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## Strategies for Developing Complex Multi-Component In Vitro Tumor Models: Highlights in Glioblastoma

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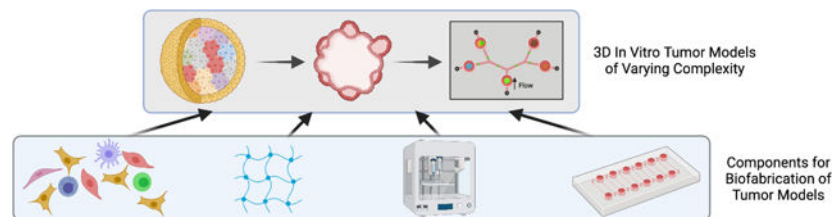
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### Abstract

In recent years, many research groups have begun to utilize bioengineered *in vitro* models of cancer to study mechanisms of disease progression, test drug candidates, and develop platforms to advance personalized drug treatment options. Due to advances in cell and tissue engineering over the last few decades, there are now a myriad of tools that can be used to create such *in vitro* systems. In this review, we describe the considerations one must take when developing model systems that accurately mimic the *in vivo* tumor microenvironment (TME) and can be used to answer specific scientific questions. We will summarize the importance of cell sourcing in models with one or multiple cell types and outline the importance of choosing biomaterials that accurately mimic the native extracellular matrix (ECM) of the tumor or tissue that is being modeled. We then provide examples of how these two components can be used in concert in a variety of model form factors and conclude by discussing how biofabrication techniques such as bioprinting and organ-on-a-chip fabrication can be used to create highly reproducible complex *in vitro* models. Since this topic has a broad range of applications, we use the final section of the review to dive deeper into one type of cancer, glioblastoma, to illustrate how these components come together to further our knowledge of cancer biology and move us closer to developing novel drugs and systems that improve patient outcomes.

### Graphical Abstract



**Conflicts of Interest:** The authors have no conflicts of interest to disclose.

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## 1. Introduction

Improvements in cell culture techniques and tissue engineering technologies have led to increased acceptance of *in vitro* models of cancer for use in basic research on disease initiation and progression and the development and testing of novel therapeutics. Compared to animal models, these *in vitro* systems have traditionally been considered relatively simplistic. For many years, researchers have used simple cell culture models to study the mechanisms of cancer and to evaluate drug compounds. However, the lack of crucial components of the tumor microenvironment (TME), including the extracellular matrix (ECM) and a myriad of tumor-adjacent and tumor-infiltrating cell types, limits the clinical and translational utility of these systems.<sup>1,2</sup> The majority of 3D tumor models are an improvement over these 2D cultures as they reproduce many of the complex cell-cell, and in some cases cell-matrix, interactions that occur *in vivo*. Advances in biomaterials development, biofabrication, and cellular engineering have promoted the development of more complex *in vitro* systems that more accurately mimic the native TME.<sup>3,4</sup> Furthermore, realization of organ-on-a-chip technology not only allows us to study mechanisms and phenomenon such as invasion and migration within a single tumor, but can also be used to create “multi-organ” systems that can be used to study phenomena such as metastasis and off target drug metabolism.<sup>5,6</sup> Techniques such as bioprinting now allow scientists to manufacture complex tissue structures outside the body with high levels of reproducibility.<sup>4</sup> With recent advances in stem cell culture, we are now able to reliably generate many human cell types found in the TME and are moving the field closer to developing personalized cancer models.<sup>7,8</sup> The development and implementation of these new techniques have led to new and exciting discoveries in the field of cancer biology. Yet, it can often be a daunting task to navigate these systems and choose the one that is appropriate for a particular research question.

Our group is one of many groups utilizing these techniques to generate *in vitro* cancer models.<sup>9–12</sup> In general, when we look to investigate a novel research question or develop a new model for a given cancer environment, we use the following workflow, which is represented in further detail in Figure 1. We first determine what cellular and extracellular matrix components are present in the tumor microenvironment (TME) of the tumor we would like to model. These include elements such as essential stromal cells, various tumor subpopulations, ECM proteins, and mechanical properties such as fluid flow or matrix stiffness. We then decide which components are most essential to answer our specific question and choose ECM-based materials needed to replicate those conditions. Finally, these components are combined in a specified model form factor. In some cases, biofabrication techniques are used to automate the process or create more complex systems. It is important to emphasize that when deciding what variables to incorporate and which form factor to use, complexity is not always better. In fact, there are many instances where simple systems have been used successfully. However, it has become increasingly clear that 2D cell culture of both cell lines and patient-derived tumor cells does not accurately mimic the patient tumor,<sup>1,2</sup> so most of the models, examples, and form factors discussed herein pertain to 3D *in vitro* models.

In this review, we will outline this approach and provide examples illustrating how a variety of tissue engineering and biofabrication technologies have been used to create multi-component *in vitro* models of cancer. We will conclude by describing how these tools have been used to study glioblastoma (GBM) in order to demonstrate the utility of these technologies in a specific cancer.

## 2. Choosing Essential Model Components: Cells and ECM

### 2.1. Cell source and cellular composition

When creating an *in vitro* cancer model, one must first consider the cell source or sources that will be used. For many decades, cancer cell lines were the preferred option for many researchers because they are easy to maintain in culture and can be propagated almost indefinitely. Since HeLa cells, cervical carcinoma cells removed from the tumor of Henrietta Lacks, were first cultured in 1951,<sup>13</sup> hundreds of cell lines derived from a variety of cancers have been produced and characterized.<sup>14</sup> These cells have proved invaluable in the study of cancer mechanisms and the development of new treatments.<sup>15</sup> However, studies using cancer cell lines have been the subject of extensive criticism because many common cell lines have been contaminated with other cell sources.<sup>16,17</sup> These problems can largely be addressed with routine genomic testing of cell lines from distributors and research labs that use the cells. More recently, many critics of cell lines have also argued that they are not accurate phenotypic and genetic representations of the *in vivo* tumor.<sup>18</sup> These concerns are valid as studies have specifically shown long term culture on plastic can lead to genetic changes in cells and that patient derived cells are better indicators of a patient specific drug response.<sup>19</sup> However, advances in genomics and proteomics and recent efforts such as the Cancer Cell Line Encyclopedia (CCLE) have made characterizing cancer cell lines much easier and more accessible.<sup>14,20</sup> With these data, scientists are better able to evaluate possible clinical implications of a study using cell lines. For example, we recently used a set of four GBM cell lines paired with a hyaluronic acid-based ECM hydrogel to evaluate how environmental elastic modulus dynamically influenced cell proliferation.<sup>21</sup> In the context of colorectal cancer, there have been numerous studies conducted in recent years developing a “multi-omics” profile of various commonly colorectal cancer cell lines.<sup>22–24</sup> In breast cancer research, several cell lines have been used. There have been multiple efforts in recent years to gain a comprehensive understanding of their genomic and phenotypic signatures to insure that these lines are used appropriately.<sup>25–27</sup> One particular study evaluated the utility of certain breast cancer cell lines for use in metastasis studies finding that some more closely matched *in vivo* signature than others.<sup>28</sup> These studies have indicated that cell lines can be used to predict drug response when proteomic and genetic profiles of the cell lines are compared to that of patient tumors. This is certainly not always the case for all cancers, such as GBM, which we will address in a future section, for which predictive *in vitro* studies and omics methodologies have failed to translate into significant improvements in patients.<sup>29–31</sup>

Especially in the context of developing personalized treatments for cancer patients, the use of cells obtained directly from patient tumor biopsies in basic and translational research applications has continued to grow.<sup>32,33</sup> However, it can be difficult to culture cancer cells from solid tumors in traditional 2D cultures, with successful culture rates reported

in around 25% of samples.<sup>32,33</sup> More recently, 3D culture techniques have expanded greatly. Higher success rates (60–90%) have been achieved for a variety of tissues using 3D cancer organoids which in this paper we will define as models in which patient derived cancer cells are grown in a 3D culture environment containing a supporting ECM hydrogel<sup>12,34,35</sup>. These 3D models will be addressed in greater detail later. Many studies have shown that these organoid cultures of primary cells replicate *in vivo* response to treatment.<sup>36</sup> For example, our group has demonstrated this using mesothelioma and melanoma patient cells grown in organoids, while other groups have demonstrated similar outcomes in gastrointestinal tumor organoids.<sup>37–39</sup> These platforms, while slightly more time consuming to generate, have proven extremely effective at replicating *in vivo* tumor response when treated with radiation, standard chemotherapeutics, and most recently, immunotherapies.<sup>20,40,41</sup> the use of patient derived *in vitro* cancer models will continue to grow, and they will serve as essential tools to aid in the development and optimization of more targeted personalized therapies.

The cellular make-up of the TME is quite heterogenous, and it has become increasingly clear that tumor cells are not alone responsible for cancer initiation, progression, metastasis, and drug resistance. The stromal cells present in and around the tumor interact with cancerous cells through a variety of mechanisms including the secretion of growth factors and other bioactive molecules and ECM remodeling.<sup>42,43</sup> These cells include cancer associated fibroblasts (CAFs), immune cells, endothelial cells, and pericytes. Inclusion of these cells in *in vitro* models, especially in 3D models which we will discuss in greater detail in the next section, has shown great promise in improving the accuracy of cancer models.<sup>8</sup> For example, support cells, such as hepatic stellate cells, added to 3D *in vitro* models induce fibrotic changes in the ECM impacting tumor cell phenotype and reaction to chemotherapeutics.<sup>44</sup> Including CAFs in 3D culture models has allowed researchers to investigate their impact on tumor cell migration and invasion.<sup>45</sup> There are several research groups that have also used a similar approach and found that by adding CAFs to their model, the model more accurately mimics the native TME and serves a better predictor of *in vivo* drug response.<sup>46</sup> The Tavana group has published a series of studies in which they used a high throughput co-culture model to investigate how the interactions between triple negative breast cancer cells and CXCL-12 secreting fibroblasts promoted cancer invasion and drug resistance.<sup>47–50</sup> Most of the studies in this area have been conducted using cell lines, but there has been a recent push to start using patient-derived CAFs and other cells within these cancer models. *In vitro* models using patient-derived CAFs have been used to show that CAFs support the survival of cancer cells via the production of essential signaling factors and metabolites in a variety of cancers.<sup>51,52</sup> Chung *et al.* created patient-derived 3D constructs containing tumor cells and CAFs isolated from a primary and a metastatic breast cancer tumor and showed that brain metastatic CAFs are a potential therapeutic target to prevent metastasis.<sup>53</sup> A more thorough analysis of heterogeneity in engineered cancer models can be found in a previous review from our lab.<sup>54</sup>

There is also increased interest in adding other cells found in the TME to *in vitro* systems including immune cells. *In vitro* human models serve as great tools to test efficacy of novel immune therapies and study the impact of immune cell infiltration versus tumor growth.<sup>41,55</sup> While maintaining and expanding many of these cells, such as T-cells, can be difficult, *in vitro* models provide a distinct advantage over more complicated animal models because all

components are human derived, and they allow for the isolation of specific variables. In one model, researchers used 3D spheroid cultures to show pancreatic tumor cells and fibroblasts induce M2 macrophage polarization which suppresses T-cell proliferation.<sup>56</sup> Importantly, this same study also indicated that the level of activation was cell line dependent, again emphasizing the need for patient specific assays.<sup>56</sup> Some types of immune cells are particularly difficult to grow *in vitro*, but recent efforts have developed systems in which patient derived tumor organoids that contain native immune cells.<sup>38,57</sup> Another recent study showed that tumor-reactive T cells can be enriched *in vitro* by patient tumor cells cultured in 3D with peripheral blood lymphocytes.<sup>58</sup> This demonstrates utility of *in vitro* models, simple and complex, for mechanistic studies investigating tumor-immune interactions and clinical applications such as personalized medicine. Of course, to completely model tumor-immune interactions, one must include an active endothelial barrier including pericytes and endothelial cells. Advances in microfluidics, which we will discuss later, have allowed for the inclusion of these cell types in physiologically relevant systems to study processes such as angiogenesis, immune infiltration, and metastasis.<sup>59–61</sup>

Determining the ideal cell populations for a given *in vitro* tumor model is an important decision. With recent advances through large scale omics studies, cell lines can provide useful information on disease mechanisms and are easy to produce. While the inclusion of multiple cell types can help make these models more physiologically relevant, extended culture on 2D plastic prevents cancer cell lines from mimicking the native tumor cells.<sup>2</sup> Patient cells are more difficult to grow, but they are a better option for personalized medicine applications, and when used in large scale studies, can provide invaluable mechanistic data about cancer biology that is impossible to achieve completely using cell lines. While not discussed in depth here, there has also been a recent push to start using induced pluripotent stem cell derived cell populations to improve model efficacy and create even better patient specific models.<sup>62</sup> These cells can be combined in a variety of 3D systems to model different cancer mechanisms.

## 2.2. Materials to model the tumor microenvironment

The ECM provides both a structure for cells to grow and interact but is also intimately involved in cell signaling and tumor behavior and progression. Compared to healthy tissue microenvironments, the TME often manifests with significantly altered expression level ratios of ECM components, including collagens, hyaluronic acid, fibronectin, and laminins.<sup>63</sup> This often leads to increased tissue stiffness and ECM density, as well as differences in composition, all of which result in altered matrix-to-cell signaling.<sup>64,65</sup> The resident stromal cells within the tumor, notably CAFs, play a major role in remodeling the ECM, often spurred on by inflammation caused by both immune and tumor cells.<sup>8,66</sup> Perhaps most importantly, changes in the ECM composition of tumors have been shown to impact treatment efficacy by physically shielding cells from therapies and initiating processes such as epithelial to mesenchymal transition (EMT) and metastasis.<sup>67,68</sup> To create more accurate *in vitro* cancer models and study the ways in which the ECM affects cancer mechanisms and drug sensitivity, researchers use a variety of biomaterials in 3D models mimic physical alterations to the TME.

The same porous scaffolds that are used in many other areas of tissue engineering and regenerative medicine are applicable to *in vitro* cancer modelling.<sup>69–71</sup> Generally, these materials are broken into 2 different categories: natural and synthetic. Natural materials are derived from polysaccharides or proteins purified from human tissue or produced by other biological processes. Synthetic hydrogels are made from polymers that are synthesized in the lab. Both natural and synthetic materials are often chemically modified to form a crosslinked network.<sup>72</sup> Each system has their own advantages and disadvantages. For one, synthetic materials allow for greater control over physical and mechanical properties since the molecular weight of the components and crosslinking density can be tightly controlled. However, cells cannot always recognize and remodel the network. Integrin binding sequences and degradable crosslinkers have been added to these synthetic networks to increase cell binding and matrix remodeling.<sup>73,74</sup> However, many cell-ECM interactions rely on specific protein conformations can be difficult to completely mimic with synthetic materials. Naturally-derived ECM biomaterials are comprised of one or more polysaccharides and proteins that occur natively in the extracellular environment which provide structure or can promote cell adhesion and cell signaling. However, these materials are not always completely defined making it difficult to control the chemical and mechanical properties of the network.<sup>75</sup> For example, Matrigel, a commonly used matrix composed mainly of collagen IV, laminin, and entactin, contains an undefined array of growth factors and cytokines that enable spontaneous formation of acini or ductile epithelial structures. This unknown component combined with batch-to-batch variabilities act as confounding variables that cannot always be accounted for.<sup>76</sup>

Natural materials such as collagen, Matrigel, or ECM from decellularized tissues were used to create the first *in vitro* 3D cancer models.<sup>77,78</sup> These were effective for early *in vitro* drug studies because most cell types are able to actively remodel these environments and the stiffness can be modulated to a certain degree by simply altering the protein concentration. However, the ECM composition cannot be modulated. Therefore, many groups have used modified versions of these natural materials which has allowed them to design systems with specific mechanical properties and ECM binding and signaling molecules.<sup>72</sup> Our group has used a modular hydrogel system composed of hyaluronic acid, gelatin, and collagen to create models of a variety of different cancers.<sup>9,12,79,80</sup> The composition of these hydrogels can be tuned based on the tissue type. For example, our models of brain tumors do not contain collagen I as it is not present in large quantities in the brain.<sup>21</sup> Recently, we have experimented with tuning the relative levels of synthetically modified fibronectin and laminin to further customize the TME, while keeping the system well-defined – an important characteristic for translation to clinically oriented applications. The stiffness of HA hydrogels can also be manipulated to match the desired tissue or study how changes affect cell behavior.<sup>81,82</sup> Chemical modifications to HA and other materials also allows for the distribution and patterning of chemical factors to form gradients across the engineered tissue which can be used to study a variety of signaling cascades in cancer.<sup>83</sup> Synthetic materials have been used in a similar manner, with researchers using them to investigate the impact of various properties of the ECM on cancer cell behavior and treatment response. In one major study, the stiffness, degradability, and ECM components in PEG hydrogels were manipulated to investigate how they affected the formation of intestinal stem cell

and colorectal cancer organoids.<sup>84</sup> It has also been shown that creating an engineered PEG hydrogel system that matches the properties of ovarian cancer accurately recapitulates *in vivo* drug response showing the importance of creating models with mechanical properties that mimic native tissue.<sup>85</sup> PEG hydrogels have also been used to study the relationship between matrix degradation on cancer cell growth. For example, PEG hydrogel cultures were used to demonstrate that treatment melanoma cells with BRAF protein kinase inhibitor leads to increased MMP mediated degradation and migration.<sup>86</sup> The modular nature of these hydrogel systems provides control over the environment allowing scientists to design systems for their specific tissue and experiment.

When designing a model, it is important to consider the composition of both the healthy and cancerous ECM. One must also consider the mechanical properties of the materials. It is likely that there are multiple options that could be used to produce a model to answer a specific set of questions. In that case, the scientists should take factors such as familiarity with the material, ease of production, cost, and ease of use into consideration. Once a subset of materials has been chosen, the scientific question that is being asked, the model system that is being used, and the ways in which that model will be fabricated will all impact the materials and the respective chemical manipulations that are needed.

### 3. Tumor model form factors

As previously stated, traditional 2D cell culture leads to genetic and phenotypic changes in cells and fails to mimic the complex 3-dimensional nature of the tumor-stroma-ECM interactions that occur within every solid tumor. As 3D culture has become more common, researchers now have many different model platforms at their disposal when moving from 2D culture to 3D systems. Each platform has its own advantages and disadvantages and are optimized to address specific scientific questions. Many of these systems were previously reviewed by Katt *et al.* in 2016.<sup>87</sup> Here we describe these form factors, along with others, and provide some more recent examples from the literature. The form factors are also highlighted in Figure 2.

The most basic 3D model system is the spheroid, or an aggregate of cells. Traditionally, spheroids are produced using the hanging drop method where cells form aggregates in a suspended drop of fluid. The development of ultra-low attachment round bottom plates has made the process much simpler and replicable.<sup>88</sup> The 3D structure of the spheroid replicates the cell-cell interactions seen *in vivo*. Early studies used spheroids to study cancer growth, and it was quickly revealed that these models were better representations of tumor biology than 2D cultures.<sup>2,87,89,90</sup> For example, spheroid culture of colorectal and ovarian cancer cells produces higher levels of E-cadherin indicating that there are more physiologically relevant cell-cell interactions in these 3D cultures.<sup>91,92</sup> However due to a lack of extracellular space to allow for sufficient diffusion kinetics, spheroids often develop a hypoxic core highlighting that these structures are limited in size.<sup>87,93</sup> However, in some cases, this scenario can be leveraged to model the necrotic core often observed in solid tumors, with one study showing that the induction of a necrotic core in colorectal cancer spheroids better replicated *in vivo* drug response.<sup>94</sup> Homogenous tumor spheroids have now been widely implemented for use in larger scale drug studies.<sup>93,95,96</sup> Since the cells grown

in spheroids secrete their own ECM, they can also be used to study ECM production and remodeling.<sup>97</sup> However, spheroids do have some limitations. They are limited in size, there is little control over newly synthesized ECM, and there are often important cell types missing. It has also been observed that some cells are not capable of forming spheroids limiting the utility of the system. For example, in our team's work with defined ECM hydrogel-based tumor organoids, we found that while we could easily encapsulate many patient-derived tumor cells in ECM hydrogels, thus forming this variety of organoid construct, Side experiments showed that many tumor types or grades did not readily form spheroids making this hydrogel based system advantageous for modeling certain tissue types.<sup>12,37,38,98,99</sup>

More complex 3D cultures have been created to address some of the problems with cancer spheroids. In these improved models, cells are suspended in a 3D hydrogel matrix as a single cell suspension, aggregates, or resected tissue chunks. Traditionally, these models were produced using Matrigel. At this point, we should note that over the last decade the term organoid has evolved, and has been used in a variety of different contexts to describe 3D culture models.<sup>12,37,38,98,100–106</sup> As we previously mentioned, we primarily use the term “cancer organoid” or “tumor organoid” to describe models in which patient derived cancer cells are grown in a 3D culture environment containing a supporting ECM hydrogel. However, we and many other laboratories have published studies using the “organoid” nomenclature to also describe cell-matrix constructs in microfluidic devices – both primary cell and cell line-derived – as well as spheroids or hydrogel-based constructs containing multiple combinations of cell lines, cell lines and primary cells, and primary cells and induced pluripotent stem cell-derived differentiated cells. The driving feature that qualifies the term “organoid” being used is not the cellular makeup, but if advanced biological composition, physiology, and function can be accurately modeled using these platforms. Cells grown in organoids are able to remodel the matrix and produce a self-organized structure that can resemble the *in vivo* tumor, especially if other cell types such as CAFs are included.<sup>44,107</sup> However as previously mentioned, the composition of Matrigel is somewhat undefined and when it is used, the researcher has limited control of the ECM composition of the tumor organoid.<sup>76</sup> Therefore, many other groups have moved towards using more defined ECM hydrogels to create their cancer organoids.<sup>9,12,79,80</sup> As previously discussed, the modular nature of these materials allows for precise manipulation of the ECM composition. By surrounding the a spheroid, tissue chunk, or cellularized hydrogel construct with an acellular hydrogel, cell migration can be studied in 3 dimensions.<sup>108</sup> These ECM embedded structures serve as useful tools to continue to investigate mechanisms of cancer growth and produce more consistent platforms that can be used for testing drugs.

Invasion and migration can also be studied using a transwell based system (Figure 2d). These model systems have two distinct compartments, separated by a porous membrane. Therefore, it is relatively straightforward to create chemical or physical gradients across the membrane. The simplest of the transwell models consist simply of cells grown on one side of the semi-permeable membrane. When exposed to the given gradient, the cells may be stimulated to migrate through the pores to the other side.<sup>87,109–111</sup> Movement of immune cells grown in suspension, such as T-cells or natural killer cells, can also be tested using a similar set-up.<sup>87,112–114</sup> In many cases, the addition of an endothelial barrier to the transwell



membrane is used to mimic the vascular endothelium. These models can be used to more accurately test factors that stimulate immune cell infiltration and cancer metastasis.<sup>115,116</sup> The addition of a hydrogel on top of the transwell membrane can be used to more accurately simulate invasion of cancer cells, immune cells, or stromal cells through the ECM.<sup>117</sup> These systems can be engineered to mimic the native tissue ECM or designed to investigate the effect of certain ECM molecules on cellular motility. As mentioned before, advances in hydrogel biomaterials engineering now allow for researchers to pattern various growth factors and proteins to form gradients in hydrogels which again could be used to investigate the mechanisms of invasion within these systems.<sup>83</sup> Transwells have many valuable uses, but they are limited by their geometry and size and, because of the presence of a relatively stiff plastic membrane, do not always accurately mimic the physiological environment.

Over the past decade or so, the use of microfluidics, or organ-on-a-chip, platforms has increased dramatically. In general, organ-on-a-chip platforms have grown because they are able to produce features of the *in vivo* tissue environment which can be reproduced in the simpler models previously discussed.<sup>118–120</sup> These conditions include fluid induced shear stresses, interstitial fluid flow, hydrostatic pressure, and controlled tissue deformation.<sup>5</sup> Many microfluidic devices have been used to investigate similar problems discussed before including cell migration. These devices also consist of narrow, defined channels through which cell migration is driven by a chemo attractive, mechanical, or electrical gradients.<sup>121–124</sup> By using a microfluidic device instead of an organoid or transwells based model, researchers have also been able to introduce other variables such as oxygen gradients and interstitial fluid flow.<sup>125,126</sup> Analysis on microfluidics in real-time is also quite easy as most devices are optically transparent and can be imaged live. A variety of sensors have also been incorporated into these devices to take real time measurements of metrics such as electrical activity, protein concentrations, and mechanical forces.<sup>127,128</sup>

Microfluidics and organ-on-chip have also been used to model processes that require flow such as metastasis. For example, our group has generated microfluidic devices with flow in which circulating tumor cells from a central cancer organoid exit, enter fluid flow, and invade distal tissue hydrogel constructs (Figure 2g).<sup>11,129</sup> This model and ones like it can be used to evaluate metastatic properties of a tumor and study tumor cell preferential homing to target tissue sites. Similar systems have also been designed to study premetastatic niche formation by evaluating the effect of cancer cells on matrix remodeling in a distal tissue hydrogel (with or without cells) in a separate chamber of the device.<sup>61,130,131</sup> Increasing complexity further, many endothelialized vessel structures have been created within microfluidic models. These vascular models are formed in a few different ways. Some systems, namely those pioneered by the Kamm and George labs, consist of endothelial cells seeded with in an ECM matrix, often collagen I or Matrigel, which then spontaneously form an interconnected network of perusable vessels.<sup>132–134</sup> Other systems have used engineered vessels formed on 2D membranes<sup>135</sup> or in ECM hydrogels via molding with a rod or needle<sup>136</sup>, viscous finger patterning<sup>137</sup>, or the use of sacrificial hydrogels.<sup>138</sup> The addition of the endothelial layer has been used to study immune cell infiltration and, of course, metastasis. For example, a self-assembling vascular model was used to investigate differences in breast cancer metastasis in different tissue types including bone and smooth muscle.<sup>139</sup> A similar self-assembling vascular model was

also used to investigate the relationship between M1 and M2 macrophages and cancer cell proliferation and tumor induced angiogenesis.<sup>60</sup> Nguyen *et al.* used a microfluidic vessel model consisting of 2 hollow lumen to study the mechanisms involved in endothelial ablation in pancreatic ductal adenocarcinoma.<sup>140</sup> The differing devices used in these studies highlight how different methods for creating mimetic vasculature are used and implemented according to the specific problem at hand. Other more specialized models of tissue interfaces have been created such as lung-on-a-chip models with an air liquid interface to study lung cancer.<sup>141</sup> There have also been several microfluidic models of the blood brain barrier (BBB) that are currently being adapted to study GBM which we will expand on later.<sup>142–144</sup>

When choosing which model format to use to investigate a given problem, it is important to consider whether the most complicated microfluidic device is necessary. As our group and many others have shown, spheroids or cancer organoids that can be produced in extremely large numbers with great reproducibility might be the best choice for a multi-drug screen or rapid personalized medicine applications. Microfluidic models may be necessary to investigate more complex questions such as factors that lead to ECM remodeling in the premetastatic niche or mechanisms of intravasation and extravasation in metastatic tumor models. A comprehensive understanding of the pros, cons, and previous uses of various biomaterials and model systems will inform the construction of a system that is problem specific. This will save time and money and produce elegant, streamlined results that can further add to the growing library of evidence supporting the effectiveness and utility of using *in vitro* models to study cancer.

#### 4. Biofabrication Techniques to create *in vitro* models

The ways in which an *in vitro* model is fabricated can be just as important as the cells, the biomaterial, and the form factor chosen. Recent advances in biofabrication, or the processes involved in engineering with purpose 3D biological constructs through the use of cells, biomaterials, and technologies such as bioprinting, now give researchers even more flexibility and make producing 3D models more efficient.<sup>145</sup> Informed use of these novel biofabrication tools can improve the model by increasing batch to batch consistency, facilitate high throughput screening of novel drug compounds, and allow for the production of models that better reproduce complex tissue structures. In the space of *in vitro* cancer modeling, bioprinting and organ-on-a-chip fabrication techniques are of particular interest. Streamlining both technologies making them accessible and affordable has the potential to increase implementation of complex *in vitro* models for research, development and production of novel therapeutics, and rapid personalized medicine.

##### 4.1. Bioprinting

Bioprinting is the use of additive manufacturing techniques to produce biocompatible, and sometimes cellularized, 3D structures.<sup>146–149</sup> Bioprinting technologies have improved significantly over the past 2 decades leading to greater ease of use and printed structures with higher resolution and complexity.<sup>150</sup> Bioprinters printers were initially extremely expensive limiting the number of laboratories that could afford to develop and utilize the technology. In part because of the “maker revolution”, additive manufacturing tools have

become much more affordable.<sup>75,151</sup> Likewise, advances in biomaterial bioink technologies has enabled the use of biomimetic hydrogels as bioinks in bioprinting while better simulating native ECM.<sup>75,152</sup> While not ubiquitous in the field of cancer engineering, more laboratories have embraced bioprinting because it allows for increased throughput of tumor model fabrication.<sup>70,104,153</sup>

3D bioprinting hardware can be broken down into 3 main categories: inkjet, extrusion, and stereolithography.<sup>154</sup> Many of the first studies using bioprinting in the early 2000's made use of commercial inkjet printers that were modified to dispense simple ECM materials and cell-laden bioinks with mediocre resolution.<sup>155,156</sup> Conversely, inkjet printers operate by ejecting material through the printhead via thermal, mechanical, or piezo electrical processes producing small droplets.<sup>157</sup> These processes are rapid and produce relatively high resolution features, but the ejection process can place high levels of stress on the cells.<sup>158</sup> The hardware is also relatively inexpensive making the process accessible. Selection of a bioink for inkjet printing can be difficult because the material needs to have a low viscosity so that it can pass through the small printheads. This makes the formation of complex 3D structures difficult because most low viscosity materials fail to hold their shape post extrusion. Utilizing materials that can be crosslinked using UV light, changes in temperature, or changes in pH can address some of these problems.<sup>158,159</sup> In the context of cancer modeling, inkjet printing has been used a variety of different systems. In one study, HeLa cells suspended in collagen matrix were printed on top of a layer fibroblasts to study how cell-cell interactions affect MMP production and drug response.<sup>160</sup> While this study likely could have been conducted without the use of a bioprinter, it demonstrates that these machines can produce highly consistent samples and have utility in high throughput studies. The addition of multiple printheads to these printers also allows for the precise patterning of multiple cell types, such as cancer cells and CAFs, further improving the accuracy of these high throughput models.<sup>161</sup>

Extrusion printing is also widely used for bioprinting as it has become relatively affordable. Early extrusion printers were built by modifying standard non-biological 3D printers with new printheads, but now there are many bioprinting specific machines available commercially.<sup>75,162,163</sup> These systems robotically control the extrusion of material which is deposited as a small bead on the print surface. The material can be pressed out of the printhead using either pneumatics or a screw piston.<sup>158</sup> Similar to inkjet printing, the properties of the material used are extremely important. The bioink must be able to flow consistently through the printhead without clogging, but also hold its shape once deposited.<sup>159</sup> Many standard biomaterials used to mimic the ECM such as Matrigel and Collagen I are not ideal for printing. Both materials have short "biofabrication windows", or length of time in which they can flow through the printhead while still holding shape once deposited. To address this short window, materials like gelatin and alginate have been used because the crosslinking is controlled by change in temperature and exposure to calcium, respectively.<sup>164,165</sup> However, these materials do not always support cell growth and/or mimic the native ECM. Therefore, there has been an increased effort to design modular bioinks that allow for successful printing and control over the chemical and mechanical properties. For example, our group has modified our hyaluronic acid-based hydrogels with alternative step-wise or reversible crosslinking methods to create shear thinning

or thixotropic bioinks with extended biofabrication windows.<sup>75,104,162,166,167</sup> Secondary crosslinking such as exposure to UV light can also be used to stabilize the extruded material.<sup>167</sup> The Feinberg lab has also pioneered a technique called Freeform Reversible Embedding of Suspended Hydrogels (FRESH) in which material is extruded into a bath of shear thinning material, which can later be removed.<sup>168</sup> These systems have still proven useful in the space of cancer modeling. As more research groups and companies begin to rely on spheroids and cancer organoids to test novel therapeutics, there has been a significant effort to develop methods of producing these constructs in a consistent high throughput manner. Our group and others have used extrusion bioprinters to create 3D constructs using patient derived tumor cells for high throughput drug screened.<sup>10</sup> These techniques have even been used with patient derived tumor samples indicating that they could possibly be used in large scale personalized medicine applications in the future.<sup>153,169</sup> However, extrusion printing is still limited by its resolution and a lack of extrudable bioinks with an appropriate level of complexity in terms of ECM components.

Light based bioprinting methods may address some of these challenges. These methods include, laser-assisted, stereolithography (SLA), digital light processing (DLP), and two-photon polymerization.<sup>158,170</sup> All of these platforms require the use of a photo-crosslinkable bioink, but they do not require the same manipulation of fluid properties as inkjet and extrusion printing because they do not need to travel through small printhead apertures. With that being said, there are still several to these techniques. In general, light-based bioprinters can create complex geometries with high resolution. For this reason, many groups have proposed using SLA or DLP printing to create small perfusable vascularized tissue constructs.<sup>158,171</sup> However, it can be difficult to achieve high resolution using softer hydrogels that are required to mimic many of the body's tissues. There have not been many examples of cancer modeling as of yet, but there appear to be myriad possibilities. In one study, SLA printing was used to produce a bone scaffold to study interactions between osteocytes and breast cancer cells in bone metastasis.<sup>172</sup> Another study used laser-assisted bioprinting to produce arrays of pancreatic spheroid organoids<sup>173</sup>, so these printing modalities can also be used to create high throughput assays for drug screening. As the cost of these printers continues to decrease, it is expected that there will be increased interest in developing compatible bioinks and use in cancer modeling.

Bioprinting for use in cancer modeling is still in its early stages. The studies highlighted above show the potential of these tools and techniques in the context of cancer engineering. As the field continues to grow, these systems will be applied to more cancers hopefully leading to the production of increasingly complex multi-component models that can be used to investigate novel questions.

#### 4.2. Microfluidic models

Microfluidic devices have traditionally been produced using photolithography techniques originally developed for developing electrical and computer chips,<sup>174,175</sup> hence the name organ-on-a-chip.<sup>5</sup> Generally, a master mold is creating using a negative photoresist such as SU-8, which is then used to cast a soft polymer, usually polydimethylsiloxane (PDMS). Channel inlets and outlets are punched, and the PDMS mold is then bonded to a glass

substrate. In these devices, cells are typically grown as a 2D monolayer after coating the channels with an ECM protein, or in 3D by injecting a hydrogel precursor solution into a specific hydrogel channel. To create more complex structures, photocrosslinkable hydrogels can be patterned in the device using a photomask.<sup>176</sup> The uncrosslinked material is then washed out before adding media. We have used this method to create metastasis-on-a-chip models,<sup>100</sup> a liver-on-a-chip for toxicity screens,<sup>177</sup> and bone marrow-on-a-chip to evaluate healthy and malignant hematopoietic stem/progenitor cell homing to the bone marrow niche.<sup>178</sup> Since the device materials – generally PDMS and glass – are transparent, images can be taken directly on the device via standard light and fluorescent microscopy, and even confocal microscopy. Endothelial barriers have also been created using variety of methods. In one commonly used device now commercially produced by Emulate, Inc., two channels are separated by a semi-permeable membrane on which cells can be seeded.<sup>179</sup> Other groups have developed microfluidic platforms in which endothelial cells self-organize into a perfusable vascular network.<sup>132–134</sup> These models have proven to be reliable, but the vessel network in each device is unique. To create more uniform vessels, many groups have taken to molding simple lumen into ECM hydrogels using needles or rods.<sup>136</sup> Lumen have also been cast using PDMS rods to create bifurcated structures.<sup>180</sup> Bioprinting can be used to create complex lumen structures either via extrusion bioprinting of a sacrificial hydrogel<sup>138</sup> or by using light based printing.<sup>171</sup> These advanced fabrication techniques have been combined to create some elegant models that have been used to study cancer. In one lung cancer-on-a-chip, a semipermeable membrane separating 2 channels was used to create an air-liquid interface. Two air channels separate from the cellular space were used create cyclical mechanical load on the membrane as it would experience in the human lung.<sup>141</sup>

Even with these incredible innovations, the advanced fabrication techniques required produce an insurmountable barrier to entry for many groups. Recent advances in low-cost high throughput fabrication techniques have allowed more researchers to apply microfluidics to their work. As 3D printing technologies continue to improve, some groups have begun using high-resolution SLA printing to produce their master molds. While these molds cannot yet produce the same high-resolution structures as traditional photolithography, they can be used repeatedly and are relatively low cost. Others have turned to more novel approaches using low-cost adhesives to form channels. One approach gaining popularity is based on patterning of adhesive films.<sup>181</sup> A computer-controlled device such as a laser cutter or razor plotter is used to create a pattern in an adhesive film. These films are then attached to a glass substrate forming small channels. Multiple patterns can be layered on top of one another to create complex fluidic devices. These low-cost devices have been used extensively to create organ-on-a-chip models of many systems including cancer. These techniques, combined with photopatterning of hydrogels, has been to study the effects of drug treatment on colorectal cancer cell migration, to generate patient-derived mesothelioma-on-a-chip systems, and to support a multi-organoid “body-on-a-chip” platform to assess the side effects of liver organoid-facilitated metabolism of chemotherapy agents.<sup>37,105,182</sup> Similar systems have also been used to create vessel lumen and could be used as an alternative to the more complex fabrication methods discussed earlier.<sup>183</sup> Bioprinting is now also being used to pattern cells and ECM on these microfluidic devices,<sup>184</sup> enabling researchers to create complex structures such as vasculature and pattern multiple cell types in physiologically

relevant orientations. These techniques can be applied in concert with microfluidics fabrication to easily create models to study metastasis, tumor angiogenesis, drug delivery, and much more.

As we have repeatedly emphasized, advances in these biofabrication techniques will continue to drive the production of more physiologically relevant tissue models that can be used for both basic and translational research. Many have envisioned a future where *in vitro* models become so advanced that animal models are not necessary. To achieve this lofty goal, we must continue to investigate ways to reproduce *in vivo* microtissue architectures and vascularization. It is also essential that we create systems containing multiple tissue types and continue progressing towards a “body-on-a-chip” platform. It has already been demonstrated that systems containing 3D tissue constructs from multiple organs can be maintained together for many weeks and that the replicate simple *in vivo* processes.<sup>185,186</sup> In the context of cancer research, developing these integrated models is key to studying areas such as pre-metastatic niche development, metastasis, and the effects drug metabolism on distal tissues. These systems should help propel us closer to the development of novel treatments and commercialization of *in vitro* models for drug testing and personalized medicine.

## 5.0 Using Multi-Component *In Vitro* Models to Study Glioblastoma

As with many cancers, the survival rate of glioblastoma patients continues to be abysmally low due in part to a lack of effective treatment. In the following section, we will outline the challenges that have faced researchers and clinicians studying GBM and highlight various examples of ways the tools we previously discussed have been used to create effective *in vitro* models. GBM specific considerations and model components are highlighted in Figure 3.

Glioblastoma (GBM, previously referred to as glioblastoma multiforme) is the most aggressive of the glial tumors and has a proclivity for necrosis, uncontrolled cellular proliferation, diffuse infiltration, increased angiogenesis, and widespread genomic heterogeneity.<sup>187</sup> Current treatment strategies include chemotherapy, surgical resection, and radiotherapy which in most cases does not completely eradicate the disseminated infiltrated cancer cells resulting in a poor prognosis. The first choice of chemotherapy agent in these patients is temozolomide (TMZ). However, TMZ treatment invariably leads to development of resistance and increased likelihood of recurrence reducing the overall survival of diagnosed patients to 1.5 to 2 years.<sup>188</sup>

The blood brain barrier (BBB) is a physical and biochemical barrier that inhibits effective drug delivery in to both diseased and normal brain.<sup>189,190</sup> Even though the BBB is disrupted in GBM, there is no evidence that this disruption increases drug bioavailability within the tumor with various studies reporting that a fraction of the tumor cells resides in areas with intact BBB which limits access to drugs completely.<sup>191</sup> The cancer cells induce neoangiogenesis with fenestrated endothelial cells lacking tight junction expression and co-opt the brain vasculature resulting in an anatomically and physiologically different barrier than the normal blood brain barrier.<sup>192</sup> It has been established that the tumor compromises

the integrity of the blood brain barrier surrounding it and promotes the formation of new poorly formed blood vasculature. This new barrier is referred to as the blood brain tumor barrier (BBTB) and it is quite different from the healthy BBB. Astrocytic endfeet displacement, aberrant pericyte distribution, and loss of astrocyte endfeet and neuronal connection along with leakier vasculature are the main characteristics of the blood tumor barrier. The BBTB/BBB structural and functional heterogeneity within the same tumor and among different patient tumors provides indicates that it is largely affected by the cellular, molecular, and the tumor subtype specific features of the glioblastoma. Various studies have demonstrated the effect of glioma on the blood brain barrier, so the inclusion of a functional BBTB/BBB in the model could help in developing more translational chemotherapeutics.<sup>193,194</sup> *In vitro* models can be used to the characteristics of BBTB/BBB for the development of anticancer agents that can effectively cross them and improve treatment outcomes.<sup>195</sup>

The high degree of genetic, epigenetic, and cellular diversity observed in GBM tumors further complicates treatment. GBM tumors are classified into subtypes based on the common grouped set of neoplastic genetic alterations observed in them. The “original” four subtypes, proneural, neural, classical, and mesenchymal, were first established by Verhaak *et al.*<sup>196</sup> Later, studies from the same group showed that the neural subtype is actually comprised of non-tumor cells which are thought to be from normal brain tissue contamination.<sup>196,197</sup> The classical subtype is characterized by amplification of the epidermal growth factor receptor (EGFR), inactivation of the retinoblastoma pathway, and high expression of neural stem markers. The mesenchymal subtype has a higher predilection for necrosis and inflammation with increased expression of families of genes involved in angiogenesis and tumor necrosis.<sup>198</sup> More recently, it has been observed that this classification is not relevant to all the cells found in glioblastoma. Snuderl *et al.* showed that there is simultaneous amplification of multiple receptor tyrosine kinases such as EGFR and PDGFRA (platelet derived growth factor receptor A) in the same patient.<sup>197</sup> Various subtypes are found in the same tumor with detrimental effects on treatment response.<sup>199</sup> However, statistical analysis done by Coleman *et al.* demonstrated that the tumors dominated by the classical subtype had the best outcome in response to aggressive chemotherapy and radiotherapy whereas the mesenchymal subtype dominated tumors had the worst outcome.<sup>200</sup> Heterogeneity is the main source of resistance observed in these tumors because certain parts of the tumor are more sensitive to treatments than others. This effect is amplified by the monotherapies usually used in the treatment of glioblastoma.<sup>201</sup> The cellular heterogeneity observed in advanced cancers such as glioblastoma is caused in part by the glioma cancer stem cells. These cells are self-renewing and contribute to disease progression, treatment, and resistance.<sup>202</sup> Interactions with the various components of the TME play an important role in glioma stem cell behavior and differentiation leading to variations in disease progression.<sup>203</sup>

Experimental *in vitro* models which are designed from a reductionistic standpoint to decipher specific aspects of tumor biology already risk having limited disease complexity relevance but simply become irrelevant in GBM because of the intense complexity involved.<sup>204</sup> Successfully targeting glioblastoma heterogeneity requires an in depth understanding of the factors driving the sub- clonal variation such as vasculature, hypoxia,

and inflammation which would require building these components into the model.<sup>205</sup> The factors stated above impress upon the need for an *in vitro* model that can faithfully recapitulate the *in vivo* components that contribute to the complexity of the glioblastoma.

### 5.1. Cells used in GBM models

For the last 30 years, several glioblastoma cell lines have been commonly used to study glioblastoma. Some have been quite valuable providing preliminary knowledge about the tumor.<sup>206</sup> The most common among these cell lines is U87 which was established in 1968 at Uppsala University in Sweden and has more than 1900 citations in PubMed.<sup>206,207</sup> Despite contributing abundant knowledge to glioblastoma research, these cell lines models are imperfect for several reasons. Namely, 2D culture of these cells has been shown to alter cell phenotype and genotype substantially so that they deviate appreciably from the *in vivo* disease conditions. The extracellular matrix that surrounds the cells *in vivo* provides cues that dictate cell differentiation, proliferation, mechano-responses, drug responses, and cell survival all of which are absent in the 2D cell culture.<sup>208</sup> The culturing of these cells in 2D monolayer has been shown to cause considerable genetic drift, chromosomal aberration, and phenotypic alterations leading to poor reproducibility and low dependability of the data derived from these cultures. The best example of this phenomenon was observed in the U87 cell line. A recent genomic analysis has revealed that it has acquired many indels and translocations from being cultured long term on plastic. The cell line is immensely different from the original cells isolated in 1960.<sup>209</sup> The *in vitro* plastic culture also applies selective pressure on these cells that does not reflect the original tumors that they have been isolated from. If these important variables are not acknowledged, the clinical utility of results obtained using cell lines in different laboratories may be incorrectly reported.<sup>210</sup>

As in other cancers, patient derived cells have the potential to serve as a more accurate representations of the *in vivo* tumor if implemented correctly. The use of patient derived tumor cells in glioblastoma models facilitates precision medicine and can be used to observe individualized tumor progression and invasion.<sup>211</sup> Patient derived tumor organoids were cultured in cerebral organoids to screen drugs, analyze tumor progression and invasion in a primitive human brain microenvironment.<sup>212</sup> Patient derived organoids are used to study individual variability in the physical properties of the tumor and the different mechanical phenotypes of glioblastoma.<sup>213</sup> Patient glioblastoma cells have been used to study the functional and genomic heterogeneity and also observe the intra-patient variability in response to various drug screens.<sup>214–216</sup> Studies have demonstrated that 3D culturing methods such as spheroids of patient GBM cells effectively maintain EGFR upregulation and mutated form of EGFR-vIII. In contrast, these were gradually lost in adherent culture.<sup>217</sup> 3D culture encourages cell to cell interactions and cell to matrix interaction and allow for a more appropriate tissue physiology, anatomy and structure compared to 2D culture.<sup>218</sup> The cells in our bodies respond to various spatial cues and stimulation from their neighboring cells and their surrounding three-dimensional environment. A model with an ECM mimetic microenvironment that provides the necessary biochemical and biomechanical cues allows researchers to study the mechanisms of cell proliferation, migration, matrix production and stem cell differentiation in the GBM microenvironment.



As with other cancer types, the inclusion of non-tumor cells in GBM models is also extremely important. The GBM microenvironment is highly complex, and it has been shown that many other cells in the GBM TME play important roles in tumor progression and growth.<sup>219–221</sup> Engineered models are now being used to study these important cell-cell interactions. For example, multiple studies have used 3D culture systems to investigate the influence of microglia on GBM cell migration and treatment response.<sup>222,223</sup> Similar studies have been conducted using co-cultures of GBM cells and astrocytes.<sup>224–226</sup> The immune environment of GBM is also heterogeneous and plays an important role in progression and treatment resistance. One study used 3D spheroid cultures to evaluate the effect of tumor infiltrating macrophages on the abundance of mesenchymal cells in different populations of GBM cells.<sup>227</sup> Another study used a bioprinted model to investigate glioma stem cell growth in the presence of various cell types including macrophages, astrocytes, and neural stem cells.<sup>170</sup> Neural stem cells (NSC) are also of particular interest in GBM initiation, progression, and treatment.<sup>228</sup> Therefore, one group created 3D *in vitro* models to investigate therapeutic efficacy of NSCs.<sup>229</sup> GBM cells interact closely with the brain vasculature so simple co-culture models have been used to study how endothelial cells impact GBM cell behavior.<sup>230,231</sup> More complex models of the blood brain tumor barrier (BBTB) will be discussed later. Finally, GBM cells also interact with the neural cells. Venkatesh *et al.* have shown that the transmission of nerve signals plays an important role in tumor progression and invasion, also evidenced by the fact that these tumors do not metastasize outside the central nervous system. Glioma cells can even form synapses with neurons, and the integrated neuron circuit provided the bidirectional signaling wherein neuron increased glioma growth and glioma cells increase neuronal activity.<sup>232</sup> This discussion on the various cell interactions that can be studied using GBM *in vitro* models is certainly not exhaustive. However, it demonstrates the utility of these multi-component systems to discrete portions of the complex GBM microenvironment.

## 5.2 Engineering the GBM ECM

The extracellular composition of the human adult brain is unique in that collagen I, which is abundant in other tissues, is practically nonexistent in the brain. The brain has low levels of fibrous proteins such as fibronectin and vitronectin but high levels of glycosaminoglycans and aggregating proteoglycans called lecticans which includes neurocan, brevican, versican and aggrecan.<sup>233</sup> An ECM structure composed of chondroitin sulfate proteoglycans, hyaluronic acid, tenascin-C and tenascin-R known as the perineuronal net (PNN), is exclusively found in the brain.<sup>234</sup> S.L. *et al.* showed that the disruption of PNN is one of the main causes of seizures in primary brain tumor patients.<sup>235</sup>

The ECM in tumoral brain contains upregulated levels of hyaluronan and other ECM components such as vitronectin, osteopontin, and tenascin-C.<sup>236</sup> Hyaluronan is increased fourfold in primary tumoral brain facilitating invasion and migration of tumor cells through its two receptors CD44 and CD168 or RHAMM (Receptor for Hyaluronan Mediated Motility). In high grade gliomas, CD44 is over expressed by some GBM subpopulations which helps CD44 positive cells to invade the brain parenchyma more efficiently.<sup>237</sup> The elastic modulus of the brain tumor tissue has a twofold increase in modulus compared to the surrounding healthy tissues due to the formation of a more tightly packed cellular network

and increased matrix deposition.<sup>238</sup> Several studies have demonstrated that the mechanical properties of the substrate that the glioma cells are cultured on affect proliferation, migration, morphology, and invasion.<sup>239–244</sup> An *in vitro* model system which was used to study the effect of hyaluronic acid and glioblastoma co-culture on endothelial cell network formation showed that this network formation in the hydrogels depended on the is dependent on the mechanical properties of the ECM and HA concentration which are critical parameters of a GBM microenvironment.<sup>230</sup>

The hallmark characteristics of glioblastoma are its infiltrative and invasive properties that enable the tumor cells to evade surgical resection and focal therapies. This invasion varies according to the subtype of GBM, and the mesenchymal subtype has been shown to harbor an increased potential to invade the tissue in comparison to proneural and classical subtypes.<sup>245</sup> It has also been observed that the ECM of glioblastoma patients with a worse prognosis and poor response to the bevacizumab varies significantly in its composition from GBM patients with a better prognosis and who respond well to bevacizumab. There has been considerable interest to use this ECM compositional difference as a prognostic factor and also be used to develop anti-invasive therapies in GBM.<sup>246</sup> Sood *et al.* have used bioengineered constructs with brain ECM and glioma cells and demonstrated that there is reciprocal signaling between tumor cells and the ECM microenvironment and an in depth understanding of this phenomenon is pertinent to understand tumor growth and invasion in the brain.<sup>247</sup>

These studies underscore the importance of accounting for the complex interplay between the tumor cells and the ECM along with the microenvironmental cues in glioblastoma models to understand tumor cell invasion and therapeutic resistance.

### 5.3 Form Factors for GBM models

As previously discussed in section 5.1, 2D culture of both cell lines and patient derived cells can lead to phenotypic and genetic changes. Therefore, there has been an increased interest in creating 3D models of GBM. By implementing many of the form factors discussed in section 3 to create complex multi-component models, many new questions have been investigated related to disease progression and heterogeneity.

Spheroids have emerged as a promising *in vitro* 3D model that can faithfully mimic many functional and structural properties of glioblastoma. Their ease of assembly and ability to provide high-throughput drug screening at considerably low cost, has made them one of the most popular scaffold free 3D models employed in cancer research.<sup>248</sup> Cancer stem cells (CSC) are the most difficult to culture *in vitro* but play an important role in relapse of glioblastoma. Spheroid cultures often help maintain the stemness of the patient derived tumor cells and study the effect of stemness on drug resistance and relapse of disease.<sup>205,218,249</sup> The tumor heterogeneity observed in glioblastoma has been replicated in spheroid cultures by creating multicellular tumor spheroids and employing them in drug studies to ascertain the effect of the heterogeneity on drug response in multiple studies.<sup>89,250–252</sup> Spheroid cell cultures provide advantages over 2D culture by better mimicking *in vivo* cell-cell interactions but still pose considerable challenges in terms of gradient access of nutrients, waste buildup and a necrotic core which prevented the

spheroids size reaching more than 300  $\mu\text{m}$ .<sup>208,253</sup> Nevertheless, using spheroids comprised of multiple GBM cell lines, each labeled with a unique fluorescent tag, allowed us to create heterogeneous GBM spheroids in which we could track evolution of individual cell populations over time with and without drug interventions.<sup>254</sup> While still a cell line-based system, this provided feasibility for current studies in which we are applying the same approach to track multiple individual GBM subtypes (classical, proneural, mesenchymal, and glioma stem cell subpopulations) within integrated organoid cultures.

To address the aforementioned hurdles using spheroids, more complex organoid models of GBM have been produced.<sup>253</sup> These systems allow for longer culturing period and better mimic the TME. Glioblastoma organoids are usually developed by embedding either intact tumor fragments or single-cell suspensions of tumor cells lines or cells obtained from patient tumors within an ECM hydrogel. Numerous studies have demonstrated that these materials provide a better in vitro environment for tumor cells mimicking the in vivo conditions. Commonly used naturally occurring biomaterials in glioblastoma models are collagen, hyaluronic acid, chitosan, alginate and Matrigel.<sup>241,255–257</sup> GBM organoids in the recent years has also been developed by genetic modulation via transposon- and CRISPR-Cas9-mediated mutagenesis of cerebral organoids.<sup>258</sup> The GLICO model, which is a GBM organoid model developed by co-culturing patient derived glioblastoma stem cells in human embryonic cells derived brain organoids, showed the deep invasion of tumor cells in the brain organoid with the aid of interconnected network of tumor microtubules.<sup>212</sup>

Transwells are used to model the neurovascular unit or the blood brain barriers in glioblastoma and also have been employed to study the effect of migration of tumor cells and microglia.<sup>259–263</sup> The transwell system is useful in measuring the effect of tumor cells on the integrity of the BBB and testing the permeability of various chemotherapeutics across the BBB.<sup>264–268</sup> Transwells have the advantage of ease of reproducibility, simple setup with minimal resources, access to apical and basal compartment for therapeutic testing, and easy visualization of cells. However, it is still only a monolayer culture setup without BBB microenvironmental features such as a mimetic ECM and physiologically accurate fluid flow and shear stress needed for proper endothelial polarization and tight junction formation.<sup>264,269</sup> To address this limitations, microfluidic technology has been employed to model BBB/BTB and it can provide a highly controlled environment with incorporation of shear stresses that faithfully recapitulates the in vivo brain endothelium conditions. These models allow for high-resolution live imaging and high-throughput screening of new therapeutics which has revolutionized CNS disease modeling.<sup>143,144,270–273</sup> To study the effect of invasive migratory tumor cells in glioblastoma microfluidic devices have been employed to determining which genetic mutations in GBM cells correlate to migration capacity in a confined space as well as assess the efficacy of drug treatment.<sup>274</sup> One study by Han *et al.* makes use of an elegantly designed microfluidic chip to identify drug resistance mechanisms much more efficiently than in previous studies.<sup>275</sup> The limitations of microfluidic devices are concerned with resolution and surface properties of many commercial materials used in devices such as acrylate, acrylonitrile butadiene styrene-based polymers, and PDMS can absorb lipids and proteins. These devices work with miniscule amount of molecules and lack of sustained nutrient and gas supply pose a challenge to long term assessment of biological processes.<sup>276</sup> As previously stated, the cost and skills required

to produce many microfluidic devices can be prohibitive to many groups, but the new low cost methods can be applied to studies of GBM, further advancing the field.

#### 5.4 Advanced biofabrication techniques to produce GBM models

Bioprinting techniques can be used to develop more refined models of glioblastoma that contain anatomically distinct regions using patient specific components such as cells and ECM mimetic materials.<sup>277</sup> In addition, bioprinting can be leveraged to ensure consistency between organoids and to enable high throughput organoid biofabrication.<sup>104,153</sup> A variety of different models have been created to study GBM. These systems each use a unique combination of cells and bioinks to answer mechanistic questions related to GBM and illustrate the utility of bioprinting as an important tool moving forward.<sup>278</sup>

The majority of existing bioprinted GBM models utilize extrusion bioprinting. In the most basic cases, a single cell type is printed within a hydrogel bioink as demonstrated by the bioprinting of human glial cells with a hyaluronic acid based hydrogel bioink.<sup>279</sup> In one series of studies, a 3D bioprinted systems was used to grow glioma stem cells in a gelatin, alginate, and fibrinogen based bioink. These studies show that these 3D constructs maintain stem cell populations, which are difficult to grow *in vitro*, and can be used to study cell differentiation and vasculogenic properties.<sup>280–282</sup> Bioprinting has also been used to create models with multiple cell types to study cell-cell interactions in GBM. For example, an extrusion printer was used to print a GBM tumor within a hydrogel system containing macrophages to facilitate the study of infiltrating immune cells on tumor cell behavior and drug response.<sup>283</sup> In another study, inkjet printing was used to pattern HepG2 and U251 GBM cells on a microfluidic device to show that prodrug metabolism into an active compound by the liver-derived HepG2 cells could induce effective U251 cell killing.<sup>284</sup> Similarly, a bioprinted co-culture model of glioblastoma cells and astrocytes was used to identify an effective novel anticancer compound, a small molecule antagonist of the N-cadherin (NCAD) which demonstrated effective killing of glioma cells.<sup>285</sup> Bioprinted glioblastoma tissue constructs have also been used to study the complex interactions between the immune cells and the tumor cells in a brain tumor microenvironment.<sup>170</sup> Heinrich *et al.* demonstrated that a bioprinted glioblastoma model can be used to study interaction between macrophages and tumor cells and to test drugs targeting this interaction.<sup>286</sup> This model was employed to show that the glioblastoma cells in this construct can recruit macrophages and polarize them in to glioblastoma associated macrophages which plays a role in tumor invasion and progression.<sup>286</sup> Even more recently, a coaxial extrusion based bioprinting system was used to model glioma angiogenesis.<sup>287</sup> All of the highlighted bioprinted models exhibit moderately high cell viability and use mainly gelatin/collagen based systems. Extrusion and inkjet printing are limited by the bioinks that can be used and often induce high shear stresses on cells. A recent study printed hyaluronic acid-based hydrogels using digital light processing (DLP) bioprinting to study the interactions of glioma stem cells with astrocytes, macrophages and neural precursor cells.<sup>170</sup> DLP printing uses a stereolithography-based approach to biofabrication, removing the need for printheads and associated shear stresses. These examples illustrate the utility of bioprinting in *in vitro* modeling of GBM. However, it is evident that the potential of this exciting technology has not yet been fully realized. As the availability of bioprinting

systems and viable bioinks that better mimic the brain/GBM ECM increases, researchers will have the ability to create precise reproducible systems that can be used in further mechanistic studies or to test drugs. 3D bioprinting can also be used to scale production of patient derived models within a shorter time frame. It can be employed with ease in a clinical setting, and given the urgency of effective treatment in glioblastoma, this could be of remarkable advantage in guiding clinical decisions.<sup>104,153,288</sup>

Various microfluidic models have also been used to study glioblastoma.<sup>289</sup> As previously discussed, microfluidic models have a wide variety of applications in the field of *in vitro* cancer modeling. GBM is no exception. Multiple studies have used microfluidic devices to create high throughput drug screening system for use with 3D spheroids or organoids composed of GBM cell lines or patient derived cells.<sup>290–292</sup> As with other tumors, migration and invasion of GBM cell populations has also been investigated using microfluidic devices patterned with small channels.<sup>293</sup> In one particular model, the microfluidic device was elegantly designed such that the effect of chemical gradients and ECM stiffness on cell migration could be studied.<sup>294</sup> Recently, this style of microfluidic device has also been used to create a diagnostic tool for GBM. Wong *et al.* developed a microfluidic device, which they refer to as a microfluidic assay for quantification of cell invasion (MAqCI), that can be used to quantify the migratory ability of GBM cells. Using these migratory outputs combined with the proliferative potential of the resulting cell populations, the researchers showed that they were able to accurately predict patient tumor recurrence.<sup>295</sup> Microfluidic devices with ECM hydrogels have also been used to study mechanisms of invasion by incorporating other important cell types including endothelial cells and other important cells found within the perivascular niche<sup>296,297</sup> and simulating interstitial fluid flow through the ECM.<sup>298</sup> Now, more groups are beginning to create 3D microfluidic models of the BBB, so logically it makes sense to continue to address these mechanistic questions on slightly more complex systems. Many microfluidic models of the BBB have been developed with the goal of using them to study BBB dysfunction and modeling effective systemic drug delivery in a variety of brain diseases.<sup>143,144,299–302</sup> Some have been implemented in studying GBM, including one model using small micro-vessel structures created using 2-photon lithography.<sup>303</sup> In another recent study, bioprinting was used to create a GBM-on-a-chip model to evaluate the effect of chemoradiotherapy on patient-derived GBM cells. Tumor and vascular bioinks were printed in a concentric ring structure using extrusion printing. This printed circular GBM model was able to sustain a radial oxygen gradient helping to mimic the central hypoxia, biochemical, biophysical characteristics observed in GBM patients.<sup>304</sup>

These models highlight the promise of these advanced biofabrication techniques for studying glioblastoma. Existing bioprinting techniques and microfluidic models can be used to answer important mechanistic questions. However, these techniques are still limited by the availability of bioinks that mimic the GBM microenvironment. Microfluidic models have already proven to be extremely useful in studying GBM. Low-cost fabrication methods combined with high-throughput fabrication techniques such as bioprinting could make these devices more applicable for large scale drug screening and personalized medicine applications.

## 6 Future Directions

In this review, we have attempted to summarize the components necessary for creating an effective multi-component *in vitro* tumor model. This engineering approach is extremely useful as it encourages researchers to utilize cells, biomaterials, and biofabrication techniques together to best mimic the native TME. Using this approach and the tools discussed has and will continue to facilitate the creation of even more complex *in vitro* models, it is also extremely useful for creating simple reductionist models that can be used to effectively screen for drugs or answer simple mechanistic questions. As *in vitro* models become more common in research and clinical applications, it is important that the field agree on a set of benchmarks for model design and data analysis. In many cases, *in vitro* have been shown to be much better representative models of human disease than small animal models. However, rigorous standards will be required for *in vitro* systems to take the place of animal models in the clinical pipeline.

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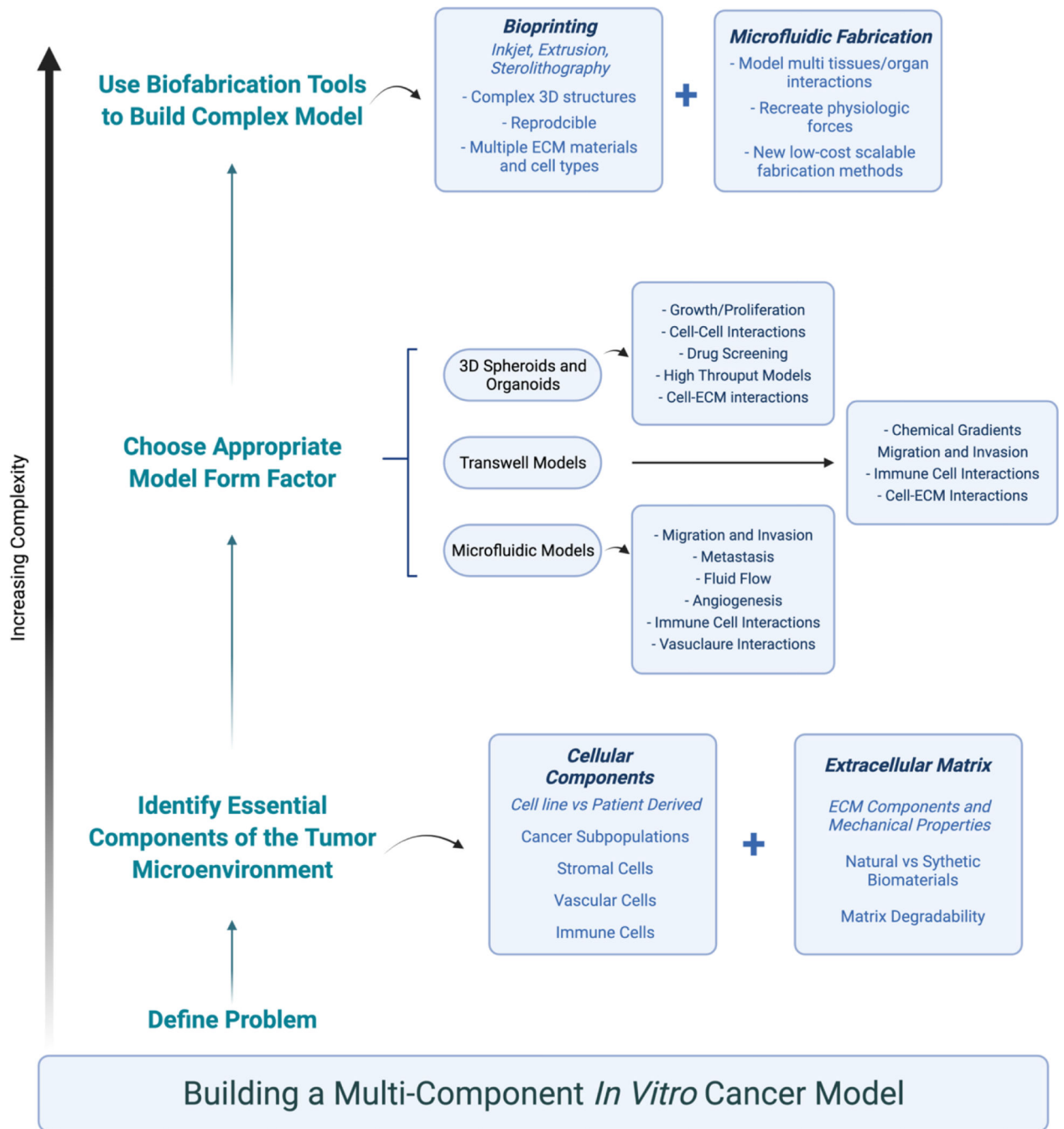
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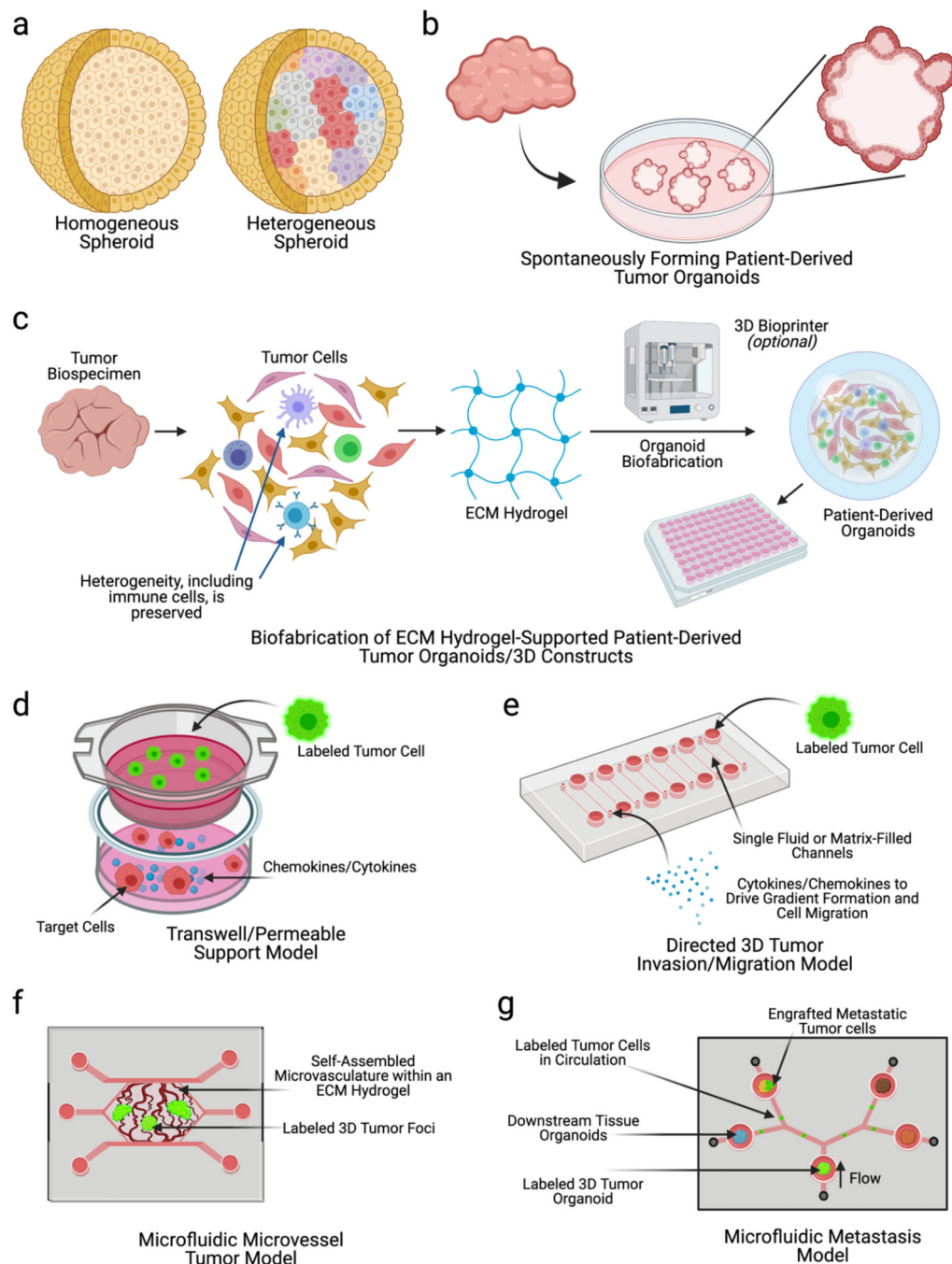
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**Figure 1.**

A visual representation of the workflow and considerations in building in vitro cancer model systems.



**Figure 2: Overview of various form factors used in in vitro cancer modeling.**

In vitro cancer models occupy a wide variety of forms. These range from relatively simple a) spheroids that can be homogeneous or heterogeneous to more complex b) organoids that spontaneously form in materials such as Matrigel. c) Other forms of organoids, or 3D tumor constructs, can be formed by encapsulating tumor cells, including those derived from a patient's tumor, in an ECM hydrogel, which can then be subsequently deposited or bioprinted in small volumes to form organoids. d) Transwell inserts or permeable support wells have been widely used to assess tumor cell migration towards cytokines, chemokines,

or other cell types. e-g) Microfluidic device technologies have rapidly advanced in cancer research, resulting in a wide variety of tumor-on-a-chip systems. These include e) directed 3D tumor cell invasion models, f) microvascular tissue chips containing self-organizing blood vessels and tumor foci, and g) metastasis-on-a-chip systems allowing tracking of tumor cells through microfluidic circulation to other tissue sites.

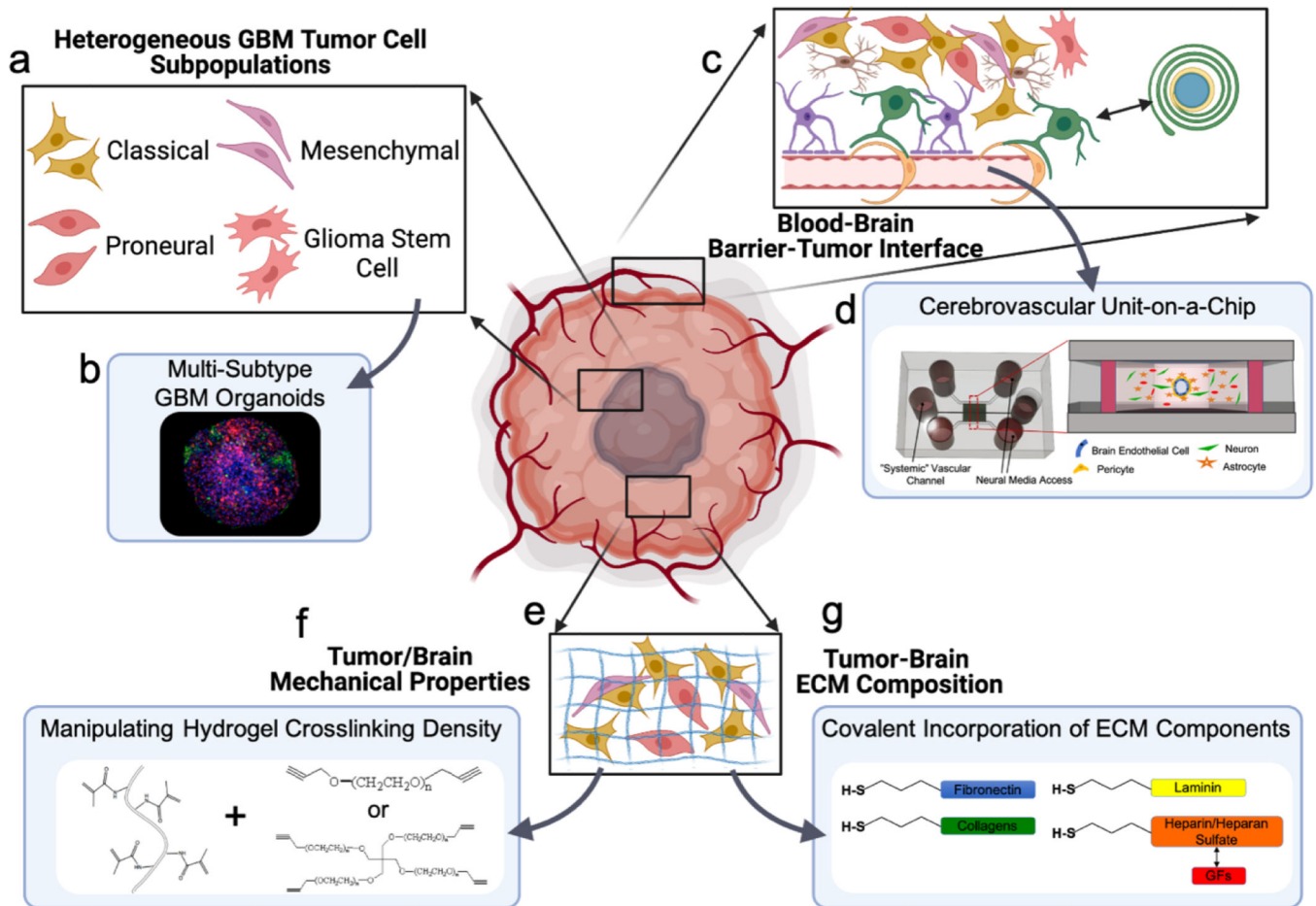
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**Figure 3. Overview of the components necessary to design complex multi-component models of glioblastoma.**

a) Like many other cancers, the GBM TME consists of many unique cell types including tumor cells, glial cells, immune cells, and vascular cells. GBM tumors cells are also composed of multiple distinct subpopulations. b) These subpopulations can be labeled and combined in a spheroid or organoid culture and their growth and proliferation studied in response to treatment. c) The blood brain barrier plays a key role in regulating tumor growth and in the delivery of therapeutics to the tumor site. d) Microfluidic models have been created to model the BBB in vitro. e) The extracellular matrix in and around the tumor in GBM is different from that of the healthy brain. There is an increase in stiffness and deposition of certain ECM molecules such as hyaluronan and vitronectin. The stiffness (f) and the relative concentration of various ECM components (g) can be controlled via chemical modification of the hydrogel biomaterials used.