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REVIEW ARTICLE

Advances in 3D culture systems for therapeutic discovery and development in brain cancer

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Highlights

- Existing models for drug discovery and development against GBM have limitations.
- We review advances in 3D systems that promise more accurate therapeutic models.
- Development of 3D cultures that can model the GBM TME is discussed.
- We describe advanced 3D systems such as organoids, 3D and 4D bioprinting and CSC.
- We identify gaps to bridge in existing 3D systems to accelerate drug discovery.

Abstract

This review focuses on recent advances in 3D culture systems that promise more accurate therapeutic models of the glioblastoma multiforme (GBM) tumor microenvironment (TME), such as the unique anatomical, cellular and molecular features evident in human GBM. The key components of a GBM TME are outlined, including microbiomes, vasculature, extracellular matrix, infiltrating parenchymal and peripheral immune cells and molecules, and chemical gradients. Current 3D culture systems are evaluated against 2D culture systems and *in vivo* animal models. The main 3D culture techniques available are compared, with an emphasis on identifying key gaps in developing suitable platforms to accurately model GBM TME including tumor stem cells, blood brain barrier models and mixed cultures with cells and molecules of the immune system, normal parenchymal cells, and microbiome models.

Teaser

In time, 3D cell culture research will lead to development of complex, multifaceted GBM models, and will enable rapid advances in precision, personalised medicine to improve patient outcomes.

Keywords: 3D cell culture, Glioma, tumor microenvironment, 3D bioprinter, Scaffolds, hydrogels

Introduction to 3D cell culture for brain cancer

Brain cancers can be divided into two types, primary and secondary brain cancer. Primary brain cancer originates within brain cells, forms in the central nervous system (CNS), and usually does not metastasis to the outside of the CNS. Secondary brain cancers are originated and metastasis from external to the CNS, such as the lung, skin, breast, colon, and kidney. Secondary brain cancers are the most common, while primary brain cancers are more lethal [1,2]. Primary brain cancers can be classifies further as gliomas (astrocytomas, oligodendrogliomas and ependymomas) and nongliomas (menigiomas, medulloblastomas) [2,3]. Gliomas are developed from glial cells, including astrocytes, oligodendrocytes, and ependymal calls or a mix of the above. Astrocytomas are the most common primary brain cancer and according to the World Health Organization (WHO), it is further classified as pilocytic astrocytoma (Grade I), low grade astrocytoma (Grade II), anaplastic astrocytoma (Grade III), and glioblastoma (Grade IV) [1,4]. Glioblastoma multiforme (GBM) is a WHO grade IV astrocytoma and is the most common, aggressive, fatal, highly vascularized, malignant primary brain tumor in adults. Treatment options remain very limited, and it has a low survival rate of less than 1 year for many patients and only about 5% survive beyond 5 years [1,3,5]. According to the most recent "central brain tumor registry of the United States (CBTRUS) statistical report", the average annual age-adjusted incidence rate of all malignant and non-malignant brain and other CNS tumors was 24.25 per 100,000 between 2014 and 2018. The total rate was greater in females than in males (26.95 versus 21.35 per 100,000). The most often occurring malignant brain and other CNS tumor was glioblastoma (14.3% of all tumors and 49.1% of malignant tumors), was more prevalent in males while the most common non-malignant tumor was meningioma (39.0% of all tumors and 54.5% of non-malignant tumors), was more common in females [6].

Patient prognosis remains poor and largely unchanged over the last 30 years due to the limitations of existing therapies such as surgical resection, followed by concurrent radiation therapy and temozolomide (TMZ) [7]. The majority of therapies fail during clinical trials due to imperfect models that

limit our ability to predict efficacy and toxicity in humans. This is particularly evident with GBM with no successful therapy that significantly improves survival since the introduction of temozolomide 20 years ago [1,3,5].

GBM is characterized by higher vascularization, significant cell heterogeneity, self-renewing cancer stem cells and the interactions between tumor and microenvironment, all of which play an important role in tumor growth (Figure 1) [8]. Tumour development, metastasis, angiogenesis, cytotoxicity resistance, and immune cell modulation are all influenced by the tumour microenvironment (TME) [9,10]. There is a urgent need for accessible GBM pre-clinical models and 3D cell culture is able to fill this gap by providing more reliable models to study the correlation between TME, tumour reoccurrence and therapy resistance.

Three dimensional (3D) cell cultures describes a wide range of *in vitro* cell culture technique used to grow cells in three dimensions using an artificially created microenvironment. Cells in 3D cell culture have physiological cell-cell and cell–extracellular matrix (ECM) component interactions which allow cells to grow *in vitro* in a tumor microenvironment that closely resembles GBM *in vivo* conditions [9,11]. Tenascins, Fibronectin, Fibulin-3 and Hyaluronic acid are the primary components of the GBM ECM [12]. These ECM components can be employed in 3D cell culture to mimic the composition and porosity of *in vivo* GBM ECM *in vitro* conditions to get better understanding of the therapeutic efficiency.

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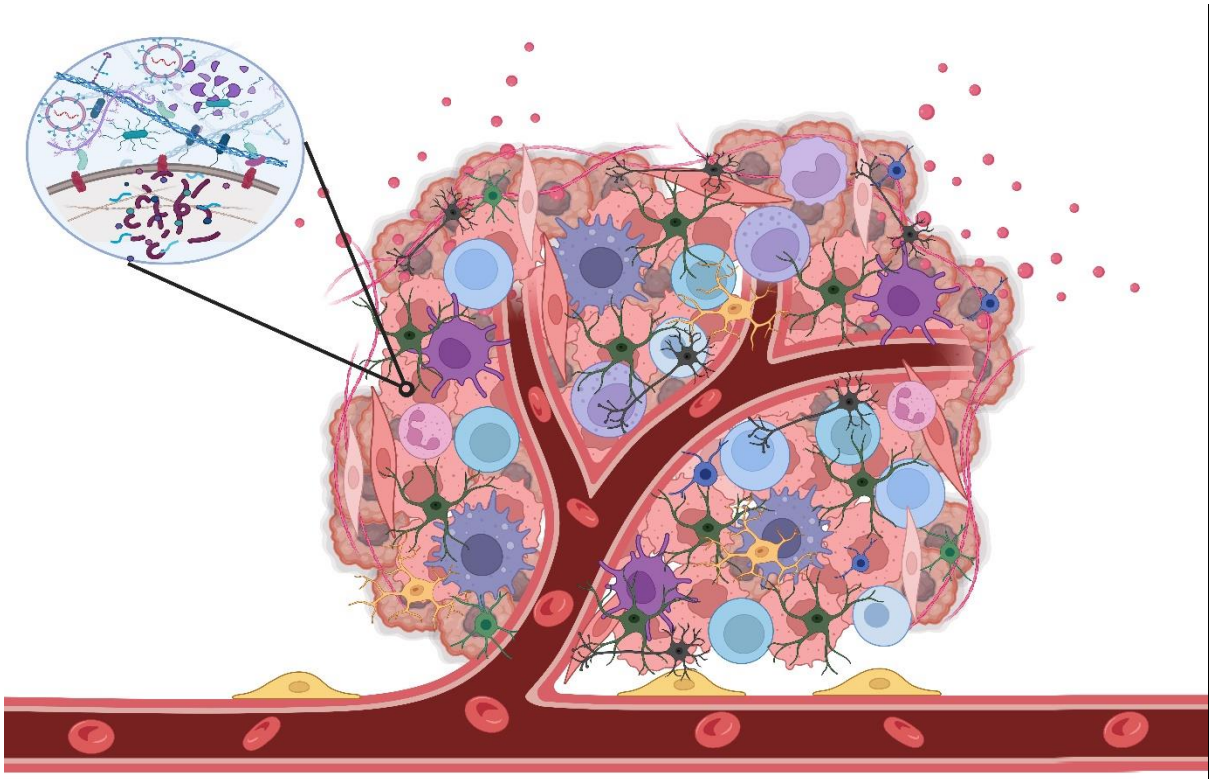

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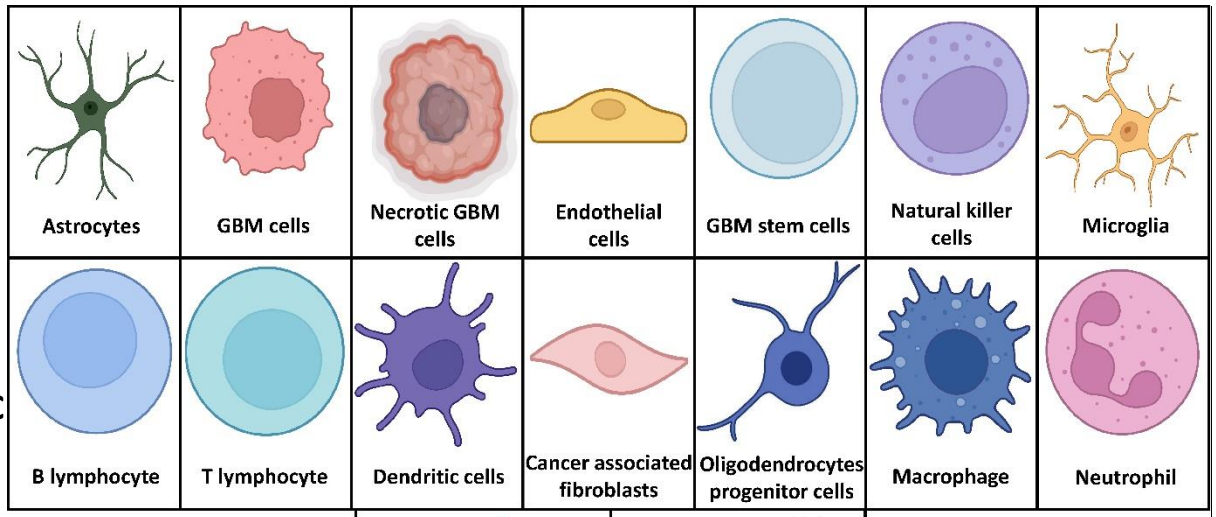

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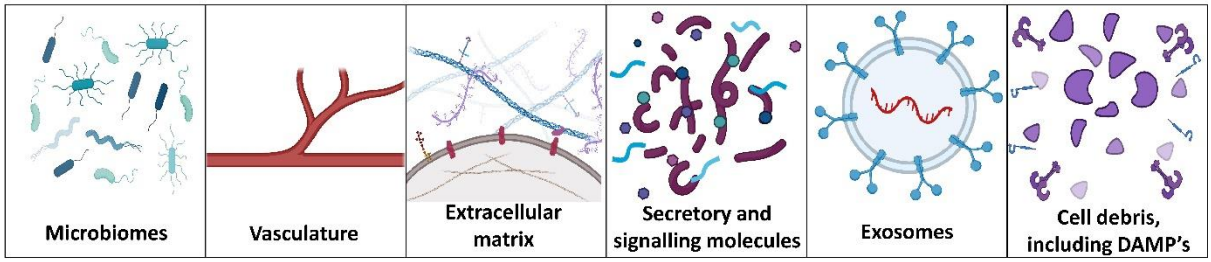


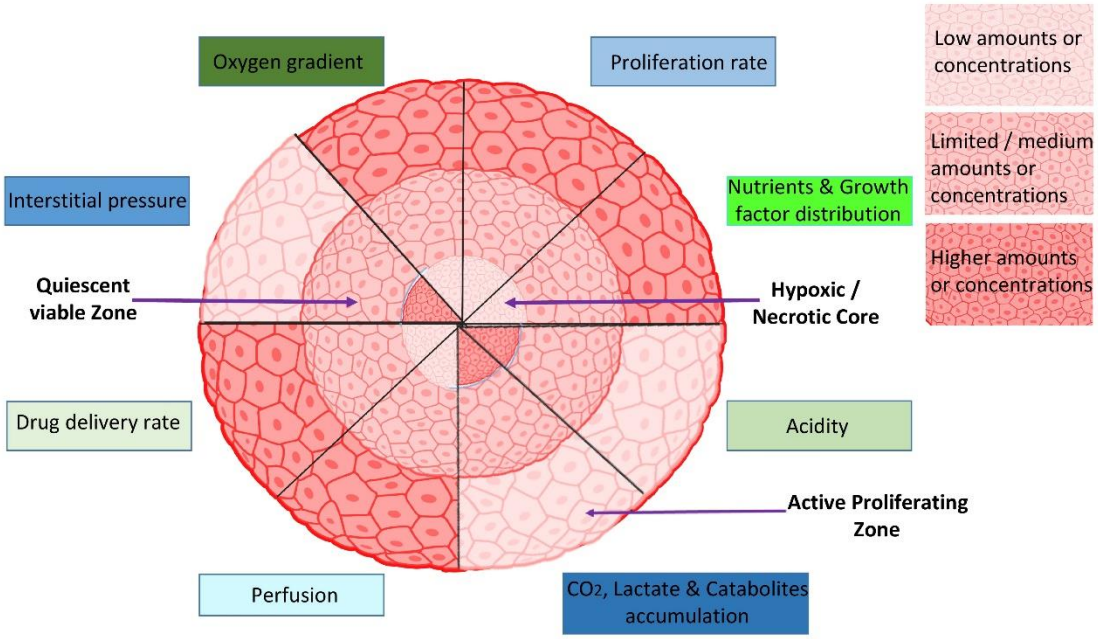
Figure 1: A) Components of GBM TME, consists of cellular and extracellular materials. B) Cells commonly found in the tumour microenvironment such as Astrocytes, GBM cells, Necrotic GBM cells, Endothelial cells, GBM stem cells, Natural killer cells, Microglia, B and T lymphocytes, Dendritic cells, Cancer associated fibroblasts, Macrophages, Neutrophil and Oligodendrocytes progenitor cells are shown here C) Non-cellular components such as Vasculature, Microbiomes, Extracellular matrix, Secretory and signalling molecules, Exosomes and Cell debris, including Damage Associated Molecular Patterns (DAMP's) that are important features of a brain tumour (Figure created with BioRender).

In Two dimensional (2D) culture, cells adhere primarily to coated surfaces of the tissue culture plate, whereas in 3D culture, adhesion is mostly with molecules of the extracellular matrix between cells along with directly interactions between adjacent cells. Matrix proteins, glycoproteins, glycosaminoglycans, proteoglycans, ECM-sequestered growth factors, vascular endothelial growth factor, platelet derived growth factor, hepatocyte growth factor, and other secreted proteins are examples of secretory and signalling molecules [12]. These proteins and growth factors have critical roles in cell proliferation, tissue morphogenesis, migration, differentiation, adhesion, survival, immunosuppression, metastasis and homeostasis [12-15]. Furthermore, the ECM can influence the cell's response to medications by altering the mechanism of action of the drug, increasing therapeutic effectiveness, or increasing the cell's inclination for drug resistance. A 3D culture model would have to imitate the microenvironment of tissue in which cells could proliferate, aggregate, and differentiate in order to predict the effectiveness of a treatment on a cell [16]. Further, Integrins and receptor tyrosine kinases are examples of cell surface receptors that can interact with ECM components. Crosstalk

between integrins and growth factor receptors regulates downstream cell signaling as well as growth factor induced biological activity, such as proliferation and invasion [9,13].

Brain tumors are surrounded and infiltrated by many noncancerous cells, including neurons, astrocytes, microglia, cancer-associated fibroblasts, tumor-associated macrophages, glioblastoma stem cells (GSCs) and endothelial cells, that provide both supporting and suppressive functions in the TME (Figure 1) [17-19]. Cancer progression and drug response are heavily influenced by cellular interactions in the TME [17,20,21]. 3D *in vitro* models can be utilized to simulate TME components and to evaluate novel therapies [14,19].

Cells in a 3D spheroids have varying microenvironment conditions due to the non-homogeneous vascular supply [22]. For example, regions of a tumour further from vasculature have restricted oxygenation, nutrients and waste removal. 3D spheroid can possess a hypoxic (oxygen-deprived) core resembling these TMEs found in solid tumours, with cells at the centre of sphere with relatively low oxygen, glucose concentration and acidic extracellular pH due to accumulation of metabolic by-products (Figure 2) [23,24]. The hypoxic cell population increase is proportional to the spheroid size also it is highly resistant to chemotherapy and radiotherapy. The outer layer of spheroid, which is highly exposed to medium and mainly composed of viable, proliferating cells. 3D spheroid has heterogeneous cellular subpopulation such as actively proliferating, quiescent, hypoxic and necrotic cells, which provides different cell proliferation zones, can be divided as proliferating zone, quiescent viable zone and necrotic core / hypoxic core (Figure 2) [11,13,25].



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146 **Figure 2**: Structure of multicellular 3D spheroid. 3D spheroids have a spherical shape with an external
147 proliferating zone and an internal quiescent viable zone that surrounds a necrotic core, resembling
148 the cellular heterogeneity seen in solid tumors. Proliferation rate, drug delivery rate, interstitial
149 pressure, perfusion, Access to O2, nutrients and acidity in different zones are shown here (Figure
150 created with BioRender).

151

152 The cellular organization, additional dimension, polarity, and geometry of 3D spheroids influence
153 cellular functions such as proliferation, differentiation, survival, morphology, gene/protein
154 expression, communication, and responses to external stimuli [16]. Ultimately this will provide a better
155 understanding of complex biological / physiological behaviour, cell-to-cell interactions, tumor
156 characteristics, drug discovery, metabolic profiling, and representation for toxicological testing
157 improve drug screening accuracy, safety, increasing the chances of finding effective therapeutic
158 methods or drug combinations to fight cancer [16].

159

The demerits of currently available 3D cell culture are that it is time consuming, expensive, lower reproducibility and limited intra-tumoral heterogeneity [26]. Further development needed in this field to assure reproducibility, high throughput analysis, compatible readout techniques and automation in order to establish validated 3D cell culture models [27]. The main strengths and weaknesses of 3D cell culture systems for cancer research applications shown in Table 1.

Comparison of 2D and 3D cell culture

In 2D cell culture, monolayer of cells adheres and grows on flat surfaces, while these cells are unable to grown in all directions. Due to this cells are flat and stretched hence it does not accurately reflect *in vivo* cellular morphology [23,25]. The monolayer is mostly composed of proliferating cells, and any necrotic cells usually detach from the surface [28]. These attached proliferating cells receive homogeneous oxygen, nutrient and growth factors from the media and uniform exposure to drug candidates in efficacy and toxicity studies [29]. The morphological changes in 2D cells influences many cellular processes such as cell proliferation, cell–cell communication, tissue specific architecture, differentiation, migration, apoptosis and gene/protein expression, which leads to inaccurate organ-specific toxicity detection and have inadequate representation of cell migration, differentiation, signal transduction, metabolism, survival and growth [16,22,30].

3D cell culture can use to overcome these problems as cells are allowed to grow in any direction without interacting with the surface, while maintaining physiological cell-cell and cell-extracellular matrix interactions, more closely mimic the natural *in vivo* environment, shape, and cellular response [16,30]. Cells in 3D cultures are not getting homogenous oxygen, nutrient and growth factors supply due to their larger size and diffusion gradient (Figure 2) leading to all major TMEs represented including proliferating, quiescent and necrotic stages found in an *in vivo* tumor (Figure 2) [25].

The proliferation rate of 2D and 3D cell culture are different and this is mostly depend on cell lines and matrix [31]. The proliferation rate of cells grown in 3D cell culture is a better represent the growth of *in vivo* tumour. When compare with 2D cell culture, additional dimension in 3D cell culture influence spatial organization of cell surface receptors engaged in interaction with other cells and induce physical constraints to cells [31,32]. Most drugs are designed either to targeting specific receptors accessible on the cell surface, or by crossing the plasma membrane and interacting with intracellular receptors to achieve therapeutic effectiveness. The availability of receptors in 2D and 3D cultures may be different due to differences in receptor expression, cell morphology, cytoskeletal and ECM arrangements, subcellular localization of receptors, modified endosomal trafficking, alterations to secretions, cell signalling and even differences in the spatial arrangement of receptors on the surface of cells [9,16].

Overall the cellular responses varying between 2D and 3D cell culture is due to several factors such as differences in physical properties, physiological conditions, spatial organization of surface receptors, gene expression levels, microenvironment and cell stages are some of them. 2D cell culture doesn't reveal toxicological resistance, accurate cellular responses to drug treatment, architecture as *in vivo* tissues, accurate depiction of cell polarisation and gene expression [33]. It also provides unreliable predictions of *in vivo* drug efficiency and toxicity, which leads to low success rate in clinical trials [33]. 3D spheroids show increased drug resistance [34](Figure 2) due to dynamic cellular interactions and restricted diffusion of nutrient, leading to activation of cell survival and drug sensitive genes [34]. Ultimately 3D cell culture can overcome the limitations of conventional 2D cell culture by providing an experimental models that more accurately represent the short- and long-term (time) effects of the drugs. The merits and demerits of 2D and 3D cell culture is compared in Table 2

Han and colleges, produced a scalable lung cancer spheroid model and carried out genome-wide CRISPR screenings in 2D-monolayers and 3D cancer spheroid cultures. CRISPR phenotypes in 3D more closely resemble those of *in vivo* tumors, and genes with differing sensitivities in 2D and 3D are highly

enriched for important mutations in malignancies. These analysis also revealed new drivers that are required for cancer development in 3D and *in vivo* but not in 2D [35]. A similar experiment utilizing GBM spheroid models will be beneficial in the future to understand which genes are essential for growth and survival in response to different environmental signals.

Comparison of 3D cell culture with animal *in vivo* models

3D cell culture plays a vital role in drug development, while it is also capable of replacing both 2D cell culture and animal trials. Initial testing stage of standard drug discovery begins with 2D cell culture, followed by animal tests and clinical trials, which resulted 95% of trial failures during clinical trials due to the insufficient prediction of the efficacy and toxicity in humans during pre-clinical studies [33,36].

3D cell cultures represent a simplified reductionist model. It highly transparent, reproducible, easy to modelling the complex processes such as growth, invasiveness and toxicity, when compared to a whole animal [30]. 3D cancer cell models are able to provide better understanding of *in vivo* cancer therapeutic efficiency and also improve the efficacy of drug discovery, due to the clear understanding the relation between cells and the ECM in which they interact [16,19]. This help to identify drugs/ therapeutic methods in early stages, which has better effects on cancer treatment and eliminating a lot of unnecessary testing.

The European REACH regulation stated aim is “To ensure a high level of protection of human health and the environment from effects of hazardous chemicals. It strives for a balance: to increase our understanding of the possible hazards of chemicals, while at the same time avoiding unnecessary testing on animals” (European Chemicals Agency, 2020). 3D cell cultures supports 3Rs principles of animal research (Replacement, Reduction and Refinement) and REACH regulation while able to reduce the number of animal usage in testing, time, cost and ethical considerations [9,37].

There are different animal models have been widely used to investigate GBM such as syngeneic implantation models (tumorigenesis is induced using carcinogens or genetic modification), genetically engineered animal models (delivery of cancer initiating genes using viral vectors to initiate tumor development), traditional xenograft models (transplanting human cancer cells into an immunocompromised rodent), patient derived xenograft and xenografts generated from patient derived cancer stem cells (direct implantation of freshly biopsied tumor tissue or cultured tumor spheres into immunodeficient animals) are some of them [38,39]. These experimental animal models have several limitations since they don't always predict efficacy and/or toxicity, don't share the same clinical features, and don't have the same receptor responses as seen in human disease. Vital genetic, molecular, immunologic and cellular differences between humans and animal models prevent it from being an effective way of researching a cancer therapies [37,40].

Animal testing is expensive and time consuming and they do not account for the whole intricacy of tumor-microenvironment interactions [19]. Also, If animal is in pain or stress during the experiment, it might change the biochemical, physiological and metabolic reactions, which can inaccurately depict the effectiveness and side effects of drugs [9,16,30,40]. Humans and animal models have distinct anatomical and physiological differences, the most apparent of which is size. The human brain is about 100 times greater in weight and more than 1,000 times larger in surface area and number of neurons, when compared with mice. Thus, in the study of GBM, well known for its infiltration of the brain parenchyma, important anatomical distinctions in the organ of origin impose potentially confounding factors in preclinical investigation [37,41]. Preclinical modeling is complicated further by an increased proportion of neocortical astrocytes, pericyte heterogeneity, and changes in vascular architecture between humans and animal models [41].

Some animal models such as mice have a short lifetime, they are less likely to development of certain types of cancers or highly penetrant cancers associated with loss of heterozygosity mutations. Animal models also have substantially greater metabolic rates than humans, which complicates

pharmacodynamic and pharmacokinetic investigations [41]. Genetic modifications initiate tumors with homogenous genetic changes whereas human GBM cells are heterogeneous. Furthermore, the genetic background of animal models can influence tumor biology, gene function, and tumor susceptibility [38].

Many variables *in vivo* are uncontrollable, and their effects are often unknown due to the complexity of organisms, whereas 3D cell culture allows for better control of variables by using a series of carefully selected reductionist models [42]. The merits and demerits of 2D, 3D cell culture and animal models are compared in Table 2.

Current *in vitro* GBM treatment regimens fail to account for a large variety of factors such as brain's unique extracellular matrix, circulatory systems, existence of resident and non-resident brain cells inside the tumour, secreted factors and nutritional sources accessible for tumor metabolism [19]. The main benefits of using 3D cell culture models for *in vitro* GBM treatment rather than animal testing are include a wider selection of techniques, leading to better measurements of outcomes, better control of variables, scalable testing, comparatively lower cost, avoidance of ethical issues and reductionist approach to accurately model a specific feature of a disease, as opposed to animal models, which are complex and often differ from human disease. It is also capable of simulating de novo drug resistance [9]. Furthermore, juxtacrine signaling, in which molecules pass directly between cells via gap junctions or other structures without being released into the extracellular environment, requires 3D tumorsphere cell–cell interactions. These receptor and juxtacrine signaling components alter a variety of intracellular signaling pathways, affecting how cancer cells react to their surroundings [9,13]. The lack of vascular and immune system in 3D cell culture techniques is a drawback when compared to animal models, that may be solved in the future by constructing advanced 3D models utilizing specialized 3D techniques such as 3D printing [42]. Ultimately 3D brain cancer models can so improve reproducibility and allow researching cellular and molecular pathways simpler to improve for personalized medicine.

Different types of 3D cell culture techniques and methods

Different elementary 3D culture techniques such as anchorage independent and anchorage dependent platforms can be used for 3D cell culture [11]. Anchorage dependent platforms can further classifies into scaffold and hydrogels based on their porosity, density and mechanical strengths [28]. These approaches are most commonly employed to create 3D spheroids, basic tumor models and multicellular tumorspheroids (Figure 3). Tumorspheroids are solid, 3D spherical formed by the proliferation of a single cancer stem/progenitor cell [43,44]. Tables 3 and 4 list the applications and merits / demerits of different 3D culture techniques / methods for the development of 3D glioma spheroids, respectively.

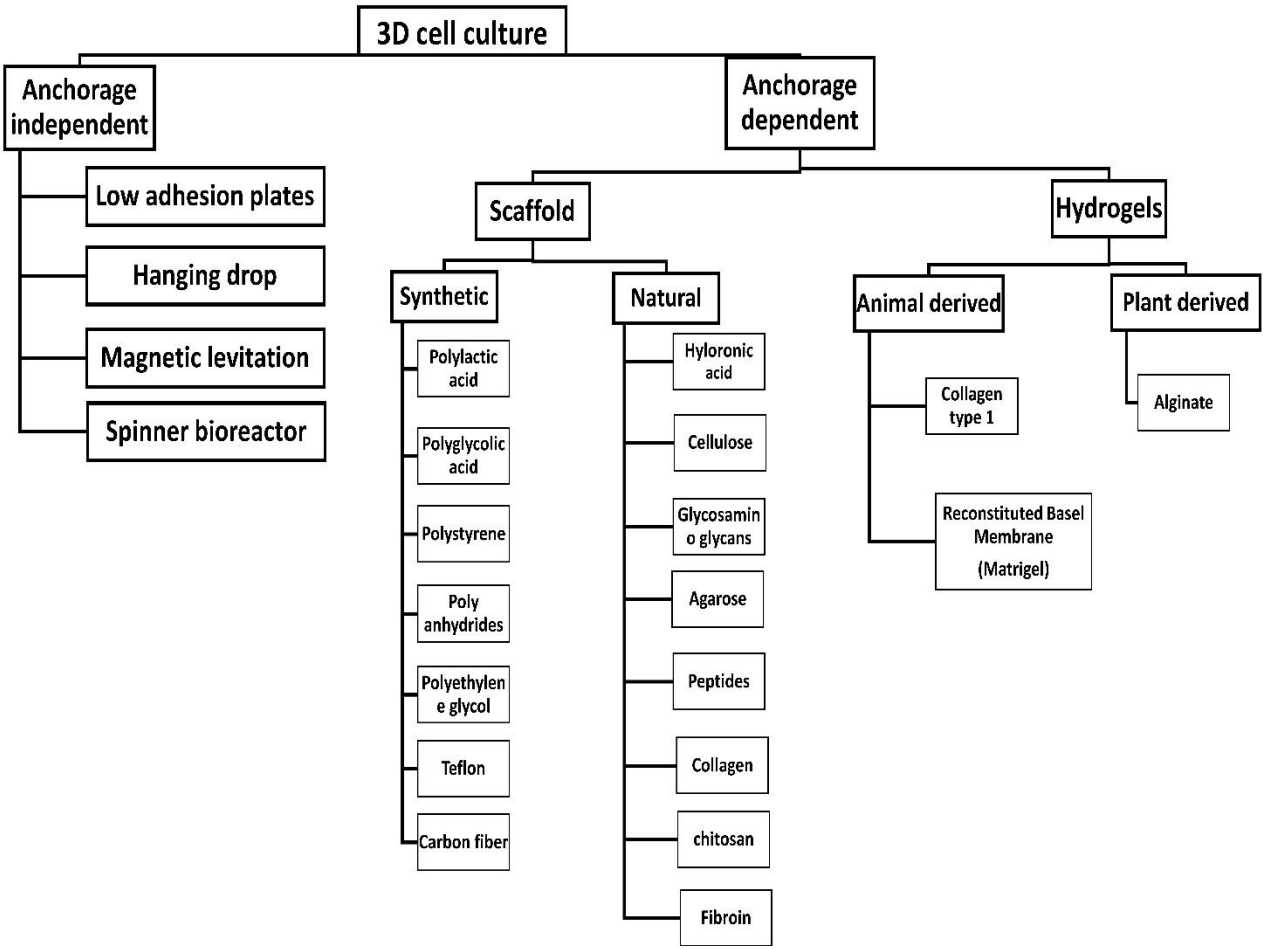

Figure 3: Different anchorage dependent and independent methods to develop 3D multicellular tumor spheroids.

Anchorage independent (scaffold free)

Anchorage independent/scaffold-free techniques rely on non-adherent cell to cell aggregation to form spheroids. Spheroids showing cell-cell interactions and secreting their own extracellular matrix. These spheroids are able to freely grow without a physical support resulting in consistency of shape and size, which provide better understanding about cellular cytotoxicity [16].

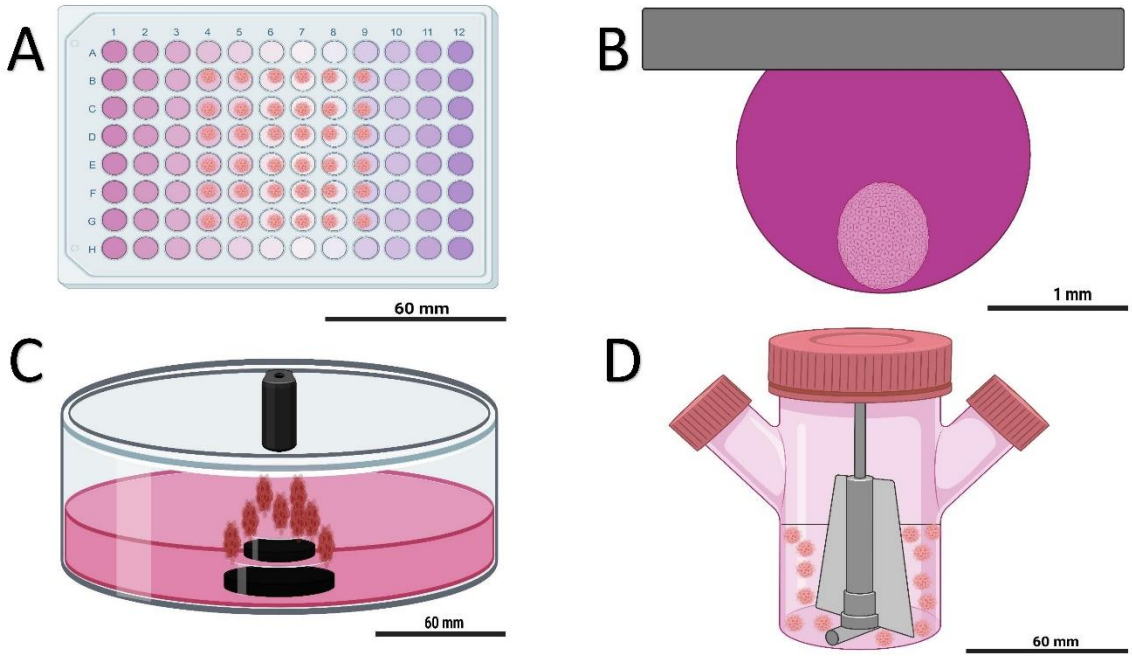

Figure 4: Anchorage independent methods available for multicellular tumor spheroids formation. These methods include, A) Low adhesion plate method; B) Hanging drop plate method; C) Magnetic levitation; D) Spinner Bioreactor (Figure created with BioRender).

Low adhesion plates

Low adhesion plates (Figure 4A) are specialised culture plates with ultra-low attachment hydrophilic polymer coating (poly-2-hydroxyethyl methacrylate (poly-HEMA), agarose, bovine serum albumin, or agar) which promote cell aggregation to form spheroids [11,45,46]. Different culture plates are commercially available (e.g. Nunclon™ Sphera™, Costar®, PrimeSurface, Lipidure®–COAT) with modified surface shapes (flat and conical shaped bottom) [11,45]. Usually ECM proteins such as collagen-I and fibronectin mediate cell attachment to the culture surface. Hydrophilic polymer coating prohibits protein adsorption to the culture ware surface, thereby minimizing monolayer cell adhesion to the culture vessel [47]. Ultimately low attachment plates promote aggregation of cells by cell-cell and cell-ECM interactions, while blocking the ECM interaction to plastic surface. Advantages of using low adhesion plates are simple, straight forward, efficient, spheroid production & handling is easy, higher reproducibility when compared to other anchorage independent methods, able to generate wide range of tumor cell types and co-culture can be incorporated [46]. Disadvantage is time consuming and relatively labour intensive, continuous passage culture is difficult, only autocrine ECM is present, success rate in long term passage is low, cells in suspension has no migration movements [16,24,46,48]. The detailed protocol for developing 3D glioma spheroids published by [49].

Hanging drop method

Hanging drop plates are open bottom-less wells that promote the formation of droplets of media (Figure 4B) that provide space to form spheroids via self-aggregation through the use of gravity and surface tension [50]. There is no surface to attach, cells grow inside a bubble of growth media and spheroids hang in open bottomless wells which are often enclosed in the bottom of the plate in order to normalize the environmental humidity of the cells [45]. Phosphate buffer saline is added to the reservoirs located on the peripheral rim of the plate and tray which are divided into sections to prevent the hanging drop dehydration during incubation [45]. Spheroid size is controlled by number of cells dispensed into each drop [11]. The droplet of media sufficient for cell aggregation and also small

enough to hold droplet by surface tension, after 3D spheroid generation it can be dispense by adding extra drop of media in to the well and spheroid loaded to adjacent plate [16]. Micro-liquid adhesion with substrate surface is greater than cellular weight; cells aggregate, proliferate, and grow in to spheroids at liquid air interface. Recommended drop volume is 10-20 µl [48]. There are currently some commercially available hanging drop plates on the market, such as Perfecta3D® and Gravity PLUSTM [45]. Multicellular spheroids also can be create by co-suspending several cell types or else consecutive addition of different cell types to form separate cell layers. The merits are: able to produce uniform spheroid size, able to control size of spheroid by seeding density, homogenous spheroids and suitable for high throughput testing, higher replicability, low cost and comfortable to handling. In the disadvantages side, plates are highly expensive, medium change and different drug treatment at different time points are impossible, not suitable for long term culture and also having small culture volume and osmolarity of the droplet will rise due to medium evaporation [16,45,46,48]. Lara and colleagues provided a thorough procedure for producing 3D glioma spheroids using hanging drop plate method [51].

Magnetic levitation

Magnetic levitation (Figure 4C) is a suspension culture technique; cells are preloaded with magnetic nanoparticles or beads in dedicated plate and external magnetic fields to provide non-adhesion, plate-like properties to facilitate cell aggregation and form uniform 3D spheroids / tumorspheres [11,45]. It can be used on a variety of cell lines, particularly those that do not self-aggregate. The amount of cells that were able to internalize the particles determines spheroid development [45]. This method is highly efficient, simple, straightforward, possibility to replicate *in vivo* microenvironments, does not require specialized media, easier spheroid collection and changing of medium with minimal disruption. It also allows for the quick generation of 3D spheroids and is scalable for higher throughput [52]. In disadvantage side this method gives slight brownish colour to spheroids and which might be not

suitable for some applications. Also some cells adhere to the bottom of plate without forming 3D spheroids and magnetic particles may alter the cellular behaviours of these spheroids [16,45,48]. There haven't been many uses of magnetic levitation for the development of 3D glioma spheroids documented.

Spinner Bioreactor

A spinner bioreactor (Figure 4D) has a container to hold cell suspension and impeller stirring continuously to minimize the cell adhesion to the surface. Bioreactors are closed systems used to strictly regulate factors such as dissolved oxygen, temperature, pH, and nutrients. Specific sensors inside the bioreactor linked to control software to monitor nutrition and metabolite input and outflow [33]. Continuous Liquid flow prevents cell adhesion contamination, time-consuming manual operations and also uniformly distributes nutrition and oxygen to form 3D spheroids [33,46]. This method is simple, able to mass production of spheroids and also suitable for long term culture [46]. While cells can be damaged by collision between cells and bioreactor wells (exposure to high shear force) and require specialized equipment's also difficult to obtain uniform spheroids [33,46,48].

Anchorage dependent (Scaffold Based)

The anchorage-dependent approach uses pre-designed porous membranes and polymeric fabric meshes called "scaffolds", which can be constructed of natural or synthetic components to offer physical support (Figure 5A) [24,53]. This physical support can provide structures from simple mechanical up to extra-cellular matrix-like structures. 3D spheres can be generated by seeding cells on three dimensional matrixes or by dispensing cells in liquid matrix followed by solidification and polymerization. Cells are embedded in extracellular components and able to initiate cell-cell and cell-matrix interactions, physical support for cell growth, adhesion and proliferation. In general, these

features, as well as structural patterns, textures, and angulations, can be manipulated in an attempt to mimic ECM traits particular to the tissue of interest [54]. There are several techniques use to create scaffold such as electrospinning (ES), stereolithography, 3D printing, solvent-casting particulate leaching (SCPL), freeze drying, shape deposition manufacturing, robotic micro assembly, phase inversion, selective laser sintering, fused deposition modelling [16,48,53].

Natural scaffolds

Biological / natural scaffolds provide physical support for cell growth as well as provide similar *in vivo* microenvironment with ECM components, growth factors, hormones and so forth. The biological scaffolds are made up of ECM components such as fibronectin, collagen, laminin, gelatin, chitosan, glycosaminoglycans (mainly hyaluronic acid), fibroin, agarose, alginate, starch (mainly additives), human decellularized ECM [14,25,48,55]. Microscale mechanical features of biomaterials, such as stiffness, porosity, interconnectivity, and structural integrity, can influence cellular function [56]. Brain tumor specific ECM components such as proteoglycans, laminins, fibronectin, tenascins, collagens I, II, IV and glioma cells overexpress ECM components like hyaluronic acid, brevikan, tenascin-C, fibronectin, thrombospondin can be employed to engineer glioma-specific scaffolds to mimic similar *in vivo* glioma TME [12,57].

The advantages of using biological scaffolds are highly similar to the *in vivo* conditions, can control similar composition/ elasticity /porosity to get better ECM presentation and also possible to combine with ideal growth factors. Also it is able to improve biocompatibility, spatial distribution and lower toxicity [55]. Natural scaffolds also have higher biocompatibility and lower toxicity when compared to synthetic polymers. Disadvantages are it is expensive, time consuming, complex process and not suitable for large scale production, difficult to dissociate cells from scaffold for experiments such as flow cytometry and risk of contaminations and disease transmission [48]. Lara and colleges provided a thorough procedure for producing 3D glioma spheroids using a natural scaffolds based method [58].

Synthetic scaffolds

Polymeric scaffolds are a useful tool for investigating cell-ECM interactions due to the_scaffold's capacity to duplicate the structure of the ECM. Polymeric hard scaffolds are also very valuable for investigating tissue regeneration and evaluating tumor cell therapies [16]. Single cell suspension can be grown in a pre-fabricated scaffold to generate 3D spheroids. These scaffold matrixes enable cellular growth, adhesion, and proliferation while also encouraging cells to create spatial dispersion and migration. These polymeric scaffolds have been designed to mimic the structure of *in vivo* tissues and easier to reproduce [55]. Matrix stiffness has been shown to have a major influence on tumour cell phenotypes and the usage of synthetic scaffolds has also been employed to investigate the effect of matrix stiffness on drug responsiveness [55]. The scaffolds can be create using polymers such as Polyglycolic acid, Polylactic acid, polyorthoesters and their co polymers or blends as well as aliphatic polyester polycaprolactone, polystyrene (PS), polycaprolactone (PCL) Polyethylene oxide (PEO), Polyethylene glycol (PEG) [25,48]. The merits of using synthetic scaffold is that the capability of controlling stiffness, elasticity, porosity and permeability, higher versatility, augment workability, reproducibility, straightforward to use and mechanical qualities of synthetic materials can be adjusted according to the cell culture required, and their chemical composition is well characterized [48]. The demerits are lack of biodegradation in most of the polymers, which might affect the cellular activity [48]. However, some synthetic polymers can be tailored to degrade and also researchers are attempting to improve biodegradability [59].

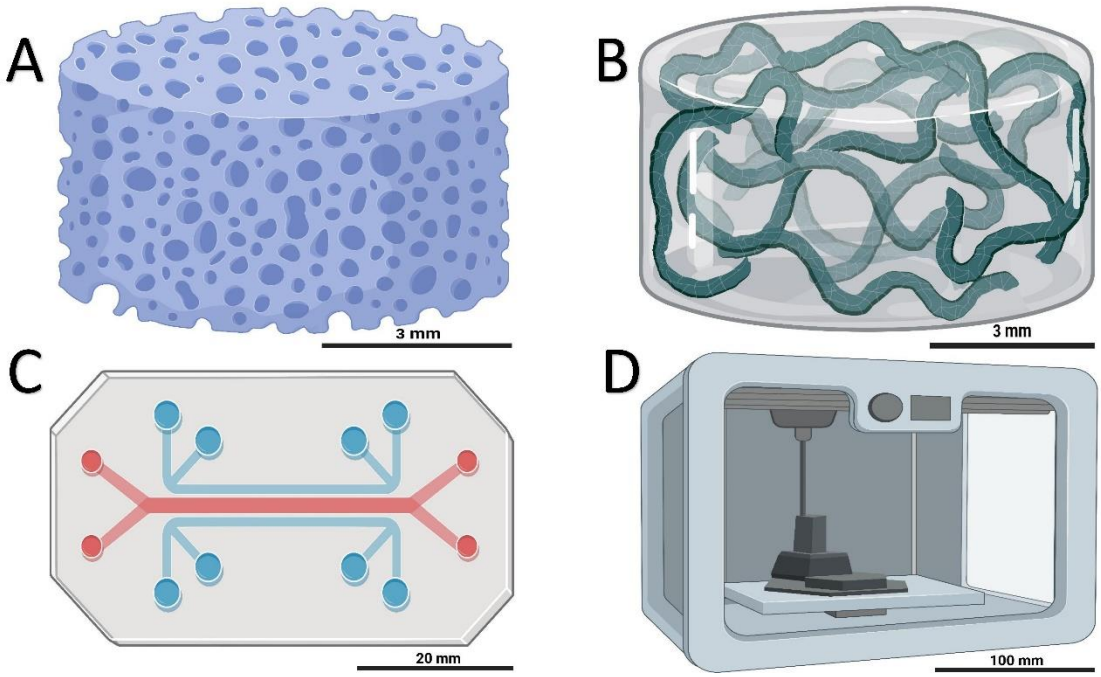

Figure 5: Anchorage dependent methods and specialized 3D culture platforms available for multicellular tumor spheroids formation. These methods include, A) Natural and synthetic scaffold based method; B) Hydrogels; C) Microfluidic devices; D) 3D Bio printer (Figure created with BioRender).

Anchorage dependent (Hydrogels)

Hydrogels (Figure 5B) provide multi-layer formats by cross-linked hydrophilic polymer chains and cells are embedded inside layers and able to grow to 3D spheroids providing cell-cell and cell-ECM interactions [33,48], which has similar biochemical, structural and mechanical properties of an *in vivo* tissue. Hydrogels are in a liquid format at room temperature which become a gel at 37 C incubation [18]. It helps cells to mix uniformly into the gel-liquid and proliferate non-destructively during the gelation process [48]. Mechanical strength, nutrition transport, topography, and degradation behaviours can all be adjusted by using polymers with varying compositions, crosslinking density, and including bioactive compounds [53]. Hydrogels are 3D matrices or porous scaffolds can be divided into synthetics and natural hydrogels [33].

There are natural hydrogels made up using natural polymers – animal/ plant -derived proteins such as aginate, hyaluronic acid, collagen, silk, fibrinogen, albumin, fibronectin, laminin, agarose, matrigel, gellan gum, gelatin, and chitosan [33]. Collagen is a major ECM component in connective tissues. Collagen type 1 animal based hydrogels are mostly used and successful since its ability to replicate the cellular microenvironment and tissue architecture. Collagen based hydrogels have good biocompatibility and cross linking pattern can be controlled by concentration and sonication time, which makes that suitable for range of tumors [48]. Alginate is another mostly using polymer derive from seaweed. The most commonly used natural hydrogel platform is reconstituted basement membrane matrix (Matrigel) derived from murine tumours [55]. Researchers used 3D Matrigel to evaluate different anti-invasive compounds (NF-kB, GSK-3-B, COX-2, and tubulin inhibitors) toxicity and invasion inhibition in U-251 MG spheroids. The results indicated that the compound effectiveness is strongly linked to intra- and inter-tumor heterogeneity in patients [60].

Synthetic hydrogels are made up with synthetic polymers such as polylactic acid (PLA), poly (vinyl acetate) (PVA), polyethylene glycol (PEG), polyacrylamide, polyacrylic acid, polyvinyl alcohol and polyvinylpyrrolidone are some of them [16,33]. Natural hydrogels are progressively being replaced by synthetic hydrogels due to higher water absorption capacity, higher strength, longer stability, and extensive availability of raw chemical resources [61].

Advantages of using hydrogels for 3D cell culture includes controllable porosity, elasticity, variation in stiffness, high water content, able to provide similar microenvironment and reproducibly, able to provides rich network of ECM signals, ability to construct combining both synthetic and natural materials and ability to couple with adhesion, proliferation, differentiation, and migration factors [33,53,55]. While demerits including physically weaker, lack of vasculature, natural gels composition can be inconstant and also lack of cross linked network for mechanical support 3D spheroid growth [48]. In future, researchers can try to develop hydrogels using similar ECM components and composition in a particular tissue / tumor site to get similar *in vivo* tumor microenvironment [48].

Hydrogels can also be designed to release therapeutics, while changing their retention period in the tissue. Scientists developed a reactive oxygen species (ROS)-responsive hydrogel (Zebularine - anti-PD1 antibody - NPs-Gel) cross-linked by combining polyvinyl alcohol and N1-(4-boronobenzyl)-N3-(4-boronophenyl)-N1,N1,N3,N3-tetramethylpropane-1,3-diaminium (TSPBA) linker to utilize the acidic TME and ROS within tumors for the controlled release of zebularine, a demethylation agent, and aPD1 antibody. This combined treatment boosted cancer cell immunogenicity, reducing tumor growth and prolonging the survival time of B16F10-melanoma-bearing mice [62].

Researchers are mostly adopting low adhesion plate and hydrogel-based approaches to construct basic tumor models and multicellular tumor spheroids. Recently scientists investigated more advanced techniques and equipment to develop more complex brain tumor models to better mimic the biochemical interplay of the brain and brain cancers as technology evolved. To facilitate spheroid formation in 3D cell culture platforms, microfluidic devices may, for example, uniformly provide oxygen and nutrients while eliminating waste. For instance, advanced brain tumor models with intact blood brain barriers may be printed using 3D bio-printers to investigate the possibility of opening the BBB and enhancing chemotherapy delivery without adverse effects. It may also be used to investigate membrane-wrapped and co-culture models.

Microfluidic devices

Microfluidic devices (Figure 5C) process/ manipulate micro liquids (usually less than 10µl) inside micro sized channels with dimension of 1-1000 µm [63]. Microfluidic channels are connected to each other by porous membranes produce spheroids and able to formation, maintenance and testing inside single device with vasculature- mimicking microfluidic channel connections [11,34,46,48]. Furthermore, this technology enables for the investigation of cell-cell interactions as well as interactions between different tissues [11].

Microfluidics are classified into two types: flow-based channel microfluidics (CMF) and electric-based digital microfluidics (DMF). Individual droplet manipulation, multistep processes, flexible electric-automatic control, and the ability for point-of-care are all benefits of DMF over CMF [64]. The physical barrier of microfluidic 3D cell culture system is composed of glass/silicon, polymers such as polydimethylsiloxane (PDMS), polymethylmethacrylate (PMMA), polycarbonate (PC) and polystyrene (PS). PDMS is the most often utilized substance due to biocompatibility, inexpensive, has good gas permeability and transparent capability, however, scaling-up process is more difficult [53]. Simple microfluidics devices are increasingly being fabricated and created by soft lithography techniques to develop patterned environments that are reasonably easy to fabricate and compatible with the majority of biological systems [16,56].

Microfluidics technique capable of continuous perfusion for faster spheroid formation, to produce uniform size and shape spheroids for high-throughput screening, It allows patterning of cells and extracellular environment to create co culturing cells in spatially controlled manner, generation of and control signalling gradients, integration of perfusion, low reagent / sample consumption, which significantly reduces costs in bioanalysis, real-time imaging and to constructing tissue-level and organ level structures *in vitro* [16,18,46,50]. In the other hand disadvantage is it is highly expensive, hard to collect cells for analysis, hard to scale-up, need complicated equipment and complexity [46,48,65].

Microfluidic devices are complex dynamic micro scale environments that simulate 3D *in vivo* environments, such as a complex chemical gradient. Its micro scale dimensions are consistent with those of numerous *in vivo* microstructures and environments [66]. Capillaries in the brain, for example, ranging from 7-10 μm in diameter, with an average intercapillary distance of about 40 μm [67]. Microfluidic devices' versatility and simplicity of fabrication allow them to be used in a wide range of applications in glioma research. These include migration studies, biomarker assessment, cell sorting from tissue samples, and treatment effectiveness testing [68,69]. The time course for culture is heavily influenced by cell type, cell density, and device type. Scientists might possibly obtain critical

519 information on tumor status from specific patient samples using microfluidic devices and recommend
520 personalized therapy within in two weeks [66].
521 Researchers demonstrated that organ-on-a-chip GBM model matched the clinical outcomes during
522 the patient-specific sensitivity against temozolomide (TMZ). This technology has also been used to
523 study the interaction within the perivascular niche, which suggests that glioma CSCs located around
524 the vasculature and presenting with the lowest motility are most likely of the proneural subtype, while
525 those with the highest invasiveness are most likely of the mesenchymal subtype; this further supports
526 the role of the tumor niche on intratumoral heterogeneity and subsequent treatment response [70]. In
527 another study, an oxygen and nutritional gradient is produced in the tumor cell embedded ECM
528 containing core chamber by delivering a regular flow via one lateral channel while shutting the other
529 [71]. This model replicates blood artery thrombosis in the brain, as seen in glioblastoma growth, and
530 allows for the observation of thrombosis-induced variables that impact invasion in real time [71]. The
531 promise of microfluidic devices as sophisticated artificial systems capable of mimicking *in vivo*
532 nutrition and oxygen gradients during tumor progression is demonstrated in this article [71].
533
534 The development of microfluidic technology has simplified, facilitated, and shortened the drug
535 discovery process [72]. It also a valuable tools for the development of wide range of biological systems,
536 from single-cell biophysical characterization to the miniaturization of a complete laboratory onto a
537 single chip (lab-on-a-chip), and lately, the recapitulation of organ physiological parameters onto a chip
538 (organ on chip / vasculature on a chip) [50,73].
539
540 ***3D Bio printing***
541 3D Bio printing (Figure 5 D) is a novel bottom-up approach to fabricate complex biological constructs
542 for 3D cell and tissue culture [24]. It is also able to control mechanical and biological properties of the
543 construct with high resolution in the X, Y and Z planes [52]. 3D bio printing is layer-by-layer deposition

of bio-inks [21] to build viable 3D constructions in a spatially specified way, guided by a computer-aided software [74,75]. It's able to enhance additional factors (cell types, materials, growth factors, differentiation factors and print the 3D construct with extraordinary spatial control at high resolution through a layer by layer process [74,76]. The main issue for bio printing is to print cells and bio-ink concurrently without impacting cell viability or substituting chemical solvents [33].

The bio-inks can be classifies as soft biomaterials (scaffold base bio-ink) and cells bio printed without an exogenous biomaterial (scaffold-free bio-ink) [75]. Layers of soft biomaterials are deposited to form an extracellular matrix, which contains live cells, arranged into a cell network that closely resembles the real tumor [77]. Single-step bio fabrication techniques including inkjet, micro extrusion, and laser-assisted bio printing uses with soft biomaterials, which can fabricate 3D structures decreasing user input mistakes [56,75]. While scaffold-free bio-ink, cells are grown up to small neo tissues that are three-dimensionally scattered and will eventually combine and develop to a more complicated structure. It is also possible to use 3D bio printing to create biosimilar acellular scaffolds and then include a cellular component using the top-down method (two-step fabrication), this approach has several limitations, including poor reproducibility, cell density control, and spatial distribution control [56,75].

3D printing can applied to develop GBM models with vascular channels to get better understanding of six core and two emergent hallmarks underpin tumour development and metastasis [78]. Research team developed of an integrated platform that allows for the generation of an *in vitro* 3D GBM model with perfused vascular channels that allows for long-term culture and drug (TMZ) delivery [79]. Glioma stem cells (GSCs) have been revealed in recent research to have a role in tumor vascularization by secreting vascular endothelial growth factor (VEGF). Wang et al. (2018a) used 3D printing to create a 3D glioma model to investigate the vascularization potential of patient-derived CSCs [80]. Heinrich et al. (2019) created a 3D-bioprinted mini-brain made up of GBM cells and macrophages to explore the interaction between glioma CSCs and other non-tumor cells. The authors discovered that glioma cells interact with macrophages and induce TAM polarization in patients' tissue [81].

Scientists used cellular and a-cellular components from the patient's adipose tissue to create a variety of customised bio-inks. After transplantation, these tailored patches will not elicit an immunological response, obviating the requirement for immunosuppression. This demonstrates the 3D printing approach's potential for organ replacement after failure or drug screening in a suitable anatomical framework [82]. Three-dimensional biological constructions are a novel and promising method of research not only in GBM but also in other diseases [77]. Recently, researchers used this techniques and tailored hydrogel as a bio-ink to construct a thick, vascularized, perfusable cardiac patch and heart-like structure. These cardiac patches are a potential field for human tissue engineering since they perfectly match the patient's immunological, biological, biochemical, and anatomical features [82]. The similar technique can be applied by using the personalized brain patches, possible to replicate the architecture of tissues to get better understanding of the therapeutic efficiency.

Advance TME models and applications

Cancer stem cells (CSC) differ from typical stem cells in several ways, including hyper-efficient DNA repair processes, the expression of multidrug resistance-related ATP-binding cassette (ABC) membrane transporters, hypoxic niche tolerance, and the over-expression of anti-apoptotic proteins. Furthermore, in the case of cancer, the difference between CSCs and non-CSCs may be linked to epithelial-to-mesenchymal transition (EMT) [46,56]. Scientists have recently focused on CSC's due to its role in tumor growth, metastasis, recurrence and drug resistance, and 3D cell culture is a vital tool to studying that due to the abundance of CSC [29,46,48]. CSC's from 3D cell culture have a distinct morphology signaling pathway profiles, cell–matrix and cell–cell interactions and gene expression pattern than CSCs from 2D culture [29,46]. Multiple genes related with stress response, inflammation, redox signaling, hypoxia, and angiogenesis are up-regulated. In comparison to 2D cultures, CSC spheroid cultures demonstrated benefits such as increased paracrine cytokine production, stronger anti-apoptotic and anti-oxidative properties, and higher amounts of ECM proteins [16,29]. Glioblastoma

stem cells (GSC) share features of GBM such as resistance to therapeutic treatments, high invasiveness, and similar epigenetic patterns. The DNA methylation pattern of GBM-derived cancer stem cells was analysed, and it was shown that these cells have the same methylation pattern as primary GBM-derived xenograft tumors [83]. It implies that GSC culture conditions preserve the majority of their original epigenetic pattern, implying that GSC are legitimate and appropriate *in vitro* model for determining the functional effect of epigenetic alteration on cellular parameters [27,83]. Researchers demonstrated that the growing GBM cells on 3D porous chitosan-alginate scaffolds greatly enhances proliferation and enrichment of cells possessing the hallmarks of CSCs. The 3D model was discovered to be more tumorigenic and to promote the expression of genes involved in the epithelial-to-mesenchymal transition, which has been linked to the development of CSCs [84].

Blood–brain barrier (BBB) prevents several chemotherapeutic drugs from accumulating to effective concentrations in glioblastoma and other brain tumors [78]. Researchers developed 3D-bioprinted GBM and BBB models, focusing on the TME compositions of GBM and BBB, appropriate biomaterials to imitate the *in-vivo* tissue architecture, and bio-printing methodologies for model fabrication. This model offer potential systems for more reliable mechanistic research and preclinical drug screens [85]. Hajal and colleagues also developed an *in vitro* model of the human BBB from stem-cell-derived / primary brain endothelial cells, primary brain pericytes, and astrocytes that self-assembled within microfluidic devices. This BBB model showed important cellular structure and morphological traits, as well as molecular permeability values that are within the predicted *in vivo* range. These characteristics, together with a functional brain endothelial expression profile and the ability to test several repetitions rapidly and inexpensively, make these advance BBB models excellent for therapeutic discovery and development [86].

TME is entails of a diverse population of **immune cells**, including microglia, macrophages, CD4+ T cells, CD8+ T cells, regulatory T cells, myeloid-derived suppressor cells, NK cells, and dendritic cells, indicating that GBM has a strong immunological component [87]. Parenchymal microglia play critical

roles in brain development, homeostasis maintenance, disorders and regulating several mechanisms such as synaptic pruning, maturation, and angiogenesis [88]. Because of their ramified motile processes, parenchymal microglia are capable of monitoring and phagocytizing any hazardous chemicals [88]. Furthermore, microglia can enhance angiogenesis, emphasizing the importance of microglia-cerebral vasculature communication [88]. Macrophages are also engaged in brain homeostasis maintenance and reside in the non-parenchymal perivascular space, subdural meningeal spaces, and choroid plexus spaces [88,89]. These Glioma associated microglia and macrophages have been demonstrated to adopt predominantly M2 phenotypes, leading to anti-inflammation/ immunosuppression and hence aiding tumor development [87,90]. Tumor cells appear to promote microglia mobility by upregulating genes involved in migration and invasion [87,90]. IL- 10, MMPs, and arginase-1 are further immunosuppressive substances released by glioma-associated microglia and macrophages [87]. Furthermore, tumor cells and glioma associated microglia and macrophages secrete chemokines like monocyte chemotactic protein-1, CCL2, capable of attracting myeloid derived suppressor cells such as immature macrophages, granulocytes, dendritic cells, and myeloid progenitors to the tumor [87,89]. Ultimately they can promote tumor growth through the release of anti-inflammatory cytokines for instance TGF-b and IL-10 [87,89]. There is, however, a lack of advanced 3D GBM models to study parenchymal, peripheral immune cell crosstalk and immune cell infiltration.

Microbiome play an important role in the human immune system's induction, preparation, regulation, and function, While Specific microbiota may also lead to immune suppression [91,92]. Gut microbiota generates metabolites such as short chain fatty acids, which inhibit pro-inflammatory cytokine release, promote regulatory T cell growth and IL10 secretion [91,92]. A portion of the circulating short chain fatty acids may potentially enter the CNS [92]. Furthermore, the integrity of the BBB is compromised during neuro-inflammation due to the actions of IL1, IL6, and TNFα [91,92]. It has to be established if the microbiome-induced mediators or metabolites also affect the BBB disruption and elicit immune suppression in the brain [92]. The brain, glands, gut, immune cells, and gastrointestinal microbiota are all part of the microbiota–gut–brain axis. Gut microbiota also influences brain function

and behaviour through neuronal, endocrine, and immunological pathways [92,93]. Researchers revealed that the gut microbiome influences the anticancer immune response and reduces the effectiveness of chemotherapeutic cancer treatment [93]. The potential impact of the microbiome on brain tumor treatment techniques should be investigated with more advance 3D co-culture models with tumour-resident bacterial strains.

Investigating **GBM / normal tissue interactions** are vital in brain cancer therapeutics hence, advanced 3D GBM co-culture models will be needed to develop, to explore the crosstalk and metabolic interactions between glioma cells and the normal glial cells such as astrocytes, oligodendrocytes, neurons and a range of normal resident brain cells. 3D cell culture also able to co culturing with different cell types, including mixed populations of tumor cells and cancer associated fibroblasts (CAF), to develop increasingly accurate *in vitro* models of disease and physiology [25]. The importance of glioblastoma multiforme cellular interaction with endothelial cells can be studied with co culture techniques to get proper understanding of the endothelial interaction on tumor progression for identify novel therapeutic approaches [25,65]. Also by adding cells such as blood vessels, can use to investigate interactions between blood vessels and cancer or how drug help to antiangiogenic effect in cancer. Researchers examined available *in vivo* data to calculate the quantities and numerical ratios of GBM and normal brain cells necessary to establish a complete and incomplete GBM resection dual co-culture model. The results indicated that drug discovery utilizing this dual co-culture methodology is feasible and provides steady and reliable drug testing outcomes [94].

GBM Organoids are a novel experimental paradigm of modern reductionists' approach. The combination of embryonic stem cells or induced pluripotent stem cells or resident stem cells, contemporary 3D culture, controlled environment and differentiation techniques has allowed us to leverage pluripotent stem cells' self-organization capacity to form human brain-like tissues known as brain organoids or mini-brains [5,77]. Brain organoids are a promising new technology that has opened up new avenues for cancer modeling, ex vivo investigation of molecular and cellular mechanisms [26,77],

while many properties of neural epithelial cells in these 3D tissues are cyto-architecturally analogous to the developing human brain [5,11]. These organoids imitate the *in vivo* cell heterogeneity present in the tumor microenvironment by resembling the *in vivo* architecture of the tissue of origin and recapitulate cell proliferation, self-organization, and differentiation [11,27]. A GBM model was created by genetically engineering brain organoids in a recent study. Researchers developed a GBM model organoid by inserting the HRasG12V oncogene into human brain organoids and using CRISPR/Cas9 to alter the fourth exon of the TP53 locus. This mutant cell, which has a characteristic similar to the aggressive mesenchymal subtype of GBM, proliferates quickly and invades the organoid. Furthermore, they revealed that primary human derived glioblastoma cell lines can be transplanted into human cerebral organoids to induce tumors [11,77,95]. Recently, Scientists also employed brain organoids to model CNS pathologies of COVID-19 and provide initial insights into the potential neurotoxic effect of SARS-CoV-2 [96]. Gunti and colleagues reviewed several tumor organoid models, procedures for establish them, recent advances and applications of tumor organoids in detail [34]. Currently, basic organoid models are being used by researchers for therapeutic discovery and development. In future we need to develop multifactorial complex models incorporating CSC, BBB, GBM tumour microenvironment, including microbiomes, vasculature, extracellular matrix, infiltrating parenchymal and peripheral immune cells and molecules, exosomes and chemical gradients to develop personalized medicine and to achieve efficient therapeutic discovery and development.

Challenges and future prospective

3D cell culture, however, has proven it has the potential to completely change the way in which new drug treatments are tested, diseases are modelled, stem cells are utilized, and organs are transplanted

[16,77]. The capacity to accurately simulate the intricacy of the TME is a major hurdle in developing physiologically appropriate *in vitro* models for drug screening and cancer biology research. By co-cultivating various cell types in a specified 3D matrix, custom-tailored ECM gels with specific amino-acid sequences, more advanced pre-clinical models must develop with cell–ECM or cell–cell interactions inside and between the TME [97]. Furthermore, combining diverse approaches, like as organotypic cultures and organoids, with 3D bio-printing, might improve the investigation of cell interactions in GBM [30,77]. In future to address this obstacle closely, researchers will develop Four-dimensional (4D) bio printing, a next generation of bio fabrication technology, involving the use of stimuli-responsive biomaterials that can be altered in a time-dependent manner (fourth dimension) in an attempt to mimic the physiological activities of TME [56,75].

If we can selectively open the BBB, then the future we could give much lower doses of powerful drugs, which would likely reduce toxic side effects and make treatment safer as well as more effective for patients. 3D cell culture and 3D printing technology can be used to create model BBB to study it effects effectively. The emerging technologies like as 4D real imaging, microfluidics, organ-on-a-chip technology, and single cell sequencing will undoubtedly be used to reveal unique insights into the biology of GB tumoroids, revealing hitherto undiscovered potentials of these models [98].

In future, Advancement in 3D cell culture will become feasible to construct entire 3D *in vitro* GB organoids, which will eventually lead to personalized treatments for glioblastoma [29,55,98]. The inclusion of patient-derived cells into standardized 3D tumor models will capture cancer heterogeneity [33], as well as repair damaged organs using patient cells to avoid rejection from the immune components [16,20]. Ultimately, 3D cell culture research has enormous potential as a cutting-edge frontier in regenerative, precision, and customized medicine [99].

TABLE 1 | The Current Three-Dimensional cell culture systems for cancer research applications: Key Strengths and Weaknesses

Strengths	**Weaknesses**	**Ref.**
Matrices contain ECM components that promote cell–cell interaction, communication, and activation of signaling pathways.	Some models generate spheroids with a wide range of sizes, resulting in a number of variation inside the same well.	[76,100,101]
Heterogeneous cell populations resemble tumor cells at various stages of the cell cycle, such as proliferating, hypoxic, and necrotic cells	Vasculature, which is critical for tumor development, survival, and medication delivery, is still missing in 3D models.	[13,16,100]
Factors/proteins identified in a certain tumor microenvironment can be added to the culture setting.	Large-scale investigations and high-throughput tests are much more expensive and time consuming.	[25,76,100]
Cellular functioning, morphological differentiation, gene and protein expression levels, and hence cellular behaviours, are comparable to those seen *in vivo*.	Variability in biological matrices can lead to inconsistent experimental outcomes.	[26,36,101]
Ability to develop multicellular systems and bridges the gap between *in vitro* and *in vivo* cancer therapeutic outcomes.	Do not reassemble the complicated TME, and the technologies that can do so can only do so for a limited time	[26,27,76]

Table 2: Comparison of 2D and 3D cell culture methods.

Characteristics	2D cell culture	Animal models	3D cell culture	References
Morphology / Cell shape	• Flat, stretched shape cells • Cells grow into a monolayer • Cells can only expand and proliferate in two dimensions	• Natural, shape of cells more representative of solid tumours • Can differ from human cells in terms of type and quantity	• Natural, shape of cells more representative of solid tumours • Cells grow into 3D spheroids • Spheroids contain multiple layers of cells similar to *in vivo*	[16,25,32,36,37,99]
Cells Interactions and microenvironment	• Cell- cell contact only on edges and mostly contact with plastic • Deprived cell extracellular environment interactions • Lack of *in vivo*-like microenvironment and "niches"	• Cell-cell and cell-extracellular matrix interaction • Interactions with the microenvironment that vary from *in vivo* human interactions • Inability to control composition of the Microenvironment	• Physiologic cell-cell and cell-extracellular matrix interaction • Cells communicate through exchange ions, small molecules, and electrical currents • Micro environment and "niches" similar to *in vivo*	[11,14,15,23,25,32]

			• Apical–basal polarization and lumen formation	
Transport	• No transport dynamics	• Complex transport dynamics	• Complex transport dynamics	[25,26]
Distribution of media /drug	• Nutrients, growth factors and drug are equally exposed to all the cells	• Similar to human cells *in vivo* • Vascularization feasible along with immune system activity	• Diffusion gradient of nutrients, growth factors, drugs and metabolic waste • Core of the spheroid received lower amount of nutrients, growth factors and oxygen making hypoxic core (mimic *in vivo* tumor structure)	[11,102,103]
Stage of cell cycle (Cell differentiation)	• Most of the cells in same stage of cell cycle • Deprived cell differentiation	• Heterogeneous cell population with proliferating, quiescent, hypoxic and necrotic cells similar to human *in vivo* • Rapid speed of reproduction	• Heterogeneous cell population with proliferating, quiescent, hypoxic and necrotic cells • The cells have a higher level of differentiation.	[11,41,102]

Phenotype and Polarity	• Forfeiture of diverse phenotype and polarity	• Similar to human cells *in vivo*	• Apical basolateral polarity is maintained • Diverse phenotype and polarity similar to *in vivo* tumor	[32,99]
Gene/ protein expression	• Not provide accurate depiction • Display differential gene and protein expression levels, mRNA splicing and cellular biochemistry compared to *in vivo* conditions	• Gene and protein expression cannot accurately reflect due to the species variations	• Provide more accurate depiction of gene and protein expression similar to those in *in vivo* tissues. • Expressed genes, proteins, mRNA, and other cellular activities are effectively identified and quantified.	[25,36,37]
Cell proliferation	• Usually cellular proliferation is faster than *in vivo* cells	• Higher proliferation rates than human *in vivo* cells	• Mostly, proliferation rates are similar to the human *in vivo* cells	[11,32,41]
Mutation	• Protracted genetic and phenotypic drifts, as well as cellular cross contamination, are common in cells.	• Complex and time consuming to identify genetic and phenotypic drifts	• Improbable to genetic and phenotypic drifts	[16]

Drug sensitivity	• Lower drug resistance • Poor drug metabolism • Misrepresentation of drug treatment efficiency	• Ability to study side effects • Higher drug resistance to treatments similar to the in vivo cells	• Higher drug resistance to treatments similar to the *in vivo* cells • Improved drug metabolism • Accurate representation of the treatment efficiency	[32,102]
Representation	• Inadequate representation	• The representation is quite intricate	• Improved models for cell migration, differentiation, survival and growth	[24,25,102]
Metabolic profiling	• Augmented sensitivity to ATP synthase	• higher metabolic rates and ATP synthase sensitivity is distinct to *in vivo* human cells	• Abridged sensitivity to ATP synthase	[16,41]
Quality and Time of culture	• Higher performance and reproducibility • Easy to interpret	• Time consuming for the study (Days) • Difficult to handle, maintain and interpret data	• Lower performance and reproducibility • Difficult to interpret data • More difficult to handle and maintain • Time consuming for culture (Days)	[16,25,36,102]

	• Culture handling is comparatively easy • Shorter time for culture (Hours)	• Lower performance and reproducibility • Long tumor latency		
Cost of maintaining culture	• Low cost maintenance • Readily available test materials and media	• Expensive when compared to both 2D and 3D cell culture	• Expensive when compared to 2D cell culture • Limited commercially available products	[11,25,32]
Apoptosis	• Lesser resistance to the drug-induced apoptosis	• Apoptosis responses may vary	• Greater resistance to the drug-induced apoptosis	[36]
Response to stimuli	• The response of cells to mechanical stimuli is inaccurately portrayed. • They are unable to respond to gravity.	• Different pathophysiology to humans	• Accurate representation of response to mechanical stimuli of cells • They are continuously able to respond to gravity.	[36,102]

co-culturing cells	• Lower benefits and inadequate representation	• Unable to control architecture of a tissue	• higher benefits and superior of co-culturing cells	[16,27]
Tumour heterogeneity	• Basic representation	• Higher due to the species differences	• Better approximation via the proliferation gradient, drug penetration and mobility variations.	[34,103]
Multi cellular study	• When studying the immunological response, this is a better option.	• Most suitable for multi cellular studies	• When there are more than two cell types in a co-culture, it becomes more challenging.	[25,65]
Genetic engineering	• Not possible	• Ease and precision of genetic manipulation	• Possible only in advance 3D models	[41]
Ethics	• No ethical concerns are required.	• Many ethical considerations arise as a result of animal suffering, international and national regulations	• A potential alternative that can eliminate animal experimentation. No ethical concerns are required but may raise due to the origins of primary and stem cells	[37]

TABLE 3 |Different types of 3D cell culture techniques and their applications, outcomes in glioma research

3D cell culture technique	**Cell line / type**	**Drug/ treatment combination**	**Outcomes**	**References**
Ultra low attachment plates	CT-2A mouse glioma	Nano formulation of atorvastatin (ATV)	Growth inhibition was more significant for the micellar – ATV formulation compared to free ATV in 3D models.	[104]
	U-87 MG and C6 glioma cells (CCL-107)	Retinoid bexarotene (BXR) derivatives with dopamine (DA) and nitroethanolamine Amide (NEA)	Tumorspheroids demonstrated higher resistance to the treatment. BXR-DA, BXR-NEA resulted in a synergetic cytotoxicity increase, induce apoptosis and inhibit cell spreading	[105]
	U-251 MG	Cold atmospheric plasma (CAP)	CAP effectively induce 3D GBM cell death in a time-, dose-, treatment frequency, and ROS-dependent manner. CAP also reduce 3D GBM spheroid growth, cell proliferation and induce damage to the tumor microenvironment.	[9]

	U-87 MG	Doxorubicin (DOX) loaded polymeric nanotubes	DOX loaded nanotubes significantly reduced the 3D cell viability in a dose dependent manner, whilst unloaded nanotubes showed no cytotoxicity.	[106]
Hanging drop plate	U87-MG	Poly(dimethylsiloxane) and resin-based drop array chip and a pillar array chip with alignment stoppers	Enhances the alignment between the chips for uniform placement of spheroids.	[107]
	LN-229	Silicon chips	Simple design elements enable high drug screening duplicates, direct on-chip real-time or high-resolution confocal imaging, and geometric control in 3D.	[108]
Spinner bioreactor	GBM 4, 8	-	Nonexistence of connexin43 (Cx43) reduces glioma invasion in 3D model	[109]
Ca-alginate scaffolds	U-251 MG	-	Gene expression profiling showed that cell cycle and DNA replication gene down-regulated, and genes involved in mitogen-activated protein kinase signaling, autophagy, drug metabolism through cytochrome P450, and ATP binding cassette transporter were up-regulated in 3D, compared to 2D cells.	[110]

Collagen Scaffold	U-87 MG	Temozolomide (TMZ), Cisplatin (DDP), Lomustine (CCNU)	With a substantially greater proportion of glioma stem cells and upregulation of MGMT, 3D grown cells also displayed improved resistance to chemotherapeutic, alkylating drugs.	[111]
Polystyrene scaffolds coated with Laminin	U-251 MG	-	The findings show that 3D context has an impact on integrin expression, particularly the upregulation of the Laminin binding integrins alpha 6 and beta 4.	[112]
Hydrogels	U-87 MG	Novel bio-inspired brain matrix (BBM) composed of an agarose base and poly-L-lactic acid 6100 (PLA) fibers	BBM able to supports tumor growth, enables rapid tracking of neural stem cells migration and therapy.	[113]
	Patient-derived GBM cells (PDCs)	HMC3 microglia	Microglia co-culture significantly inhibited GBM invasion but enhanced proliferation	[114]

	D-270MG, U-87 MG	Three patient-derived cell lines were compared including adult glioblastoma cells (aGBM), pediatric glioblastoma cells (pGBM), and diffuse pontine intrinsic glioma (DIPG).	The findings imply that brain tumor behaviour is influenced by both patient age and tumor site. (Tumor proliferation, invasion and morphology)	[115]
	U-251 MG	shRNAs targeting human LIMK1 and LIMK2	LIM kinase isoforms LIMK1 and LIMK2 strongly regulate GBM invasive motility and tumor progression and support.	[116]
Microfluidic device	Triple co-culture of U-87 MG, hCMEC/D3 cells and astrocytes.	Antibody-functionalized nutlin-3a loaded nanostructured lipid carriers (Ab-Nut-NLCs)	The approach successfully blocks dextran diffusion through the bioinspired BBB while enabling Ab-Nut-NLCs to pass through.	[117]
	U-251 MG, U-87 MG	TMZ and simvastatin (Simva)	Cells were significantly less sensitive to drugs and induction of apoptosis in the 3D model as compared to 2D. Autophagy inhibition had no effect on TMZ and Simva-induced apoptosis.	[118]

3D bio printer	U-87 MG	N-cadherin (NCAD)	NCAD prevented spheroid formation and induced cell death in the 3D model	[119]
	Glioblastoma stem cells (GSCs)	Compared the growth of GSCs alone or with astrocytes and neural precursor cells in a hyaluronic acid-rich hydrogel, with or without macrophage.	Whole-genome CRISPR screening using bio printed complex systems revealed distinct molecular dependencies in GSCs, relative to sphere culture.	[120]
	U87, SU3 glioma stem cell line	hydrogel scaffolds were printed (Gelatin/alginate/fibrinogen Hydrogel)	The 3D bio printed *in vitro* glioma model provided novel alternative tool for researching gliomagenesis, stem cell, , anticancer drug susceptibility and treatment resistance, while showed higher resistant to TMZ compared to the 2D glioma model.	[121]
	U87-MG	3D model including alginates, MM6 monocyte/macrophages, ECM proteins (collagen-1, hyaluronic acid), and glioma associated stromal cells.	Glioblastoma stem cells demonstrated greater resistance to chemotherapeutic drugs in 3D printed tumor than in 2D monolayer cultures.	[122]

TABLE 4 | Comparison of different 3D cell culture techniques and equipments, highlighting their respective merits and demerits for both 3D tumor model production and applications.

3D culture method	**Benefits**	**Drawbacks**	**References**
Low attachment plate	Relative simplicity	Relatively labour intensive	[22,25,33,45,47,48]
	Reproducibility	No support or porosity	
	Relatively low cost	Only autocrine ECM existing	
	Faster spheroid production	Difficulty in mass production	
	Suitable for long-term culture	Lack of uniformity (size / shape)	
	Suitable for multicellular spheroids (MCS) and co-culture	Continuous passage culture is challenging	
	Possible to use a high-throughput screening	Not suitable for migration or invasion assays	
	Cells can easily be removed from the media and utilized in subsequent experiments.	Cell aggregates form as a result of cell motility in the media.	
	Uniform spheroid size control		

	Availability of pre-coated plates	Some cell lines need expensive plates coated with specific materials	
	Plates are optically transparent		
	Useful for drug screening, as well as direct visualization and analysis.		
Hanging drop plate	Relative simplicity	Long term culture difficult	[22,25,45,48]
	Uniform spheroid size control	Smaller culture volume	
	Co-culture feasibility	Impossible to medium exchange	
	Suitable for high-throughput testing	Not suitable for migration, invasion or cell viability assays	
	Relatively low cost	Smaller size of spheroids	
	Reproducibility	Labour intensive	
		Not suitable for drug testing	
		Tedious spheroid handling and transfer	
Magnetic levitation	Relative simplicity	3D culture is coloured brown	[45,48]
	Efficient	Limited applications	
	Not required specialized media	Cellular behaviour might affect	
	Easy to collect spheroids and change media	Numerous cells also attach to the plate's bottom	

	Capable of being employed on non-self-aggregating cells	Magnetic beads need pre-treatment and can be expensive	
Spinner bioreactor	Suitable for mass production	Difficult to change media	[25,33,45,48,123]
	Relative simplicity	Larger medium volume needed	
	Suitable for long-term culture	Special apparatus needed	
	Homogeneous media composition	Higher variability in size and shape	
	Customizable and controllable culture parameters	Exposed to high shear force	
	Possible to use a high-throughput screening	Not suitable for drug testing	
	Minimum labour	Higher costs	
	Stimulated metabolite transport		
	Higher similarity to the *in vivo* conditions		
Scaffold based	Mimic *in vivo* microenvironment	Difficulty of cell retrieval	[25,27,33,45,48,53,55]
	Relative ease handling	Low optical transparency	
	Suitable for long-term culture	Not suitable for drug testing	
	Suitable for co-culture	Variation in scaffold-to-scaffold	
	Compatibility with all types of cells and well plates	Limited high-throughput screening	
	Properties can be modified according to the study	Expensive for large scale production	

	It is simple to prepare for immunohistochemistry analysis.	Lack of uniformity (size/shape)	
	Higher similarity to the *in vivo* conditions	Scaffold materials may affect the cellular adhesion, growth and behaviour	
	Direct visualization	Restricted control over self-assembly	
	Availability of wide range of materials, including a decellularized matrix	Cells connected to the scaffolds flatten and proliferate in the same way as cells cultured 2D	
Hydrogels	Cells can be easily recovered for further analysis	Low repeatability depending on cell line	[18,22,27,33,48,55]
	Possible to use a high-throughput screening	Difficulty of cell recovery from hydrogel	
	Wide variety of polymers availability	Poor mechanical properties	
	The ability to customize properties	Low optical transparency	
	Higher similarity to the *in vivo* conditions	Natural hydrogel's components are variable and undefined	
	Cellular attachment, proliferation, and differentiation are all stimulated.	Bioactive ingredients in hydrogels may influence the structural formation	
	Suitable for study the aggressiveness of the cells and metastasis	Labour intensive and time consuming	
	Mimic *in vivo* microenvironment	Batch to batch variation	

Microfluidic device	Ability to control spheroids size and parameters	High cost for the microfabrication and devices	18,33,48,55,72,73,76,123
	Continuous perfusion aids in the development of spheroids	Difficult to collect cells for further analysis	
	Real time imaging possible	Required expertise	
	Capable of incorporating vascular and circulation like components	Limited high-throughput screening options	
	Mimic *in vivo* microenvironment	Issues with contamination	
	High-throughput assays regarding toxicity, targeting, efficacy, and organ distribution	Design dependant outcomes	
	Commercially available		
	Higher gas permeability		
	Higher optical transparency		
	Large amounts of data may be obtained from small samples.		
	Able to construct *In vitro* organ specific device		
	Replicate the complex 3D tissue architecture	Higher Cost of bio printer and bio inks	72,76,77

3D bio printing	Possible to use a high-throughput screening	Low accuracy of cell positioning	
	Complex interactions between TME or ECM and cells	Printing resolution can yet be enhanced	
	Mimic *in vivo* microenvironment	Need photo crosslinking	
	Suitable for study the invasiveness of the cells and metastasis	Effective biomaterials are required.	
	Suitable for study the drug efficiency, cell signaling, immunologic interactions and cellular crosstalk		

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