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

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1 REVIEW ARTICLE

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3 **<u>Advances in 3D culture systems for therapeutic discovery and development in brain cancer</u>**

4

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27 **Highlights**
28
29 - Existing models for drug discovery and development against GBM have limitations.
30 - We review advances in 3D systems that promise more accurate therapeutic models.
31 - Development of 3D cultures that can model the GBM TME is discussed.
32 - We describe advanced 3D systems such as organoids, 3D and 4D bioprinting and CSC.
33 - We identify gaps to bridge in existing 3D systems to accelerate drug discovery.

34 **Abstract**

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

44

45 **Teaser**

46 In time, 3D cell culture research will lead to development of complex, multifaceted GBM models, and
47 will enable rapid advances in precision, personalised medicine to improve patient outcomes.
48

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

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53

54 **Introduction to 3D cell culture for brain cancer**

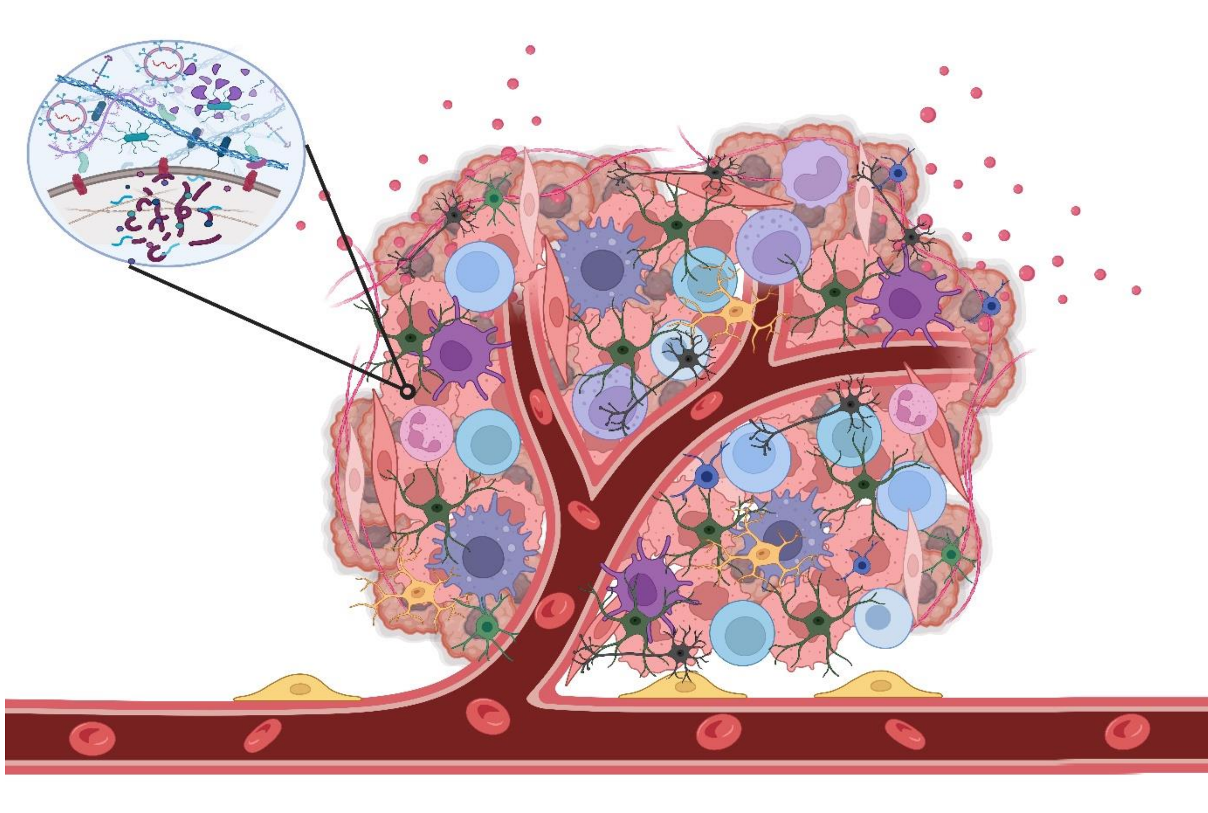
55

56 Brain cancers can be divided into two types, primary and secondary brain cancer. Primary brain cancer
57 originates within brain cells, forms in the central nervous system (CNS), and usually does not
58 metastasis to the outside of the CNS. Secondary brain cancers are originated and metastasis from
59 external to the CNS, such as the lung, skin, breast, colon, and kidney. Secondary brain cancers are the
60 most common, while primary brain cancers are more lethal [1,2]. Primary brain cancers can be classifies
61 further as gliomas (astrocytomas, oligodendrogliomas and ependymomas) and nongliomas
62 (menigiomas, medulloblastomas) [2,3]. Gliomas are developed from glial cells, including astrocytes,
63 oligodendrocytes, and ependymal calls or a mix of the above. Astrocytomas are the most common
64 primary brain cancer and according to the World Health Organization (WHO), it is further classified as
65 pilocytic astrocytoma (Grade I), low grade astrocytoma (Grade II), anaplastic astrocytoma (Grade III),
66 and glioblastoma (Grade IV) [1,4]. Glioblastoma multiforme (GBM) is a WHO grade IV astrocytoma and
67 is the most common, aggressive, fatal, highly vascularized, malignant primary brain tumor in adults.
68 Treatment options remain very limited, and it has a low survival rate of less than 1 year for many
69 patients and only about 5% survive beyond 5 years [1,3,5]. According to the most recent "central brain
70 tumor registry of the United States (CBTRUS) statistical report", the average annual age-adjusted
71 incidence rate of all malignant and non-malignant brain and other CNS tumors was 24.25 per 100,000
72 between 2014 and 2018. The total rate was greater in females than in males (26.95 versus 21.35 per
73 100,000). The most often occurring malignant brain and other CNS tumor was glioblastoma (14.3% of
74 all tumors and 49.1% of malignant tumors), was more prevalent in males while the most common
75 non-malignant tumor was meningioma (39.0% of all tumors and 54.5% of non-malignant tumors), was
76 more common in females [6].

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

80 limit our ability to predict efficacy and toxicity in humans. This is particularly evident with GBM with
81 no successful therapy that significantly improves survival since the introduction of temozolomide 20
82 years ago [1,3,5].
83 GBM is characterized by higher vascularization, significant cell heterogeneity, self-renewing cancer
84 stem cells and the interactions between tumor and microenvironment, all of which play an important
85 role in tumor growth (Figure 1) [8]. Tumour development, metastasis, angiogenesis, cytotoxicity
86 resistance, and immune cell modulation are all influenced by the tumour microenvironment (TME)
87 [9,10]. There is a urgent need for accessible GBM pre-clinical models and 3D cell culture is able to fill this
88 gap by providing more reliable models to study the correlation between TME, tumour reoccurrence
89 and therapy resistance.
90
91 Three dimensional (3D) cell cultures describes a wide range of *in vitro* cell culture technique used to
92 grow cells in three dimensions using an artificially created microenvironment. Cells in 3D cell culture
93 have physiological cell-cell and cell–extracellular matrix (ECM) component interactions which allow
94 cells to grow *in vitro* in a tumor microenvironment that closely resembles GBM *in vivo* conditions [9,11].
95 Tenascins, Fibronectin, Fibulin-3 and Hyaluronic acid are the primary components of the GBM ECM [12].
96 These ECM components can be employed in 3D cell culture to mimic the composition and porosity of
97 *in vivo* GBM ECM *in vitro* conditions to get better understanding of the therapeutic efficiency.

98 **A**



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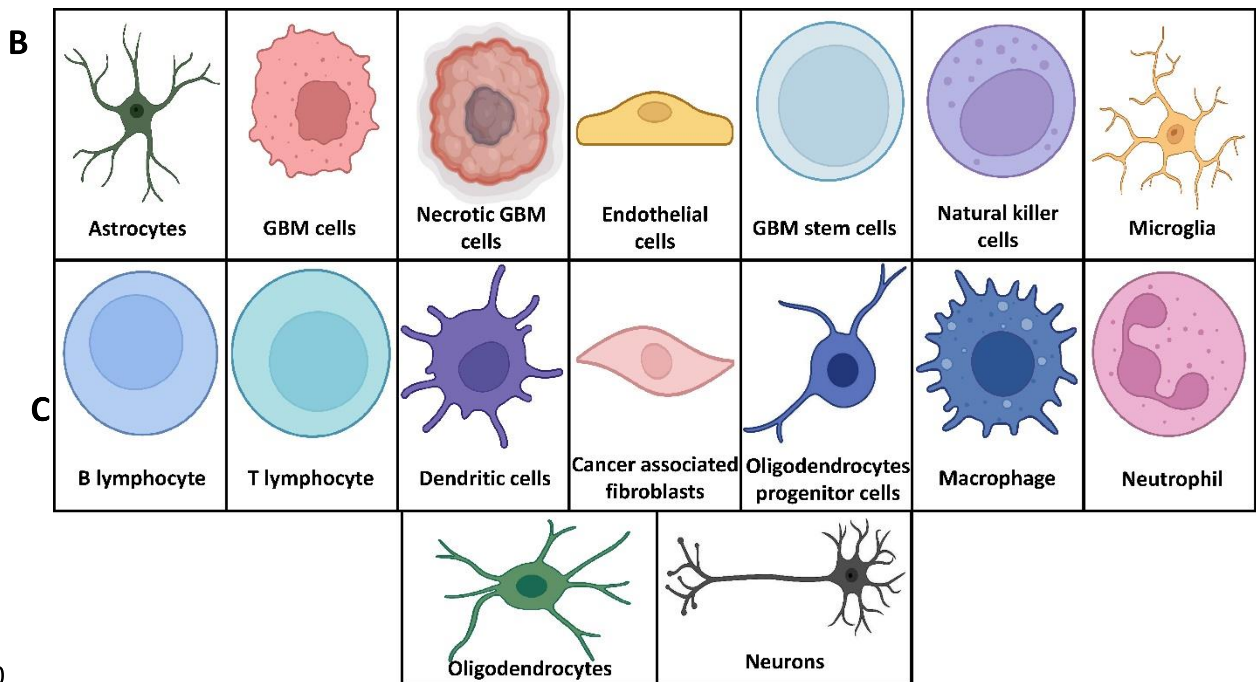

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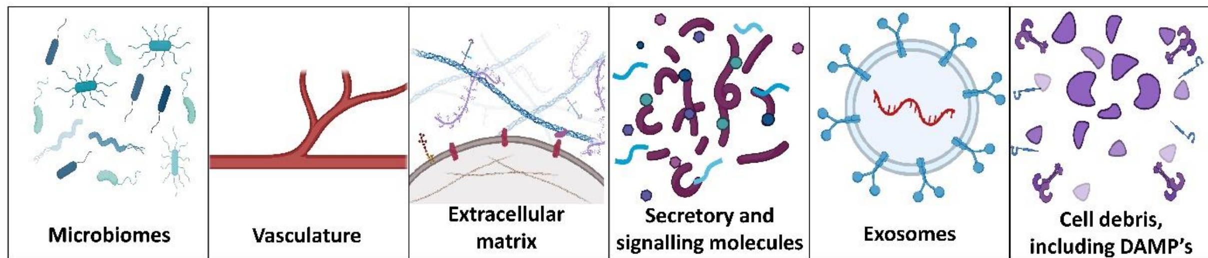


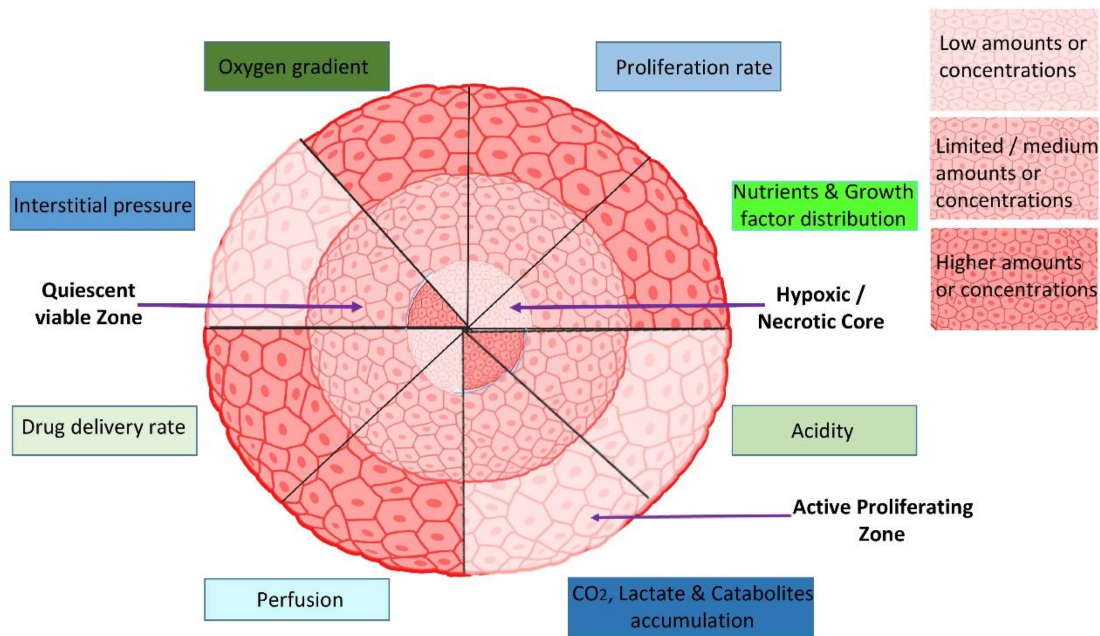
102

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

110

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

124 between integrins and growth factor receptors regulates downstream cell signaling as well as growth
125 factor induced biological activity, such as proliferation and invasion [9,13].
126
127 Brain tumors are surrounded and infiltrated by many noncancerous cells, including neurons,
128 astrocytes, microglia, cancer-associated fibroblasts, tumor-associated macrophages, glioblastoma
129 stem cells (GSCs) and endothelial cells, that provide both supporting and suppressive functions in the
130 TME (Figure 1) [17-19]. Cancer progression and drug response are heavily influenced by cellular
131 interactions in the TME [17,20,21]. 3D *in vitro* models can be utilized to simulate TME components and to
132 evaluate novel therapies [14,19].
133 Cells in a 3D spheroids have varying microenvironment conditions due to the non-homogeneous
134 vascular supply [22]. For example, regions of a tumour further from vasculature have restricted
135 oxygenation, nutrients and waste removal. 3D spheroid can possess a hypoxic (oxygen-deprived) core
136 resembling these TMEs found in solid tumours, with cells at the centre of sphere with relatively low
137 oxygen, glucose concentration and acidic extracellular pH due to accumulation of metabolic by-
138 products (Figure 2) [23,24]. The hypoxic cell population increase is proportional to the spheroid size also
139 it is highly resistant to chemotherapy and radiotherapy. The outer layer of spheroid, which is highly
140 exposed to medium and mainly composed of viable, proliferating cells. 3D spheroid has
141 heterogeneous cellular subpopulation such as actively proliferating, quiescent, hypoxic and necrotic
142 cells, which provides different cell proliferation zones, can be divided as proliferating zone, quiescent
143 viable zone and necrotic core / hypoxic core (Figure 2) [11,13,25].



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145

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

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

165

166 **Comparison of 2D and 3D cell culture**

167

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

178 3D cell culture can use to overcome these problems as cells are allowed to grow in any direction
179 without interacting with the surface, while maintaining physiological cell-cell and cell-extracellular
180 matrix interactions, more closely mimic the natural *in vivo* environment, shape, and cellular response
181 [16,30]. Cells in 3D cultures are not getting homogenous oxygen, nutrient and growth factors supply due
182 to their larger size and diffusion gradient (Figure 2) leading to all major TMEs represented including
183 proliferating, quiescent and necrotic stages found in an *in vivo* tumor (Figure 2) [25].

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

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

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

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

213

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

215 3D cell culture plays a vital role in drug development, while it is also capable of replacing both 2D cell
216 culture and animal trials. Initial testing stage of standard drug discovery begins with 2D cell culture,
217 followed by animal tests and clinical trials, which resulted 95% of trial failures during clinical trials due
218 to the insufficient prediction of the efficacy and toxicity in humans during pre-clinical studies [33,36].

219 3D cell cultures represent a simplified reductionist model. It highly transparent, reproducible, easy to
220 modelling the complex processes such as growth, invasiveness and toxicity, when compared to a
221 whole animal [30]. 3D cancer cell models are able to provide better understanding of *in vivo* cancer
222 therapeutic efficiency and also improve the efficacy of drug discovery, due to the clear understanding
223 the relation between cells and the ECM in which they interact [16,19]. This help to identify drugs/
224 therapeutic methods in early stages, which has better effects on cancer treatment and eliminating a
225 lot of unnecessary testing.

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

232

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

244 Animal testing is expensive and time consuming and they do not account for the whole intricacy of
245 tumor-microenvironment interactions [19]. Also, If animal is in pain or stress during the experiment, it
246 might change the biochemical, physiological and metabolic reactions, which can inaccurately depict
247 the effectiveness and side effects of drugs [9,16,30,40]. Humans and animal models have distinct
248 anatomical and physiological differences, the most apparent of which is size. The human brain is about
249 100 times greater in weight and more than 1,000 times larger in surface area and number of neurons,
250 when compared with mice. Thus, in the study of GBM, well known for its infiltration of the brain
251 parenchyma, important anatomical distinctions in the organ of origin impose potentially confounding
252 factors in preclinical investigation [37,41]. Preclinical modeling is complicated further by an increased
253 proportion of neocortical astrocytes, pericyte heterogeneity, and changes in vascular architecture
254 between humans and animal models [41].

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

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

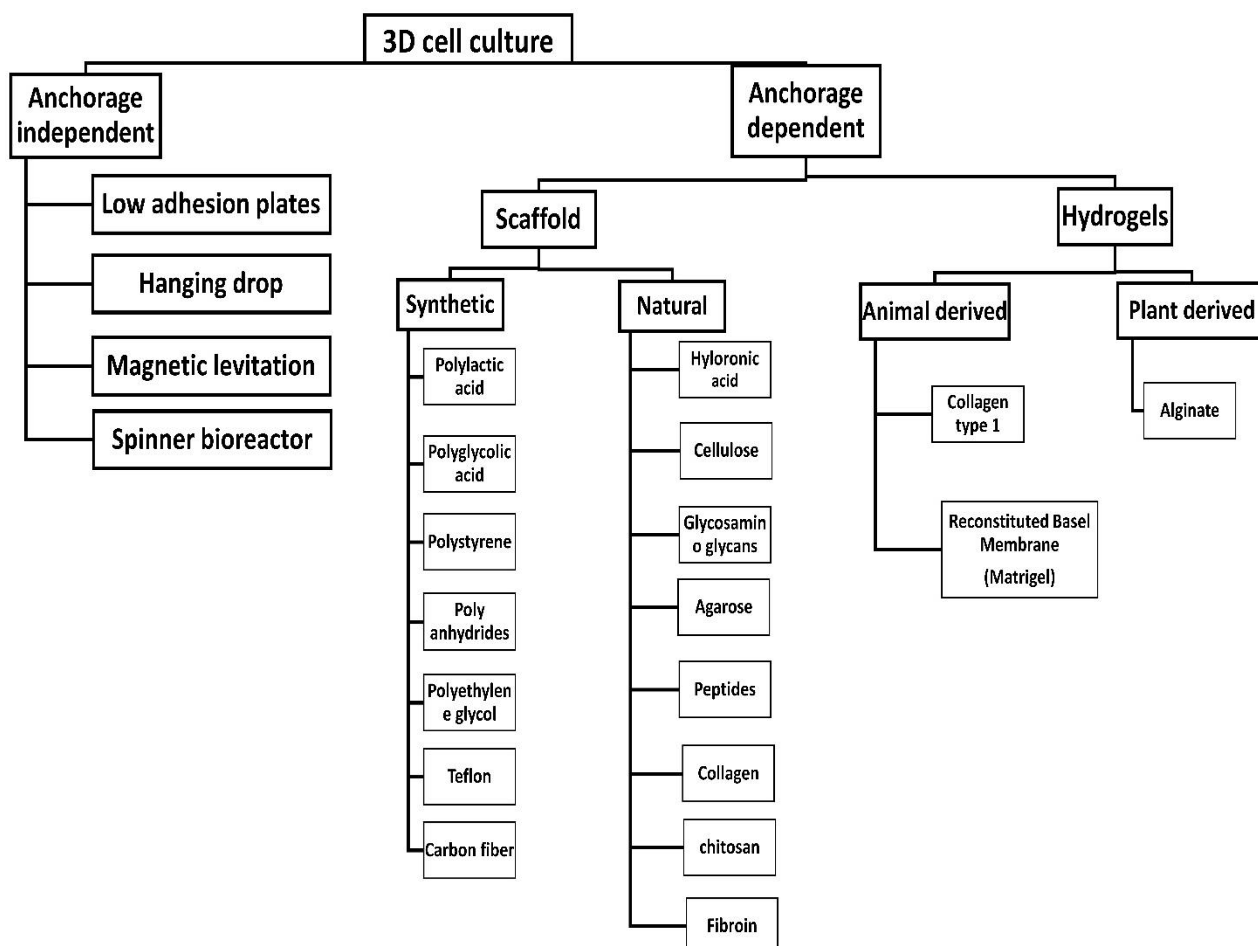
262 Many variables *in vivo* are uncontrollable, and their effects are often unknown due to the complexity
263 of organisms, whereas 3D cell culture allows for better control of variables by using a series of carefully
264 selected reductionist models [42]. The merits and demerits of 2D, 3D cell culture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

266 Current *in vitro* GBM treatment regimens fail to account for a large variety of factors such as brain's
267 unique extracellular matrix, circulatory systems, existence of resident and non-resident brain cells
268 inside the tumour, secreted factors and nutritional sources accessible for tumor metabolism [19]. The
269 main benefits of using 3D cell culture models for *in vitro* GBM treatment rather than animal testing
270 are include a wider selection of techniques, leading to better measurements of outcomes, better
271 control of variables, scalable testing, comparatively lower cost, avoidance of ethical issues and
272 reductionist approach to accurately model a specific feature of a disease, as opposed to animal
273 models, which are complex and often differ from human disease. It is also capable of simulating de
274 novo drug resistance [9]. Furthermore, juxtacrine signaling, in which molecules pass directly between
275 cells via gap junctions or other structures without being released into the extracellular environment,
276 requires 3D tumorsphere cell–cell interactions. These receptor and juxtacrine signaling components
277 alter a variety of intracellular signaling pathways, affecting how cancer cells react to their surroundings
278 [9,13]. The lack of vascular and immune system in 3D cell culture techniques is a drawback when
279 compared to animal models, that may be solved in the future by constructing advanced 3D models
280 utilizing specialized 3D techniques such as 3D printing [42]. Ultimately 3D brain cancer models can so
281 improve reproducibility and allow researching cellular and molecular pathways simpler to improve for
282 personalized medicine.

283

284 **Different types of 3D cell culture techniques and methods**

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



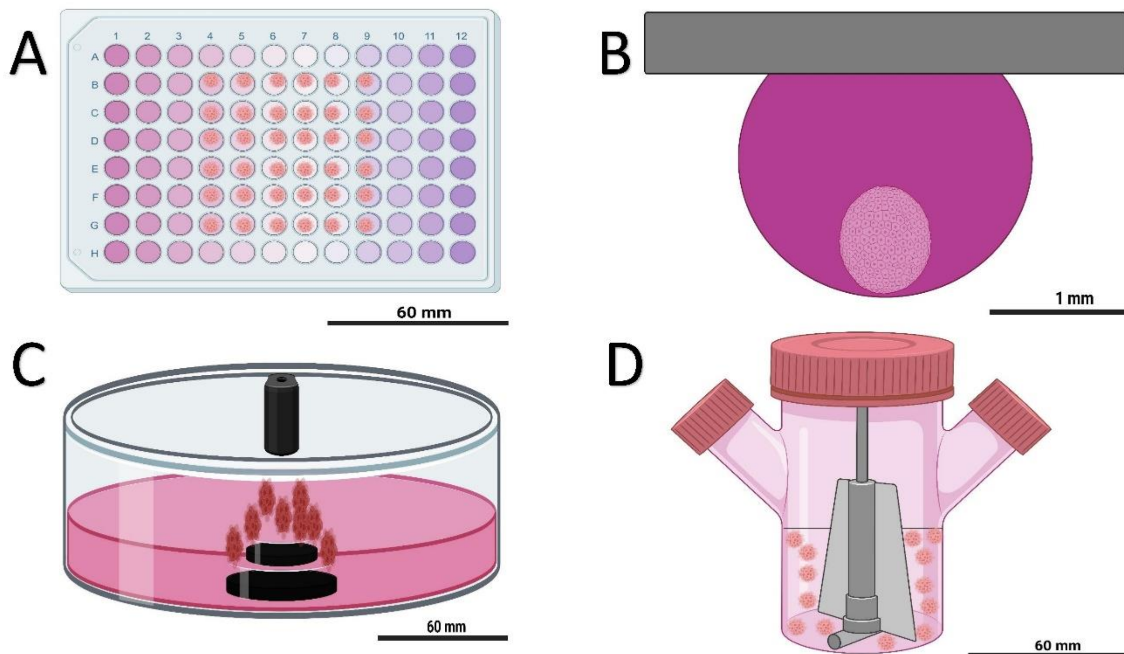
293

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

296

297 ***Anchorage independent (scaffold free)***

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



302

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

306

307 *Low adhesion plates*

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

323

324 *Hanging drop method*

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

333 enough to hold droplet by surface tension, after 3D spheroid generation it can be dispense by adding
334 extra drop of media in to the well and spheroid loaded to adjacent plate [16]. Micro-liquid adhesion with
335 substrate surface is greater than cellular weight; cells aggregate, proliferate, and grow in to spheroids
336 at liquid air interface. Recommended drop volume is 10-20 µl [48]. There are currently some
337 commercially available hanging drop plates on the market, such as Perfecta3D® and Gravity PLUSTM
338 [45]. Multicellular spheroids also can be create by co-suspending several cell types or else consecutive
339 addition of different cell types to form separate cell layers. The merits are: able to produce uniform
340 spheroid size, able to control size of spheroid by seeding density, homogenous spheroids and suitable
341 for high throughput testing, higher replicability, low cost and comfortable to handling. In the
342 disadvantages side, plates are highly expensive, medium change and different drug treatment at
343 different time points are impossible, not suitable for long term culture and also having small culture
344 volume and osmolarity of the droplet will rise due to medium evaporation [16,45,46,48]. Lara and colleagues
345 provided a thorough procedure for producing 3D glioma spheroids using hanging drop plate method
346 [51].

347

348 *Magnetic levitation*

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

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

362

363 *Spinner Bioreactor*

364 A spinner bioreactor (Figure 4D) has a container to hold cell suspension and impeller stirring
365 continuously to minimize the cell adhesion to the surface. Bioreactors are closed systems used to
366 strictly regulate factors such as dissolved oxygen, temperature, pH, and nutrients. Specific sensors
367 inside the bioreactor linked to control software to monitor nutrition and metabolite input and outflow
368 [33]. Continuous Liquid flow prevents cell adhesion contamination, time-consuming manual operations
369 and also uniformly distributes nutrition and oxygen to form 3D spheroids [33,46]. This method is simple,
370 able to mass production of spheroids and also suitable for long term culture [46]. While cells can be
371 damaged by collision between cells and bioreactor wells (exposure to high shear force) and require
372 specialized equipment's also difficult to obtain uniform spheroids [33,46,48].

373

374 ***Anchorage dependent (Scaffold Based)***

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

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

387

388 *Natural scaffolds*

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

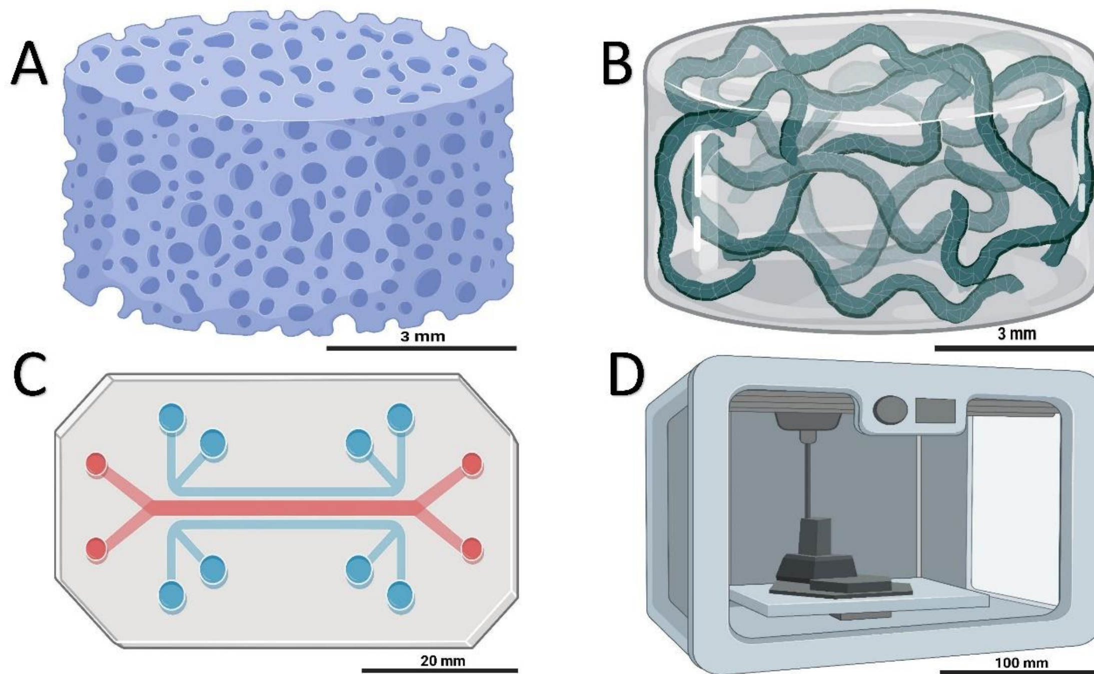
399 The advantages of using biological scaffolds are highly similar to the *in vivo* conditions, can control
400 similar composition/ elasticity /porosity to get better ECM presentation and also possible to combine
401 with ideal growth factors. Also it is able to improve biocompatibility, spatial distribution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

407

408 *Synthetic scaffolds*

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

427



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

432

433 *Anchorage dependent (Hydrogels)*

434 Hydrogels (Figure 5B) provide multi-layer formats by cross-linked hydrophilic polymer chains and cells
435 are embedded inside layers and able to grow to 3D spheroids providing cell-cell and cell-ECM
436 interactions [33,48], which has similar biochemical, structural and mechanical properties of an *in vivo*
437 tissue. Hydrogels are in a liquid format at room temperature which become a gel at 37 C incubation
438 [18]. It helps cells to mix uniformly into the gel-liquid and proliferate non-destructively during the
439 gelation process [48]. Mechanical strength, nutrition transport, topography, and degradation behaviours
440 can all be adjusted by using polymers with varying compositions, crosslinking density, and including
441 bioactive compounds [53]. Hydrogels are 3D matrices or porous scaffolds can be divided into synthetics
442 and natural hydrogels [33].

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

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

460 Advantages of using hydrogels for 3D cell culture includes controllable porosity, elasticity, variation in
461 stiffness, high water content, able to provide similar microenvironment and reproducibly, able to
462 provides rich network of ECM signals, ability to construct combining both synthetic and natural
463 materials and ability to couple with adhesion, proliferation, differentiation, and migration factors
464 [33,53,55]. While demerits including physically weaker, lack of vasculature, natural gels composition can
465 be inconstant and also lack of cross linked network for mechanical support 3D spheroid growth [48]. In
466 future, researchers can try to develop hydrogels using similar ECM components and composition in a
467 particular tissue / tumor site to get similar *in vivo* tumor microenvironment [48].

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

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

484

485 ***Microfluidic devices***

486

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

493

494 Microfluidics are classified into two types: flow-based channel microfluidics (CMF) and electric-based
495 digital microfluidics (DMF). Individual droplet manipulation, multistep processes, flexible electric-
496 automatic control, and the ability for point-of-care are all benefits of DMF over CMF [64]. The physical
497 barrier of microfluidic 3D cell culture system is composed of glass/silicon, polymers such as
498 polydimethylsiloxane (PDMS), polymethylmethacrylate (PMMA), polycarbonate (PC) and polystyrene
499 (PS). PDMS is the most often utilized substance due to biocompatibility, inexpensive, has good gas
500 permeability and transparent capability, however, scaling-up process is more difficult [53]. Simple
501 microfluidics devices are increasingly being fabricated and created by soft lithography techniques to
502 develop patterned environments that are reasonably easy to fabricate and compatible with the
503 majority of biological systems [16,56].
504 Microfluidics technique capable of continuous perfusion for faster spheroid formation, to produce
505 uniform size and shape spheroids for high-throughput screening, It allows patterning of cells and
506 extracellular environment to create co culturing cells in spatially controlled manner, generation of and
507 control signalling gradients, integration of perfusion, low reagent / sample consumption, which
508 significantly reduces costs in bioanalysis, real-time imaging and to constructing tissue-level and organ
509 level structures *in vitro* [16,18,46,50]. In the other hand disadvantage is it is highly expensive, hard to collect
510 cells for analysis, hard to scale-up, need complicated equipment and complexity [46,48,65].
511 Microfluidic devices are complex dynamic micro scale environments that simulate 3D *in vivo*
512 environments, such as a complex chemical gradient. Its micro scale dimensions are consistent with
513 those of numerous *in vivo* microstructures and environments [66]. Capillaries in the brain, for example,
514 ranging from 7-10 µm in diameter, with an average intercapillary distance of about 40 µm [67].
515 Microfluidic devices' versatility and simplicity of fabrication allow them to be used in a wide range of
516 applications in glioma research. These include migration studies, biomarker assessment, cell sorting
517 from tissue samples, and treatment effectiveness testing [68,69]. The time course for culture is heavily
518 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

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

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

559 3D printing can applied to develop GBM models with vascular channels to get better understanding
560 of six core and two emergent hallmarks underpin tumour development and metastasis [78]. Research
561 team developed of an integrated platform that allows for the generation of an *in vitro* 3D GBM model
562 with perfused vascular channels that allows for long-term culture and drug (TMZ) delivery [79]. Glioma
563 stem cells (GSCs) have been revealed in recent research to have a role in tumor vascularization by
564 secreting vascular endothelial growth factor (VEGF). Wang et al. (2018a) used 3D printing to create a
565 3D glioma model to investigate the vascularization potential of patient-derived CSCs [80]. Heinrich et al.
566 (2019) created a 3D-bioprinted mini-brain made up of GBM cells and macrophages to explore the
567 interaction between glioma CSCs and other non-tumor cells. The authors discovered that glioma cells
568 interact with macrophages and induce TAM polarization in patients' tissue [81].

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

580

581 **Advance TME models and applications**

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

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

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

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

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

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

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

650 Investigating **GBM / normal tissue interactions** are vital in brain cancer therapeutics hence, advanced
651 3D GBM co-culture models will be needed to develop, to explore the crosstalk and metabolic
652 interactions between glioma cells and the normal glial cells such as astrocytes, oligodendrocytes,
653 neurons and a range of normal resident brain cells. 3D cell culture also able to co culturing with
654 different cell types, including mixed populations of tumor cells and cancer associated fibroblasts (CAF),
655 to develop increasingly accurate *in vitro* models of disease and physiology [25]. The importance of
656 glioblastoma multiforme cellular interaction with endothelial cells can be studied with co culture
657 techniques to get proper understanding of the endothelial interaction on tumor progression for
658 identify novel therapeutic approaches [25,65]. Also by adding cells such as blood vessels, can use to
659 investigate interactions between blood vessels and cancer or how drug help to antiangiogenic effect
660 in cancer. Researchers examined available *in vivo* data to calculate the quantities and numerical ratios
661 of GBM and normal brain cells necessary to establish a complete and incomplete GBM resection dual
662 co-culture model. The results indicated that drug discovery utilizing this dual co-culture methodology
663 is feasible and provides steady and reliable drug testing outcomes [94].

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

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

688

689

690

691 **Challenges and future prospective**

692 3D cell culture, however, has proven it has the potential to completely change the way in which new
693 drug treatments are tested, diseases are modelled, stem cells are utilized, and organs are transplanted

694 [16,77]. The capacity to accurately simulate the intricacy of the TME is a major hurdle in developing
695 physiologically appropriate *in vitro* models for drug screening and cancer biology research. By co-
696 cultivating various cell types in a specified 3D matrix, custom-tailored ECM gels with specific amino-
697 acid sequences, more advanced pre-clinical models must develop with cell–ECM or cell–cell
698 interactions inside and between the TME [97]. Furthermore, combining diverse approaches, like as
699 organotypic cultures and organoids, with 3D bio-printing, might improve the investigation of cell
700 interactions in GBM [30,77]. In future to address this obstacle closely, researchers will develop Four-
701 dimensional (4D) bio printing, a next generation of bio fabrication technology, involving the use of
702 stimuli-responsive biomaterials that can be altered in a time-dependent manner (fourth dimension)
703 in an attempt to mimic the physiological activities of TME [56,75].

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

710 In future, Advancement in 3D cell culture will become feasible to construct entire 3D *in vitro* GB
711 organoids, which will eventually lead to personalized treatments for glioblastoma [29,55,98]. The inclusion
712 of patient-derived cells into standardized 3D tumor models will capture cancer heterogeneity [33], as
713 well as repair damaged organs using patient cells to avoid rejection from the immune components
714 [16,20]. Ultimately, 3D cell culture research has enormous potential as a cutting-edge frontier in
715 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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