–17)* much focus has been put on accurately modeling TME interactions *in vitro* and *in vivo*.

The tumor stroma, which represents non-neoplastic part of the TME, is composed of extracellular matrix (ECM) and multiple support cells *(14)*, including cancer-associated fibroblasts (CAFs), endothelial cells, pericytes and immune cells, such as lymphocytes, neutrophils, dendritic cells (DCs), and monocytes. There are also some other less prevalent cell types such as myeloid-derived suppressor cells (MDSCs) and mesenchymal stromal cells (MSCs), as well as platelets *(18,19)*. Stromal cells are interacting with tumor cells and the ECM, and by secreting chemokines, growth factors (GFs), enzymes, extracellular vesicles, and miRNAs they influence metabolic pathways related to cancer *(20)* meaning that some cell types can either promote or suppress tumor growth depending upon the cellular context *(21)*. This effect of ECM on cancer cells is also reciprocated the other way around, meaning that cancer cells effect ECM by initiating different processes, like ECM deposition, degradation and remodeling which can impact tumor progression and invasiveness *(22)*.

Taking all this into consideration, 3D models seem inherently more biomimetic compared to 2D cell monolayers cultured on tissue-culture plate *(23)*. Therefore, there is an increased demand for bioengineering 3D models for better understanding of tumor growth. Three-dimensional systems offer the opportunity to model the cancer mass together with its surrounding stroma, evidently the key factor in promoting and directing cancer invasion *(23,24)*. 3D models can also be useful for co-culturing various types of cells in one model. In addition, ECM of a 3D model reflects more suitable environment to access oxygen, nutrients and growth factor transport as well as drug uptake and response which is then affecting the therapeutic potential and benefit of anticancer drugs *(16,25)*. 3D models not only provide a useful platform for the identification of the biological features of cancer cells but they offer a useful screening platform for novel antitumor agents and provide an interesting link between the 2D model and animal experiments *(26)*. All these differences between 2D and 3D are quite crucial and represent difficulties in understanding complex mechanism in cancer development *(27,28)*.

As already stated, three-dimensional (3D) cell culture technology can better recapitulate the *in vivo* organization and microenvironment of *in vitro* cultured cancer cells. Cells in the 3D culture environment differ morphologically and physiologically from cells in the 2D culture environment therefore making the behavior of 3D-cultured cells more reflective of *in vivo* cellular responses. Additional dimensionality of 3D cell cultures is the crucial feature which makes the difference in cellular responses because it influences the spatial organization of the cell surface receptors engaged in interactions with surrounding cells, as well as it induces physical constraints to cells. These spatial and physical aspects affect the signal transduction from the outside to the inside of cells, influencing gene expression and cellular behavior *(29)*. Therefore, 3D cell culture systems have gained increasing interest in drug discovery and tissue engineering due to their evident advantages in providing more physiologically relevant information and more predictive data for *in vivo* tests *(29)*.

Even though all these facts became clear in the recent years, 2D cell cultures still remain the main model in cancer research, not only because they are cost-effective but also because many analyses are optimized for use with 2D models, therefore leaving drug screening quite limited for certain cancers *(30)*. This in particular includes the majority of imaging systems/protocols designed to be compatible with 2D culture plates, as well as a range of automated fluidic systems compatible with 2D culture systems *(30)*. Latter features have not yet been efficiently integrated into 3D culture systems, so for instance, hy-

drogel matrix-based 3D cultures are still costly, they face a problem of significant 3D tissue size heterogeneity, and harvesting from the gel is necessary for many forms of analysis *(30,31)*.However, bioengineering humanized 3D models of cancer is the future in studying cancer and will eventually replace 2D monolayers and the need of animal models as well. This is in concordance with the 3R (replacement, reduction and refinement) goal to establish more humane animal research *(1,23)*.

TYPES OF 3D CANCER MODELS

In the recent years, a variety of 3D culture systems has been developed and adopted in drug discovery, cancer and stem cell biology, engineering functional tissues for implantation, and other cell-based analysis (Figure 1) *(29)*.

Today, there are a number of engineering approaches to generate 3D cancer models, with the specific goal to increase and accurately model the biomimetic complexity within the tumor microenvironment (TME) *(32)*. Here the most crucial aim is to achieve accurate compartmentalization of both the tumor and the stroma with clear boundary for cellular crosstalk and migration between these two parts. In this fashion, it is important for ECM to mimic and reproduce the biomechanical properties of the native tissue or organ since this is essential for cell attachment within each specific compartment *(23)*. In this sense, it is also necessary that there is no contact with tissue culture plate and optimal permeability of oxygen and nutrients should be provided *(33–37)*.

Spheroids

The simplest and most represented 3D models are spheroids. They are aggregates of cancer cells grown on low-attachment plates or in hanging drops which are in immediate proximity to one another in this 3D formation *(23,38)*. They are the simplest 3D model, most often generated from a single cell type, which can be commercially available cell lines or primary tumors cells from patients. They are one of the most used 3D models for the study of tumor biology, especially for testing of anticancer drugs *(3)*. Their sizes, shapes and properties can vary depending on the cells of origin.

Spheroids accurately recapitulate important tumor features including cellular heterogeneity, cell signaling pathways, cell–cell/cell–ECM interactions, gene expression patterns similar to *in vivo* conditions, and a tumor morphology composed of different cell layers *(39)*. These models are valuable in terms of low cost, ease of use, reproducibility, and high throughput capabilities with flexibility to integrate multiple cell types or different types of gradients *(3,40)*. They exhibit oxygen and nutrient permeability which is the highest at the surface. However, there is no space for stroma compartments and therefore the tumor-stroma interactions are limited. Other limitations are contact with surface plastic, no presence of the ECM, no collagen to attach to and furthermost no compartmentalization between cancer and stromal cells *(41)*.

Due to the limited diffusion of nutrients and oxygen, larger spheroids (500 μm in diameter) accurately mimic the microenvironment of micro-metastases and avascular tumors representing a proper model for studying the effects of hypoxia on cancer development *(42)*. In this way, three concentric zones are formed on a spheroid – an anoxic core which is in the center containing necrotic cells, a middle hypoxic zone with a low concentration of oxygen and nutrients, and an outer zone containing highly proliferative cells *(43)*. Despite being more time-consuming and more expensive than 2D cell culture, spheroids are a widely used 3D culture model which can be combined with 3D bioprinting technologies, microfluidics, and scaffold-based platforms to generate more physiologically representative tumor models (Figure 2) *(3)*.

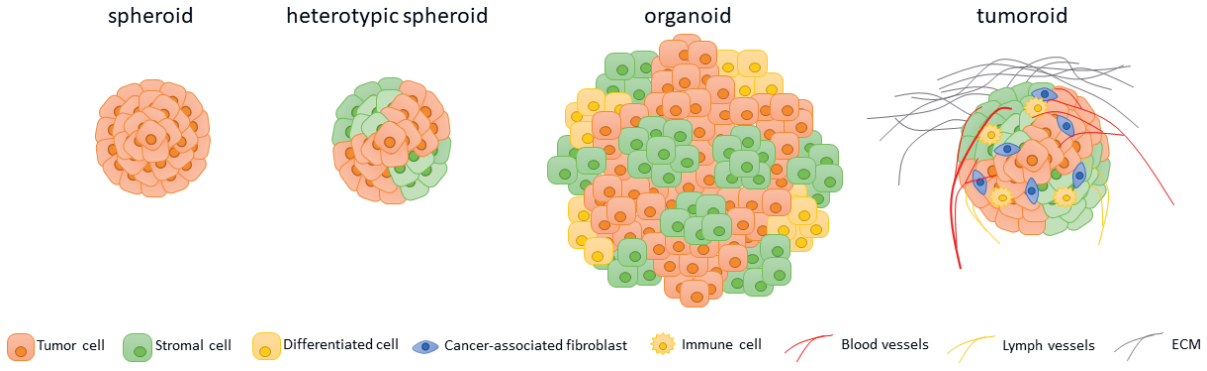

Figure 1. *Types of 3D cultures, shown by increasing order of complexity: single-cell spheroid, heterotypic spheroid containing two cell types, organoid, and tumoroid. Spheroids are generally composed of a single population of tumor cells. If two cell types are combined within the same spheroid (e.g., tumor cells and stromal cells) it is called a heterotypic spheroid. Organoids are more complex structures which contain multiple cell types usually found within the tissue of origin and are usually generated from patient material. The most complex system is the tumoroid, which contains multiple cell types, vasculature and components of the immune system and the ECM.*

Tumor cell spheroids are especially useful for studying cancers that form tumor embolus, or a closely packed tumor cell cluster, as it happens in inflammatory breast cancer *(43)*. They incorporate various levels of TME complexity and can be useful to analyze the influence of ECM stiffness on cancer. In breast cancer, cancer cells interacted with stromal cells and inhibited preadipocyte differentiation and maturation only in high-stiffness tissue which could not be replicated in a 2D monolayer culture system. This example again highlighted the importance of a 3D environment to mimic TME interactions *(44)*. Another example are the macrophages that play a crucial role within the TME. Their natural ECM remodeling behavior has been particularly challenging to integrate into spheroid-based models so far, however, different strategies have been applied in the recent years to better understand the role of macrophages in the TME using spheroids *(45–47)*. These examples showed that the interplay between the ECM, tumor, stromal and immune cells promotes the activation of TAMs, thus mimicking aggressive tumor stages *(3)*.

Another important feature to mention when talking about spheroids are cancer stem-like cells (CSCs) which have the ability to form multicellular three-dimensional (3D) spheres *in vitro* (*24,25*). In general, stem cells are undifferentiated cells that provide the source of all types of specialized cells in the body *(48)*. They can self-renew and differentiate into different downstream cell lineages. Similar to this, CSCs are small sub-population of poorly differentiated cancer stem-like cells, identified in most tumors, also known as cancer initiating cells, that are responsible for the recurrence, metastatic potential, and resistance of different tumors *(48,49)*. First such subpopulation was identified in acute myeloid leukemia (AML) in 1994 *(3)*. This has given a possibility to isolate similar tissue-specific CSCs and progenitor cells from other tumors *(5)*. Tumor growth is dependent on CSCs, giving rise to more differentiated tumor cells, similar to the role of stem cells in normal tissue *(9)*. Along with that, another important feature of CSCs is their resistance to cytotoxic chemotherapy and ionizing radiation *(10,11)*. Taking into consideration all their characteristics, it is essential to assess the presence and self-renewal ability of CSCs in different tumors. Therefore, *in vitro* models to investigate the properties of CSCs are highly required. Sphere-formation assay is one of the *in vitro* methods commonly used to identify CSCs and study their properties *(48)*. This model is based on the ability of stem cells to grow in non-adherent serum-free gel matrix, and it has been proven reliable to assess the presence and self-renewal ability of CSCs in different tumors. This is a useful tool to evaluate the effect of conventional or novel agents on the initiation and self-renewing properties of different tumors. The effects can be directly evaluated through assessment of the sphere-forming efficiency (SFE) over five generations or other downstream assays such as immunohistochemical analysis of the generated spheres *(48)*.

Just growing the cell lines as spheroid cultures results in enrichment of cancer stem cell population, so spheroid cultures can be used as a method for their preparation *(50)*. Such spheroids are more resistant to therapeutics compared to the same cells grow as a monolayer *(51)*.

Overall, spheroids proved to be useful models in understanding tumor biology. However, they exhibit some limitations related to reproducibility because of poor uniformity in size/morphology and/or low throughput and difficulty in retrieving cells for analysis, which prevents the development of standard models *(52)*.

Self-Assembled Organoids

Unlike spheroids, organoids represent a more complex 3D architecture. They are generated from the progenitor cells and can closely mimic the 3D structure and architecture of the tissue from which they are derived *(53)*. Tumor organoids are usually developed from a single cell into a 3D construct, and follow different development stages of natural tumors, therefore they are capable of retaining the natural cancer cell heterogeneity of the native tumor to a greater extent, preserving the pathophysiology of the tumor *in vitro (54)*. Unlike spheroids that are formed by the forced aggregation of multiple cells into a 3D construct, tumor organoids develop a 3D shape on their own based on their genetic programming, which more closely represents the actual development of a tumor and gives them an advantage over spheroids or other 3D cultures *(55)*. Working with organoids has some drawbacks because the process is more time-consuming, there is difficulty in reaching *in vivo*-like maturity, the variability can be high between the experiments, and there is a lack of vasculature and stroma *(56)*. However, organoids can be efficiently cultured from the patient's own cells *(57–60)* and they are more cost-effective.

By combining multiple cell types together, multicellular heterotypic spheroids can be generated which provide advantage over organoid models that usually only contain progenitor cells of epithelial origin, leaving the organoid model without an immune-competent microenvironment and stromal components *(61,62)*. Nevertheless, there are studies where co-culture with stromal cells was established and activation of CAF was observed *(63,64)*. Additionally, it was demonstrated that organoids derived from cell lines cultured in a monolayer or passaged as mouse xenografts are phenotypically distinct from primary organoids which retain the tumor architecture, cell–cell interactions, stemness, and cellular heterogeneity. This once again demonstrates the advantages of directly culturing cells in 3D models over 2D cultures with loss of the native tissue phenotype. 3D bioprinting can also be used with organoids to form sophisticated organoid culture systems which then can be generated by

combining cancer cells and a self-gelling hydrogel derived from ECM of different origin (decellularized rat or human mammary tissue for instance) *(36)*. In these dECM hydrogels, structural and signaling profiles were retained eliciting distinct responses when cultured with normal cancer cells. This is important because usually the disposition of cells without any predetermined organization typically leads to uncontrollable cellular behaviors *(36)*. In this way, the combination of 3D bioprinting and organoids has the potential to promote the adequate spatial arrangement of cells in complex 3D system while still maintaining the hierarchical-like architecture of TME and increasing the reproducibility of the model *(3)*.

Tumoroids

The terms organoid and tumoroid are sometimes used as synonyms in the literature *(65, 66)*. However, there is a difference between organoid and tumoroid in the sense that organoids lack the full complement of cells and factors found in the patient's tumor, while tumoroids retain the full architecture of the TME and ECM. These models can recapitulate the cancer mass itself but also the stromal environment. The tumor boundary is accurately modeled by implanting a cancer mass within a stromal compartment. The distance of invasion can be measured directly from the origin *(67)*. Whilst these models can be high cost depending on the tumor type, they have a high level of reproducibility and oxygen and nutrient permeability is sufficient. Within such models the diffusion coefficient of both glucose and oxygen are high *(68, 69)*. Modeling and validation of oxygen consumption shows this measure to be cell-specific, signaling the distinct metabolic profile of different cell populations *(70)*. The tumoroid model can be used in combination with other systems to increase stromal biomimicry.

Microfluidic Models

Stromal biomimicry can be achieved by using microfluidic models. They are platforms consisting of a network of microfluidic channels which permit the continuously perfused cell culture. With this model it is possible to design complex 3D culture systems in which various parameters can be modified and controlled independently *(3)*. This system has revolutionized the ability to mimic the natural biophysical/chemical conditions of cells in *in vitro* models with the goal to model the (patho)physiological functions of tissues and organs – so-called organ-on-chip devices.

These microfluidic organ-on-a-chip models have been used with increasing popularity. In the case of these models, the contact between the cells and tissue-culture plastic cannot be eliminated, but these models are beneficial because they have exceptionally high permeability to oxygen and nutrient flow. In a number of organ on-a-chip models, the distance of invasion can be measured between tumor and stromal cells. Cost is still relatively low and high throughput outcome can be set up, however, these models can lack ECM components unless gels such as collagen or Matrigel R are injected into the channels as well *(71)*.

Various factors can be specifically controlled in these models, including the mechanical forces applied, the orientation of tissue interfaces, the types and localization of cells, and the chemical gradients, thus allowing optimized levels of cell survival *(72)*. Additionally, these devices use microscale volumes which is less expensive while still high-throughput screening compared to other 3D culture methods and bioreactors *(13,16,39,73)*. Nevertheless, it should be taken into account that the specialized skills are necessary to fabricate chips *(72)* so the fast development of new fabrication techniques, such as 3D bioprinting, has helped in this regard *(74–76)*.

Some additional limitations include lower reliability and robustness, edge effects and high shear stress which can also affect the performance and consistency of the device by hindering laminar flow in channels, which can harm cells and/or affect their distribution, resulting in inconsistent results. *(77)*. Finally, there is a need for new materials for the chip fabrication because polydimethylsiloxane (PDMS), the most used material, can nonspecifically absorb small molecules *(78,79)*.

Multiple cell types can be cultured in a microfluidic chip to analyze specific interactions, which is particularly interesting when investigating communication between cancer and stromal cells with several examples being available so far, for instance in breast cancer–immune cell interactions *(80)*. In this example greater T cell infiltration was observed when monocytes were present in the culture, and also when higher levels of hypoxia were emulated by using tumor spheroids instead of dispersed cancer cells *(80)*. These findings demonstrate the important role of microfluidics in generating heterotypic 3D models to study different cell types and specific tumor–stroma interactions.

Stroma-driven ECM remodeling is a crucial consequence of tumor–stroma activation that sustains cancer progression. There have been several studies in this direction which provided a unique way to monitor the switch between healthy and pathological stroma *in vitro* and represents an alternative to the ectopic *in vivo* experiments that are typically used to analyze such events. The design of the microfluidic chip also allowed precise control of cell confinement and interaction, continuous perfusion, and assembly of cell produced ECM *(3)*.

Recently, innovative models have been produced that combine tumor organoids/spheroids and microfluidic chip systems. By incorporating organoids into a microfluidic device, organoids-on a-chip inherit the combined benefits of both microfluidics and 3D organoid models,

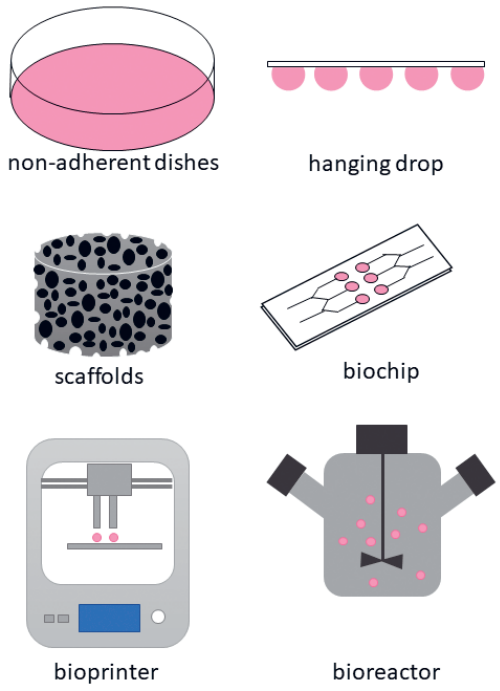

Figure 2. *3D cultures can be cultivated using different approaches and methodology. The simplest systems are the cultivation in non-adherent plastic dishes and the hanging drop method, followed by the use of scaffolds. The more complex systems include microfluidic chips, bioprinting and the use of bioreactors for large-scale cultivation.*

thus providing a unique way to study tumor–stroma interactions and their systemic effects *(78, 81–83)*. From the studies done so far it was visible that cells displayed morphological features similar to *in vivo* conditions, contrasting to poorly aligned cells grown in 2D cultures or deprived of flow. Microfluidic device promoted the perfusable culture of different cells types and also proved to be an appropriate platform for monitoring cancer cell extravasation in real time showing how distinct microenvironments can influence cancer progression *(3)*.

BIOPRINTING SCAFFOLDS AND CELLULARIZED BIO-INKS

Matrigel R and Hydrogels

More complex 3D models include decellularized matrices, Matrigel R and collagen which are used to provide an extracellular matrix for the cancer cells to populate in order to achieve biomimicry for the initial cancer mass *(37)*. Together with collagen hydrogels, there are other options of biomaterials to recreate the ECM, such as decellularized human tissue, 3D bioprinted hydrogels *(36)*, hyaluronan printed hydrogels *(34)* and self-assembling peptide hydrogels *(35)*. These are beneficial for co-culture with stromal cells, incorporating chemical factors and extracellular matrix proteins and good oxygen/nutrient permeability *(84–86)*. These models are also useful because they allow compartmentalization and generation of a tumor-stroma boundary *(87)*. However, they are costly and prone to variability in terms of added ECM components. Some synthetic polymers such as poly(ethylene glycol) (PEG), poly(n-isopropylacrylamide) (pNIPAAm), and poly(caprolactone) (PCL) which are less variable can serve as an alternative approach *(88)*.

Scaffolds

Another more complex 3D environment is provided with scaffolds. Ideal scaffold offers an appropriate environment for cell adhesion, proliferation/differentiation, and migration to allow the generation of *in vitro* tumor models that closely recapitulate essential cell–ECM interactions. Tumor cells can be cultured within biomaterials, including decellularized native tissues, or on 3D scaffolds based on ceramics *(89,90)* or synthetic and/or natural polymers *(3)*. Hydrogel-based scaffolds are preferred because their mechanical properties closely mimic the tumor ECM, while scaffolds produced from synthetic polymeric biomaterials including polyethylene glycol (PEG), polycaprolactone (PCL), poly(hydroxyethylmethacrylate) (PHEMA), poly(lactic-co-glycolic acid) (PLGA), and ceramics (such as hydroxyapatite or bioglass) *(89,90)* allow more controlled environment and the ability to modulate them as required.

The surface of synthetic polymers can be modified to incorporate peptides or fibrinogen, that promote protein adsorption and cell adhesion *(17)*. Hybrid scaffolds can combine soft hydrogels with polymeric scaffolds and cells *(91)*. Therefore, the choice of biomaterials as well as the physical/chemical conditions of the scaffold determine how the cells will react to the substrate and what will be the experimental outcome.

Natural biomaterials originated from tissues and cells would include collagen, fibrin, alginate, and chitosan *(92-94)*. An alternative choice would be decellularized ECM (dECM), which does not compromise the tissue-specific architecture and the ECM, and offers the advantage of recreating natural biochemical environments, generating scaffolds that have biochemical and structural cues similar to those present *in vivo (95,96)*. The most commonly used ECM substitutes, such as Matrigel, incorporate undefined and highly variable factors that can affect the experimental results and the reproducibility of the model *(53)* so because of close resemblance to the native matrix structure, cell–ECM interactions can be more easily replicated in dECM-based models after cellularization and are also promising alternatives to better control the TME *in vitro*. They have advantages over scaffolds that focus only on individual ECM components and not on the ECM environment as a whole *(97)*. However, the decellularization process has its limitations because it is challenging to ensure tissue intactness after treatment with detergents and enzymes *(3)*.

Taking all this in mind, multiple modifications of scaffold-based cell culture supports have been optimized for tumor modeling. One of the examples is a tissue matrix scaffold (TMS) which uses native ECM *(91)* and consists of a multilayered tissue culture platform prepared from decellularized mouse mammary tissue. Cancer and stromal cells are cultured in a compartmental fashion that induces the expression of intracellular and extracellular biomarkers of breast cancer cells, thereby confirming correct tumor growth and proliferation. This example of TMS mimics the structure of the mammary tissue while providing a simple-to-use tool for screening specific tumor biomarkers *(91)*.

Another approach used the anisotropic collagen scaffolds seeded with adipocytes and tumor cells. In this model, culturing breast cancer cells in collagen pores aligned perpendicular to the surface allowed examination of adipocytes in the tumor stroma. This example reflected the *in vivo* microenvironment and 3D spatial configuration. The invasion of tumor cells into the stroma was then monitored. The presence of adipocytes increased the migration of cancer cells and promoted cancer cell invasion, while reducing the overall number of migratory cells, which demonstrated the heterogeneity of cellular behavior in this model *(94)*.

To summarize, scaffolds provide an inexpensive and easily analyzable platform which has tunable and instructive properties that can recapitulate relevant biochemical and structural cues *(91)*, offering a proper ECM-mimicking environment for culturing cells. Scaffolds can be integrated in most current 3D *in vitro* models and have been used to produce complex 3D bioprinted models *(98, 99)*, to induce the assembly of cell spheroids, and to promote the 3D culture of cells in microfluidic platforms. Therefore, scaffolds have advantages over 2D models and *in vivo* models.

Decellularized Scaffolds

Decellularized scaffolds are of porcine, bovine and human origin and can be stripped of all animal components, thus avoiding the trigger of an immune reaction *(100-102)*. Additionally, decellularized scaffolds model the native tissue more closely than a tissue-engineered one, however the limitation lies in a number of decellularization methodologies which may alter the stiffness and porosity of the native tissue, which in turn affects cellular response. Some of the methods for decellularization include the use of harsh chemicals and enzymes, which effectively strip the cellular components within tissues, thus leaving behind intact matrix *(103)*. The limitation here is the impossibility to remove some of these chemicals from tissues which leads to either physical alteration of the matrix or trace amounts of these chemicals affect the viability of newly added cells. However, some methods like the vacuum-assisted osmotic shock have been proven effective in removing cellular components an in the same time maintaining ECM integrity and allowing for the new highly viable cell infiltration *(104)*.

3D Bioprinting

As mentioned before, 3D bioprinting is a novel technique that uses scaffolds in the fabrication of more complex models with well-defined architecture, composition, and high reproducibility *(105)*. Cell bioprinting is a unique approach for 3D cancer cell patterning that facilitates the control of spatial and temporal distribution of cells *(15)*. 3D bioprinting techniques include extrusion-, inkjet-, and stereolithography-based bioprinting, as well as laser-assisted and electrospinning-based bioprinting *(106)*. The choice of biomaterial largely depends on its biocompatibility, the shape-fidelity of the material, and the level of instructiveness required and the process generally must avoid damaging pressure/heat sensitive fluids, especially when printing living cells *(15)*. Some recent examples of bioprinted 3D model enabled the incorporation of cancer cells into a complex microenvironment where interactions between tumor and stromal cells, ECM deposition, and self-organization of the tissue could be observed *(106)*.

3D printed models now also allow 4D manipulation of variables where the time represents a fourth dimension which is crucial for evaluating the dynamics or kinetics of GFs, drugs, or the metastatic spread of tumor cells over time. One of the good examples of this attempt was bioprinted 3D tumor constructs developed to recapitulate the TME that leads to metastatic dissemination of lung cancer *(107)* which enabled precise placement of cells and spatiotemporal control of molecular gradients that locally modulate dynamic cellular events.

In general, spatially defined 3D *in vitro* models have been improved by 3D bioprinting techniques. The microscale resolution, high precision in forming 3D constructs, the ability to use multiple materials, and commercial availability have potentiated utilization of the 3D bioprinting techniques. However, there are some limitations which include slow printing speeds, development of nontoxic and printable bioinks, and insufficient reproducibility to create standard models *(106,108)*.

3D bioprinting, as well as a wide range of bio-inks such as alginate and polyvinyl alcohol (PVA) have been established for the printing of scaffolds to desired structures. There is also the possibility of high manipulation of these bio-inks, leading to tuning of the concentration *(34)*, porosity and stiffness and provide 100% viability of the cells seeded onto the scaffolds for up to 12 days *(109)*. There are also bio-inks with pre-mixed cells in blends of agarose, gelatin and collagen *(110)*. These systems have a number of benefits, although cell viability and attachment can be low whatsoever.

Patient Derived Xenograft (PDX)

Lastly, there are two models to be discussed: *patient-derived xenografts (PDX)* and *patient derived organoids (PDO)*, both derived from a patient's cancer cells. Patient-derived xenografts (PDX) are models in which the tissue or cells from a patient's tumor are implanted into an immunodeficient or humanized host animal, usually mouse. The advantage of this model is that the ECM is biomimetic and intact with the achieved compartmentalization of tumor and stromal tissue, whilst oxygen and nutrients are readily available through the host's circulatory system *(23)*. However, the disadvantage lies in the fact that only limited amount of stromal cells can be explanted together with the tumor sample taken from the patient. Those are usually cancer associated fibroblasts and endothelial cells and over time they are replaced by the host cells *(111)*. Additionally, taking into consideration that the implanted explants/fragments are generated in immunodeficient mice in which the inherent immune response is not fully functional *(112)*, the adaptive immune response cannot be accurately modeled, even though there are some recent attempts which now incorporate immune cells to allow for this process *(113)*. PDX model, even though, not entirely *in vitro* model, is a good example of the thin line between 3D *in vitro* and *in vivo* models and the possible combination of the two.

Patient derived organoid (PDO) or Patient-derived tumor organoid (PDTO)

Patient derived organoids (PDOs), or Patient-derived tumor organoids (PDTOs) are described as a miniature three-dimensional (3D) cell cultures derived from a patient's cancer cells that stably retain key characteristics of the respective organs *(114)*. These models are generated from surgically resected tumor specimens, biopsied tissues or circulating tumor cells from the patient, and they are grown into tumor organoids after embedding into a 3D matrix *(115)*. They have been subjected to extensive investment and experimental validation to understand patient-specific drug responses and investigate cancer cell growth *(114)*.

As mentioned before, patient-derived xenograft (PDX) models are models in which tumor biopsies are injected into an animal. They were developed to improve the predictive capacity of preclinical animal models. Patient-derived organoids (PDO) on the other hand, use patient tumor samples to create *in vitro* models that represent conservation of the cellular composition and maintain aspects of tumor structure and heterogeneity *(116)*. Their main advantage when compared to PDX is that this method does not rely on animals for propagation and it is more human-relevant approach for understanding human diseases, therefore the support for new research projects using PDO is gradually increasing *(116)*. When comparing PDX and PDO, there are several improvements when using PDOs. Firstly, unlike PDX which are rather expensive, PDOs offer moderate, acceptable cost. They are suitable for high-throughput drug screening which was not achieved with PDX. PDOs in clinical trials have served as a tool for personalized medical decisions to predict patients' responses to therapeutic regimens and potentially improve treatment outcomes *(117)*. They reconstitute tumor-stroma interactions and are suitable for clinical application, since there are no ethical concerns in question when compared with PDX *(115)*.

Also, there have been some improvements in using complex immune-organoid systems as testing platforms to facilitate precision cancer immunotherapy. Maintaining the TME and ECM is of great importance especially in drug testing studies because it provides the true response to conventional chemotherapeutic and targeted therapies *(118)*. It is of great importance to retain the full architecture of the tumor microenvironment (TME) and extracellular matrix (ECM). It is necessary to mimic the natural growth of cancer more closely, and in this sense cancer masses should be engineered separately as well as biomimetic stromal compartments containing appropriate cell populations (e.g., fibroblasts, endothelial cells, immune cells and other ECM components). Therefore, heterotypic organoids are models of increasing complexity which take into account the contribution of ECM and supporting cell populations as well. Moreover, there have been some intriguing applications of tumor organoids with novel multi-omics in preclinical cancer research, using genetic editing, proteomics, and liquid biopsy. *(119)*.

Moreover, living organoid biobanks encompassing several cancer types have been established, providing a representative collection of well-characterized models that will facilitate drug development. There are several attempts to include these models into the cancer research. One of them is an example of the colorectal cancer liver metastasis (CRLM) where there is no effective method to predict chemotherapy response and postoperative prognosis of CRLM patients *(120)*. Patient-derived organoid (PDO) has become an important preclinical model which has shown that organoid platform for CRLM could capture intra- and interpatient heterogeneity. Also, it has been shown that PDOs could be used to predict chemotherapy response and clinical prognosis of CRLM patients, leading to a potential application for personalized medicine *(120)*. Another, quite relevant example is the application of PDO in ovarian cancer which is the leading cause of death from gynecological malignancies *(121)*. Despite great advances in treatment strategies, therapeutic resistance and the gap between preclinical data and actual clinical efficacy remain the main problem in OC. Therefore, the role of PODs in the assessment of high-grade serous OC (HGSOC) cells-of-origin, illustrate their use as promising preclinical OC models and highlight the advantages of organoid technology in terms of disease modelling and drug sensitivity testing *(121)*.

DISCUSSION

Cancer studies are mainly relying on *in vitro* models, and continuous improvement of these models is crucial for the further development of cancer research. Without any doubt, 3D models represent a big step in the right direction. However, some improvements are much needed. On-line resources are being developed to facilitate interpretation of experimental conditions and results, such as the MISpheroID database *(122)*. In the future it would be important to develop more physiologically relevant cancer environment within 3D model systems (Figure 3). This would indicate that both the extracellular matrix and stroma should be programmed to mimic native tissue in the more realistic manner *(123, 124)*.

Even though there is an increasing interest in conducting studies on cancer using 3D models, some features regarding stiffness, stromal complexity and compartmentalization remain limited *(23)*. The problem of low collagen density and the ECM stiffness, which is directly impacted by its concentration, is present in almost every 3D model discussed in this review. Moreover, native tumor tissue is characterized by the gradient of stiffness and collagen concentration during tumor development which could not be achieved in 3D models so far. However, some improvements have been done in this regard concerning scaffolds *(125)* and tumoroids *(126)*, while microfluidic devices *(127)*, spheroids and hanging drops *(128)* still either do not have any or have very limited collagen concentration. From the 3D models discussed so far, only tumoroids are able to achieve physical stiffness and a level of biomimetic ECM adequate to promote cellular growth and invasive phenotype. Also, it should be noted that stiffness is not only dependent on collagen itself, but rather on a combination of all ECM components as a whole *(129)*. Therefore, other ECM components essential for cell growth will have to be included within 3D set-ups as well. They include various surrounding cells such as laminins and fibronectin, as well as additional collagen subtypes, the stromal cell population such as fibroblasts or the highly differentiated cancer associated fibroblasts (CAFs) *(130)* and even endothelial cells *(131)*. This is important for studying cellular cross-talk between cancer and stromal cells as well as for studying vascular network formation, remodeling and disruption in relation to a growing tumor mass. This is crucial in understanding the cancer's ability for nutrient acquisition and metastasis *(132)* as well as in identifying future drug targets *(133)*. Other factor often missing in this 3D modeling is immune cell population which is rather important to understand further cancer progression *(23)*.

Additional important feature that is missing within a number of 3D models of cancer is the compartmentalization between cancer mass and stroma and often these cells are mixed together in co-cultures, and expected to self-aggregate *(134)*. It is of high importance to have this physical, chemical and cell barrier since in number of invasive cancers, it often signifies the staging and aggressiveness of the cancer *(135)*. Human tissue is well defined by barriers and compartments (organs) and a number of cancers arise at these borders where cells change from one type to another or tissue environments change *(136)*.

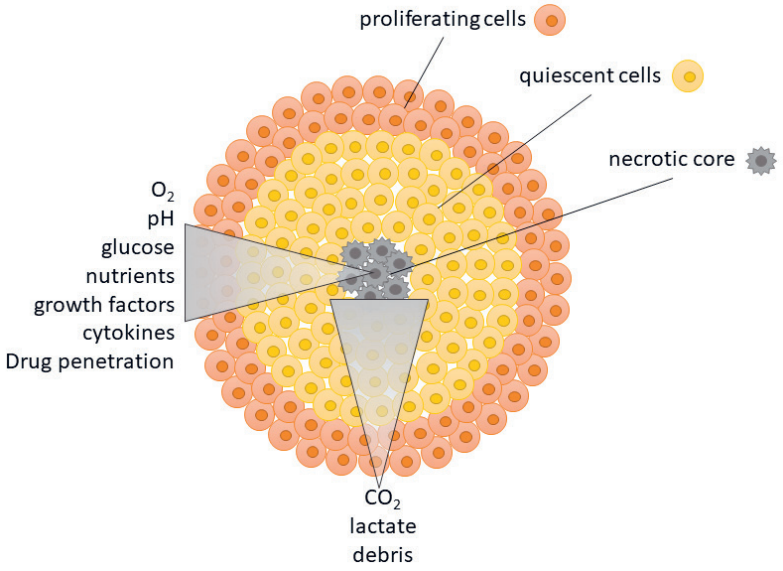

Figure 3. *3D cultures consist of the outer layer of proliferating cells (orange), middle region of quiescent live cells (yellow), and the hypoxic/necrotic core (grey). Due to this structure, there is a gradient of molecules and nutrients through the structure, resulting in different bioavailability of these molecules depending on the depth. The concentration of oxygen, glucose, nutrients, growth factors, cytokines and drugs is higher on the surface of the structure and drops with the depth, while waste products such as carbon dioxide, lactate and cell debris accumulate in the middle of the structure.*

CONCLUSION

So far, only tumoroid models can be considered as matrix relevant models, with controlled density and composition. It is of great importance to mimic the natural growth of cancer more closely and in this sense it is necessary to engineer separate cancer masses and biomimetic stromal compartments containing appropriate cell populations (e.g., fibroblasts, endothelial cells, immune cells and other ECM components) and then bring them together *(134)*.

It is also crucial to include patient samples in all 3D models *(130)*. Further attention should be given to the primary cancer cells because this could lead to the development of personalized drug-screening platforms. The future of 3D cancer research lies not only in investigation of tumor growth but also in studying invasion, migration, cancer stem cell's plasticity and cancer cell dormancy which can model the interaction between cancer and stromal cells more accurately. Finally, a physiologically relevant ECM regarding composition and stiffness will allow for more defined barrier between the cancer mass and surrounding stroma. With all these improvements in mind, 3D models as a pre-clinical tool could lead to a more ethical approach to research with limited need for animal studies *(23)*.

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