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Review

3D Neural Tissue Models: From Spheroids to Bioprinting

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Abstract: Three-dimensional (3D) *in vitro* neural tissue models provide a better recapitulation of *in vivo* cell-cell and cell-extracellular matrix interactions than conventional two-dimensional (2D) cultures. Therefore, the former is believed to have great potential for both mechanistic and translational studies. In this paper, we review the recent developments in 3D *in vitro* neural tissue models, with a particular focus on the emerging bioprinted tissue structures. We draw on specific examples to describe the merits and limitations of each model, in terms of different applications. Bioprinting offers a revolutionary approach for constructing repeatable and controllable 3D *in vitro* neural tissues with diverse cell types, complex microscale features and tissue level responses. Further advances in bioprinting research would likely consolidate existing models and generate complex neural tissue structures bearing higher fidelity, which is ultimately useful for probing disease-specific mechanisms, facilitating development of novel therapeutics and promoting neural regeneration.

Keywords: 3D printing; nerve regeneration; neurons; glial cells; traumatic nerve injuries; neurodegenerative diseases;

1. Introduction

Disorders of the nervous system are estimated to affect more than one billion people worldwide [1]. Typical examples of neural disorders include acute traumatic injuries (e.g. traumatic brain injury (TBI), spinal cord injury (SCI)), neurodegenerative diseases (e.g. Parkinson's disease, Alzheimer's disease, Huntington's disease) or neurodevelopmental disorders (e.g. microcephaly and autism). In almost all cases, effective treatments are lacking. Although great efforts have been devoted to promote functional restoration and neural regeneration [2-5], our molecular understanding of the pathogenic mechanisms remains very limited. This hinders the development of novel therapeutic interventions. Such dismal progress likely results from the lack of suitable models that recapitulate the complex cell-cell and cell-environmental interactions *in vivo*.

Animal models provide the greatest extent of physiological relevance and therefore, are still considered to be the gold standard [6]. Yet animal experiments are time consuming, costly, and usually cannot fully reflect the actual conditions in human patients due to the apparent genetic, biochemical, and metabolic differences between species [7]. Moreover, it is technically challenging to monitor what is going on inside the animals, and ethical issues are frequently raised. Alternatively, *ex vivo* models using slice cultures of nerve tissues have been widely adopted [8-10]. Compared with intact animals, tissue slices are easier to manipulate experimentally. In addition, they are more easily amendable to image analysis, and preserve the local cellular organization [11]. However, numerous limitations exist; notably, significant functional loss occurs rapidly once slices are separated from the body.

Apart from animal models and *ex vivo* culture systems, cell-based *in vitro* models are extensively explored through both two-dimensional (2D) and three-dimensional (3D) cultures. Here, 2D and 3D refer to the dimension into which cells grow over time. 2D monolayer cultures, which culture cells on a thin surface-coated petri dish, are most commonly used, largely owing to its cost effectiveness, ease of handling, and robustness across diverse cell types. Indeed, 2D neural culture studies have been very popular and successful, particularly in the areas of axon/dendrite growth, neuronal survival and synapse formation [12]. However, 2D cultures are generally inadequate in recapitulating specific physiological features due to insufficient cell-cell and cell-extracellular matrix (cell-ECM) interactions [13-15]. In contrast, 3D cultures, which culture cells in an artificially established 3D environment, provide more complex environment with longer lifespan, and tend to

be more informative and predictive. Given its closer physiological relevance, 3D neural models are thought to be a better *in vitro* complement to the animal models.

Different 3D culture systems, such as cell biology-based models (spheroids and organoids), and engineering-based models (scaffold and microfluidic platforms), have been widely explored for their ability to generate more faithful neural tissue-like structures that incorporate diverse cell types and materials, and both physical and biochemical signals [16-19]. Driven by cell intrinsic organization, cell biology-based models are superior at mimicking early developmental details. On the other hand, engineering-based models excel in controlling the organization and composition of materials to achieve the optimal properties (e.g. mechanical properties, porosities and degradability), that are critical for reconstructing tissues in a controlled and consistent manner. This allows desired tissue constructs to be fabricated consistently. It is worthy to note that the convergence of organoids and scaffolds has generated models with improved tissue architecture and increased reproducibility. This validates the ability of engineering methods to enhance the utility of cell biology-based models with the preservation of self-organization property [20]. However, despite the great progress in engineering engineering-based models, cells within these conventional engineered constructs are frequently flooded with a bulk of materials or exogenous signals, and fail to fully capture physiologically relevant cell-cell and cell-ECM interactions. This has triggered the introduction of bioprinting. Bioprinting has developed into a promising tool to construct reproducible and flexible models automatically, with precisely arranged living cells, biomaterials and instructive biomolecules based on pre-designed patterns [21]. Exquisite control over these elements may better imitate the intricacies of the natural physiological environment. Unfortunately, despite the great potential of bioprinting, several limitations remain to be addressed; these include the need to: improve the printing resolution; formulating cell/printing-permissive bioink with fully defined components; obtain good spatiotemporal control over signaling gradients and, supply sufficient nutrients and oxygen.

This review intends to provide an in-depth discussion and analysis of current 3D neural tissue models. In the first part of the article, we describe the fundamental design principles of constructing neural tissue models. Following this, we review existing 3D *in vitro* neural tissue models, evaluating the major advantages and limitations of each model. A clear understanding of the pros and cons of these existing models will pave the way for appropriate 3D bioprinting design specifications. With this in mind, we describe recent applications of bioprinting for *in vitro* neural tissue modeling, with a particular focus on printing systems, cell types, materials,

structures, and functionality. Finally, we present a perspective on the potential of bioprinting to develop better *in vitro* neural tissue models.

2. Design principles for developing neural constructs

A major caveat of 2D cultured cells is the absence of a 3D environment that native cells grow in naturally. In fact, accumulating studies have demonstrated that 3D *in vitro* cell models yield better cellular functions and elicit more appropriate physiological responses [13, 22]. Hence, in order to generate 3D tissues, instead of building an entire organ, it is often more pragmatic to focus on building the essential parts of the organ, a miniature organ, or a functional unit with sufficient complexity that captures the key features of native tissues. However, to what extent these 3D *in vitro* models are recapitulating the true biofunctionality of tissues awaits further evaluation. Characteristic features of the native tissues should be considered so as to recapitulate the complicated tissue physiology [23]. Here, we describe some salient features that are shared by neural tissues as guidelines for designing neural tissue constructs.

The nervous system contains a large assortment of cell types. There are various types of neurons and glia cells that are distributed throughout different regions of the nervous system. Take the brain as an example, it contains approximately 86 billion neurons and 85 billion non-neuronal cells [1]. Within the central nervous system, the non-neuronal cells, referred to as glia cells, include astrocytes, microglia, oligodendrocytes, endothelial cells, and pericytes. Therefore, *in vitro* 3D models should embrace cells in a very high density and diversity to provide sufficient cell-cell interactions, which enable the cells to have the appropriate phenotypes.

Besides the diverse cell types, ECM composition in the nervous system is also very unique. Components such as collagen, fibronectin and laminin, which are abundant in other organs actually exist in much lower amounts in neural tissues. Instead, proteoglycans of the lecticans, hyaluronan and tenascins are copious [24]. In addition, the ECM is also composed of a myriad of soluble factors, such as growth factors, cytokines and chemokines, which function in a concentration-dependent manner [25]. Many fundamental processes of neural development and circuit formation, including dendritic outgrowth and axonal targeting [26], operate on finely graded growth factor concentrations. Accordingly, the re-establishment of such gradients will be an essential component in promoting nerve regeneration [27-30].

Besides serving as a structural support for cells, the ECM also provides extrinsic signals to regulate cell behavior. Specifically, the mechanical property of the ECM is an essential parameter that should be considered when designing *in vitro* tissue surrogates. Neural tissues possess very distinct biophysical properties, such as low elastic modulus (neonatal brain tissue 110 Pa and adult brain tissue <1 kPa, 90~230 kPa for spinal cord) [12], which is much lower than the heart, cartilage and bones. Mounting evidence has shown that matrix stiffness exerts great effects on cell morphologies and behaviors [31-33]. In particular, NSCs are likely to differentiate into glial cells when embedded in matrices with higher modulus (>1 kPa), while softer gels (100~500 Pa) tend to enhance cell migration and differentiation into neurons [34-37]. Similar to NSCs, mesenchymal stem cells (MSCs) tend to differentiate into the glial lineage on stiffer matrices (10 kPa), but adopt neuronal phenotypes on softer substrates (1 kPa) [38]. Likewise, matrix porosity greatly influences cell migration and metabolic efficiency [39]. For example, large pore sizes (more than 100 μm) and a better interconnectivity enhanced nutrient exchange, and proper pore size that is similar to the native tissues leads to better cell migration [19]. Additionally, neural cells have intricate responses to topological cues (e.g. microgrooves, pits, pillars) [40-44], which have been extensively investigated and proven to better facilitate cell viability [45], migration [46], proliferation [37], differentiation [47] and neurite outgrowth [33, 48-50]. The identification and recapitulation of these essential parameters (e.g. multiple cell types, mechanical property, porosity, biocompatibility, biochemical and physical gradients) be useful in reconstructing neural tissues with maximal authenticity.

3. Current status of conventional 3D *in vitro* models

Both cell biology-based models and engineering-based models have advanced our understanding of neural tissue development and neuropathology significantly. Recognition of the advantages and limitations of these models will be informative to developing novel 3D bioprinting approaches that could enhance the functionality and utility of current models. Herein, we describe most prevalent 3D *in vitro* neural models (Figure 1) in the context of how they have been used in reconstructing neural networks, and modeling and understanding neuropathological development and present the major differences between these methods .

3.1. Cell biology-based models

3.1.1. Spheroids

Spheroids are multicellular aggregates formed by either spontaneous self-assembly or forced cell-cell adhesion without scaffolds [51]. Spheroids can be fabricated through the hanging drop method, by using spinner flasks, and centrifugation and non-adherent surfaces [52]. Typically, spheroids secrete their own ECM instead of requiring other foreign ECM-mimicking materials, hence, maintaining the native ECM composition [53]. Table 1 shows a summary of recent developments in neural spheroid models for various applications.

Spheroids have been employed to recapitulate the fundamental features of brain tissues in terms of cell diversity, electrophysiology, ECM production and mechanical stiffness. Dingle *et al.* used micro-molded agarose wells with a diameter of 400 μm to facilitate spheroid formation. They generated 3D spheroids with rat neonatal cortical cells at four different initial seeding densities (1k, 2k, 4k, 8k cells/spheroid) [53]. The spheroids reached a stable size after 14 days, with cell densities similar to *in vivo* density ($2 \sim 4 \times 10^5$ cells/ mm^3). Immunohistochemical analysis revealed the presence of not only neurons, but also multiple glial cell types including astrocytes, oligodendrocytes, and even microglia. Remarkably, after 14 days *in vitro*, patch-clamp recordings showed that the neurons within the spheroids were electrically active, and participated in synaptic networks, as evidenced by the detection of spontaneous postsynaptic currents (sPSCs). In addition, the cortical spheroids secreted laminin, and exhibited elastic modulus that was similar to that of newborn rat cortex.

Figure 1. An overview of current *in vitro* neural tissue models and various applications. There are two main approaches to construct *in vitro* neural models: cell-biology based and engineering-based. Cell biology-based models include spheroids and organoids, which are heavily dependent on cell spontaneous organization, and are able to capture the fundamental features of the native tissues, in a highly

variable form. Engineering-based models include scaffold-based and microfluidics, which impose better control over matrix organization and provide desired tissue structures reproducibly. However, both of them lack of tight control over cells/signaling factors organization. Bioprinting is uniquely poised to harness the strengths of each field and exploit the collective efforts. As an enabling tool, bioprinting enables the combination of spheroids, scaffold and microfluidics into one integral platform to construct neural tissue models with better quality and consistency.

3.1.1.1. *Mimicking neuronal networks formation*

When combined with engineering approaches, spheroids can be used as building blocks to form neural networks [54]. The nervous system functions by signal transmission between different regions and between local circuits in the same region. Signal transduction relies on highly complex 3D networks. Understanding the signal transduction may provide deep insights into the pathophysiology of neurological disorders [55, 56]. Therefore, a number of studies have been conducted to mimic neuronal networks [17, 57-59]. One notable study recently published by Shoji Takeuchi's group further developed the spheroid system to build larger neural constructs [17]. Specifically, they packed pre-formed spheroids into millimeter-sized PDMS chambers and allowed the spheroids to fuse and connect to form macroscopic neural building blocks (NBBs) ($6\text{ mm} \times 1\text{ mm} \times 0.5\text{ mm}$), as shown in Figure 2 (i). It is noteworthy that these millimeter-sized NBBs were formed entirely with rat cortical cells without any scaffold. Correspondingly, a high cell density, that was equivalent to that of the cortex *in vivo* ($1 \times 10^6/\text{mm}^3$), was achieved after 13 days of culture. In addition, neurites reaching 1 mm in length were observed, and dendritic spines were formed, indicating the establishment of long range connections within these NBBs. Consistent with that, Ca^{2+} transients were also recorded via calcium imaging, which demonstrated that functional neuronal networks existed within the NBBs. To explore the possibility of assembling inter-block neuronal networks, the authors attempted to connect two NBBs that comprised of rat cortical cells and rat hippocampal cells respectively. Remarkably, neurons extended their neurites into the other block, and the resulting synchronized calcium oscillation indicated the establishment of synaptic connections between the NBBs. This pioneering study provided proof-of-concept evidence for *in vitro* construction of functional neural network at the macroscopic level. However, many critical concerns remain. In particular, these NBBs could be cultured to a maximum of 15 days only. This would, therefore, prevent its use in long term studies of maturation and maintenance of the neural network. This is probably due to extensive necrosis in the center of the NBBs, which resulted from inefficient nutrient and/or oxygen penetration. In addition, it was unclear whether the cells inside the NBBs were organized in any structures or layers similar to the real cortex.

Figure 2. Cell biology-based models closely resemble natural brain tissue with greater details. (i). Millimeter-sized NBBs resembling 3D heterogeneous neural network. A. Schematic of co-culture of 3D neural components by assembly of cortical-NBB and hippocampal-NBB. B. Immunocytochemistry imaging of NBB formed by neurospheres on day 13. Neurons, glial cells, synapses and nucleus are shown in green, red, green, and blue colors respectively. Results indicated that the cells are in good condition and synaptic connections were established after 13 days culture [17] (reproduced with permission). (ii). Size-controllable spheroid models to mimic the cytoarchitecture of the cortical region of the brain. A. Neurites extension and their connection among the neurospheres. B. Calcein AM stained neurospheres and their networks. C. Neurosphere without treatment of amyloid beta (top) and with the treatment of amyloid beta (bottom). White arrows point to healthy neuronal cell bodies and yellow arrows point to apoptotic neuronal cell bodies. D. Confocal images of immunostained neurosphere, (Nuclei (DAPI, blue), neurofilament (anti-neurofilament, red), and tubulin (anti- β 3 tubulin, green)). Scale bars are 50 μ m [54] (reproduced with permission). (iii). Midbrain-like organoid comprising distinct cell layers. Left: Cryosection of an hMLO at day 35 stained for Ki67 and MAP2. Right: a zoom in view of the white box. White scale bar, 200 μ m. Yellow scale bar, 10 μ m (reproduced with permission from Ref [18]).

3.1.1.2. Understanding of neuropathology

Other than modelling normal brain tissues, people have also drawn great insights into tumor development and neurotoxicity based on spheroid models [60-65]. Terrasso *et al.* differentiated the human embryonic carcinoma cell line, NT2, in a spinner system [62]. After a month of culture, spheroids containing both neurons and astrocytes were produced, and the resulting system was used to study the cytotoxic effects of a set of chemicals (including acrylamide and chloramphenicol) on neurons and astrocytes. This system appears efficient

and scalable, but suffers from inconsistency in the spheroids obtained because of the random fusion that occurred among the neural spheroids, which in turn produced aggregates with highly variable dimensions.

Using low-attachment microwells, Ivanov *et al.* generated relatively uniform sized spheroids of UW228-3, a human medulloblastoma cell line, and investigated the dose-response relationship of the cytotoxic antitumor drug, etoposide [64]. A notable feature of the study is the co-culture of normal human fetal brain cells with the cancer cells within the same spheroid. Indeed, separate color-labeling of the two cell populations enabled the authors to evaluate the differential effects of the drug on each cell type. More intriguingly, this method also allowed them to observe that tumor and normal cells mostly segregated into different domains within the spheroids, with occasional foci of tumor cells being observed inside a normal cell domain. Whether this phenomenon represented any features of tumor microinvasion remains to be tested. Nevertheless, this study illustrated a potential utility of the 3D co-culture system to study aspects of brain tumors, in addition to cell death.

In addition to brain tumor studies, spheroids have been extended to model degenerative neurological diseases such as Alzheimer's disease (AD) [54, 66, 67] and Parkinson's disease (PD) [68] in recent years. Choi *et al.* seeded dissociated prenatal rat cortical cells into polydimethylsiloxane (PDMS) microwells with four different diameters (300 μm , 400 μm , 500 μm , 800 μm) to generate uniform sized neurospheres and used them as an *in vitro* 3D model to study the neurotoxic effect of amyloid- β ($\text{A}\beta$) [54], the putative toxin in AD (Figure 2 (ii)). The authors employed several different assays, including MTT, immunostaining, and electron microscopy, to catalogue the differences in terms of cell viability, neurite morphology, and synaptic integrity between no $\text{A}\beta$ treated and $\text{A}\beta$ treated groups over a 10-day culture period. Furthermore, the authors reported decreased acetylcholine amount in $\text{A}\beta$ treated group. This work adds onto a growing list of studies aiming to model neurological disorders in a dish using 3D cultures [13, 67, 69]. Nevertheless, most of such studies hitherto have been limited to morphological assays and neuronal survival. Functional measurements, especially electrophysiological characterizations of the cells, would be needed to make them truly useful for modeling diseases.

3.1.2. Organoids

Organoids are large 3D cellular aggregates that resemble organ cell types, structures and functions. By utilizing PSCs and adult stem cells (ASCs), organoids have been demonstrated to capture some fundamental

features of human tissues [70-76]. In many ways, organoids are advanced spheroids, with a major distinction: in organoids, stem cells self-organize through cell sorting and spatially defined differentiation [77], while in spheroid models, **stem cells or progenitor cells are usually lacking, leading to a deficiency in self-renewal and differentiation. Consequently, organoids typically produce more stable 3D cultures that can be maintained for longer periods of time than spheroids, as shown in Table 3.** Table 2 provides a summary of the recent developments in brain organoids research. The first entire organoid was generated by Eiraku *et al* [75]. Specifically, optic cups were developed by culturing mESCs (mouse embryonic stem cells) in a mixed media (Matrigel, laminin and entactin proteins, nodal protein) in a floating culture condition, which was different from previous static cultures on coated dishes. This highlighted the importance of culture condition for the formation of organoids.

Another notable feature of organoids is the use of Matrigel as a permissive niche for embedding PSCs, which was also validated by a recent breakthrough in brain organoids as reported by Lancaster and Knoblich *et al* [71]. By culturing embryonic bodies (EB) embedded Matrigel in a growth-factor free medium using a spinner flask, large cerebral organoids (1 ~ 4 mm) containing cells that were characteristic of different brain regions were obtained. These ‘mini-brains’ recapitulated the essential elements of the developing human cortex including cortical layers, and have been since adapted for modeling diseases such as microcephaly [70, 71]. This study provided a foundation for further development towards specific brain regions [73, 74, 78, 79].

Along this line, Jo *et al.* have recently succeeded in generating human midbrain organoids from hPSCs through self-organization (Figure 2 (iii)) [78]. Specifically, the human midbrain-like organoids (hMLOs) grew to more than 2 mm by 30 days and contained layered neuroepithelia that was similar to the developing midbrain. Furthermore, gene expression and immunostaining analyses, along with electrophysiological recording of pacemaking firing indicated the presence of cardinal midbrain dopaminergic neurons. Most importantly, hMLOs released dopamine, and over a long term culture, (more than 2 months) reliably and robustly produced neuromelanin, a dark pigment found in human substantia nigra. Given that none of the previous studies reported the production of neuromelanin *in vitro*, their 3D midbrain organoid models uniquely allowed the preservation of important physiological features of the *in vivo* tissues. Taken together, these organoids represent a useful platform to study human midbrain development and associated diseases.

In general, organoids preserve the intrinsic organization capability of various cell types and are better at recapitulating the developmental details. In particular, organoid models have been shown to be quite successful in capturing some fundamental structures and physiological features of native tissues. Nevertheless, these self-assembly models also suffer from several limitations. Firstly, they are extremely variable in size and shape. In addition, as the composition of Matrigel is not completely understood, many organoids that involve the use of Matrigel appear to be unstable and suffer from batch to batch inconsistency. This in turn restricts their use in high throughput screenings. Moreover, as indicated in Tables 1, 2 and 3, it is obvious that in general organoid models exhibit larger dimensions than spheroid models. Correspondingly, necrosis has been consistently observed in most studies due to insufficient nutrient/oxygen exchange in the center of these structures. Thus, proper vascularization that integrates capillary networks into the organoid structures may be necessary to facilitate gas and nutrient exchange.

Table 1. Overview of 3D spheroid neural models

Application	Cell type	Fabrication method	Size of spheroids (d) / well (D)	Seeding density	Culture period	Culture medium	Functional evaluation	Ref
Mimicking neuronal networks formation	Rat cortical cells	Microwell array from micropatterned agarose wells	D = 400 μ m, d = 200 ~ 300 μ m (size controlled by initial seeding density)	1k, 2k, 4k, 8k cells/spheroid	14 d to reach a stable size	Neurobasal A medium supplemented with 1 $\times$ B27	<ul style="list-style-type: none"> Neurons within the spheroids were electrically active Formation of synaptic networks Laminin secretion 	[53]
	Rat cortical cells and hippocampal cells	mm-sized PDMS chamber	D = 6 mm $\times$ 1 mm $\times$ 0.5 mm	5×10^6 cells/ml	Max. 15 d	DMEM/F-12 (1:1) supplemented with 10% heat-inactivated FBS and B27 serum-free additive	<ul style="list-style-type: none"> Functional networks were observed inside the NBB Synaptic connection between NBBs 	[17]
	Rat cortical cells	PDMS chamber	D = 50 ~ 300 μ m	4×10^6 cells/ml	14 d		<ul style="list-style-type: none"> Formation of centimeter sized neurospheroid network 	[57]
	Rat primary NPC	PDMS chamber	d < 500 μ m	2×10^7 cells/ml	7 d	Neurobasal media containing B-27 Supplement	<ul style="list-style-type: none"> Satellites spheroid were found around the host spheroids Bundle networks were found between host spheroid and satellite 	[58]
Understanding of neuropathology	Human medulloblastoma cell line and human neural stem cells	Ultra-low attachment 96-well plates	Both d around 400 ~ 500 μ m	50 ~ 40000 cells/well	3 d reach 400~500 μ m	DMEM/F-12 (1:1) with B27 (2 ml) hEGF (20 mg/ml, 100 ml), bFGF(10 mg/ml, 100 ml), Heparin (5 mg/ml, 100 ml) for 100 ml.	<ul style="list-style-type: none"> Feasibility study Etoposide level has varied effect on stem cells and tumor spheroids 	[65]
	Human medulloblastoma cell line and human neural stem cells	Ultra-low attachment 96-well plates	D = 600 μ m	3500 cells/well from each type	7 d	DMEM/F-12 (1:1), B27 (2 ml) hEGF (20 mg/ml, 100 ml), bFGF(10 mg/ml, 100 ml), Heparin (5 mg/ml, 100 ml) for 100 ml.	<ul style="list-style-type: none"> Feasibility study Spheroid (co-culture of tumor cell and stem cell) was treated with Etoposide in different levels, led to ratio change of tumor cell and stem cell 	[64]
	NT2 cells	Stirred spinner system	d = 179.0 $\pm$ 76.5 μ m	6.7×10^5 cell/ml	24 d	DMEM, 10% FBS d3-d23: DMEM supplemented with 10% FBS and 20 μ M retinoic acid	<ul style="list-style-type: none"> Differentiated neurospheres enriched in mature neurons Functional astrocytes was obtained by 	[62]

						d24 onwards: DMEM, 5% FBS	stirred suspension culture	
	Rat cortical cells	PDMS microwells	D = 300, 400, 500, 800 μ m	N.A.	10 d	Neurobasal media supplemented with B-27	<ul style="list-style-type: none"> Disrupted synaptic integrity in Aβ treated group Decreased acetylcholine amount in Aβ treated group 	[54]
	SH-SY5Y cell line	6-well plates	d ~125 μ m (after 4~5 d)	2×10^6 cells/well	7 d	DMEM medium supplemented with 15% FBS	<ul style="list-style-type: none"> Neural differentiation , increased levels of degeneration were achieved compared to 2D system 	[67]
	Human midbrain-derived NPC	Stirred culture systems	D = 300 ~ 400 μ m	2×10^5 cells/ml	Max. 39 d	d0-d6:AM d7:DM d21-d39 MM	<ul style="list-style-type: none"> Spheroids contained mature neurons with abundant synaptic connections Functional dopaminergic neurons were observed. 	[68]

Table 2. Overview of 3D organoid models

Organoid type	Cell type	Culture conditions	Size of organoids	Culture period	Key findings	Ref
Retinal organoid	mESCs	SFEBq floating culture	0.6 mm ~ 0.7 mm	24 d	<ul style="list-style-type: none"> Progressive optic cup self-formation in 3D Four phase of 3D eye-cup morphogenesis reproduced <i>in vitro</i> 	[75]
Cerebral organoid	hPSCs	Spinning bioreactor	1 mm ~ 4 mm	2 ~ 4 months	<ul style="list-style-type: none"> First derivation of cellular structures comprising of entire human brain <i>in vitro</i> 	[71, 72]
Midbrain Organoid	hPSCs	Orbital shaker	2 mm ~ 4 mm	3 ~ 6 months	<ul style="list-style-type: none"> Functional midbrain neurons are present Presence of neuromelanin 	[78]
Forebrain/Midbrain/Hypothalamic organoid	hPSCs	3D printed mini bioreactor	1 mm ~ 2 mm	2 ~ 4 months	<ul style="list-style-type: none"> Evidence of cortical layering Modeling ZIKA virus effect 	[70]
Forebrain organoid	hESCs	40% O ₂ / 5% CO ₂ conditions static culture	~1 mm	More than 3 months	<ul style="list-style-type: none"> Self-organization of axial polarity, inside-out layer pattern Human-specific neural progenitor features reproduced 	[79]
Hippocampal-choroid plexus organoid	hESCs	40% O ₂ static culture	1 mm ~ 2 mm	~75 d	<ul style="list-style-type: none"> Generation of functional hippocampal granule- and pyramidal-like neurons 	[73]
Cerebellar organoid	hESCs	Floating culture	~1 mm	More than 35 d	<ul style="list-style-type: none"> Self-formation of cerebellum-like structure with ESC-derived cerebellar plate and rhombic lip neurons 	[74]

Table 3. Comparison between 3D *in vitro* models for neural tissues

	Cell types	External ECM	Size	Cell density	Matrix mechanical property	Culture time	Scalability	Consistency	Strength
Spheroid	<ul style="list-style-type: none"> Primary cells Immortalized cell lines Stem cells 	Typically free of foreign material	<1 mm	Up to a few thousand cells per well	N.A.	Up to 2 months	++	++	<ul style="list-style-type: none"> Uniformity Ease of handling
Organoid	<ul style="list-style-type: none"> Pluripotent stem cells Adult stem cells 	Matrigel	0.5 ~ 4 mm	Up to a few thousand cells per well	~500 Pa	Up to 1 year	+	+	<ul style="list-style-type: none"> Long term culture Preservation of some parts of tissue structures Recapitulate early developmental events
Scaffold-based	<ul style="list-style-type: none"> Primary cells Immortalized cell lines Stem cells 	Hydrogel (natural and synthetic): e.g. collagen, hyaluronic acid, PEG Fibers	Building platform dependent, up to a few cm	$10^5 \sim 10^8$ cells/ml	Tunable, depend on biomaterials used ~100 MPa	Depend on materials degradation property (1 day ~ several months)	+++	+++	<ul style="list-style-type: none"> Spatial control over ECM organization Automation Reproducibly generate desired tissue architectures
Microfluidic	<ul style="list-style-type: none"> Primary cells Immortalized cell lines Stem cells 	Hydrogel (natural and synthetic): e.g. collagen, hyaluronic acid, PEG	< 1mm	$\sim 10^8$ cells/ml	Tunable, depend on biomaterials used Typically < 1 MPa	Depend on materials degradation property (1 day ~ several months)	+	+++	<ul style="list-style-type: none"> Dynamic culture via perfusion flow Co-culture Control over cell placement
Bioprinting	<ul style="list-style-type: none"> Primary cells Immortalized cell lines Stem cells 	Printable biomaterials, e.g. gelatin, alginate, hyaluronic acid, collagen, PEG	Building platform dependent, typically can be up to a few cm	$10^6 \sim 10^8$ cells/ml	Tunable, depend on biomaterials used ~ 3 MPa	Depend on materials degradation property (1 day ~ several months)	+++	++++	<ul style="list-style-type: none"> Exquisite control over cells, signals patterning and ECM organization. Automation Enable integration of cell-based models (organoid, spheroid) and microfluidic technology into one platform.

The number of “+” indicates the level of performance

3.2. Engineering-based models

Cell biology-based models rely on cell intrinsic organization and exogenous cues that are supplied during the culture period, while engineering-based models aim to reconstruct living tissues by assembling the key signals that are necessary for tissue regrowth and reformation [23], as shown in Table 3. Instead of the poorly defined Matrigel and the highly variable structures that are formed in cell-biology based models, engineering-based models typically utilize fully defined components as matrix and present cells with desired tissue architecture. We draw on some specific examples to elaborate their applications.

3.2.1. Engineering-based scaffolds for neural tissue construction

3.2.1.1. Reconstructing ECM of neural tissue microenvironment

Since Matrigel has been used frequently in organoid cultures, this material has also been widely utilized in tissue engineering approaches [70, 71, 78]. However, Matrigel is poorly defined and the batch-to-batch variation is not conducive for high throughput screening applications. Hence, several notable research endeavors have been carried out to explore fully defined materials to replace Matrigel [80-87]. However, although these materials are largely based on the native ECM components, they were mainly formulated to served as matrices for PSC instead of neural cell cultures [82]. Extensive materials have also been investigated and modified to incorporate diverse chemical or physical cues to reconstruct the neural tissue environment with enhanced authenticity. Briefly, both hydrogel and solid materials, such as poly(ϵ -caprolactone) (PCL) and poly(lactic-co-glycolic acid) (PLGA) [88-90], have been widely explored for constructing neural tissue models [48, 91-95]. As compared to hydrogels, solid materials are more easily amendable to impart topographical cues to direct cell alignment and axonal growth. However, it is less straightforward to use these solid materials to provide support for 3D (vertical) cell/tissue growth. Depending on the specific biological interest, both natural and synthetic hydrogels have been carefully defined to mimic the physiological environment [96, 97], including alginate [80, 98-100], gelatin [101], polyethylene glycol (PEG) [31, 102, 103], GelMA [104], collagen type I [19, 32, 80, 81, 105], Matrigel [106, 107], chitosan [101, 108, 109], agarose [32, 110], silk fibroin [33, 111], Hyaluronic acid [82, 104, 112], Methylcellulose [113], etc. In addition, functionalized peptides such as RGD, IKVAV and YIGSR or proteins including laminin (LN) and fibronectin (FN) have been assembled with bioinert hydrogels such as PEG to provide specific binding sites for cell attachment growth [114-117].

Each material has its own pros and cons. In this respect, readers are referred to [48, 91, 118] for further details regarding the development in materials used for neural cell culture.

Chandrasekhar *et al.* investigated the synergistic effects of matrix (type, composition, stiffness, architecture) and signaling molecules (retinoic acid (RA) and sonic hedgehog (Shh)) on mESC differentiation [119]. Specifically, they compared neuronal response when cultured on 2D substrates (namely collagen type I; gelatin; laminin; poly-D-lysine (PDL), fibronectin and non-coated control group) vs 3D scaffolds (namely 2 mg/mL collagen-type I (pH 5.5, 7.4, 9); HA (1, 2, 5 mg/mL); and Matrigel (100%, 50%, 25%); gelatin and peptide-RAD16-II) in the presence or absence of RA. Correspondingly, robust neural differentiation and neurite outgrowth were observed in 3D Matrigel and 2D collagen coated surfaces. When the effects of hydrogel moduli were considered, individually, collagen (pH 7.4 and pH 9), matrigel-100% and HA 5 mg/mL, which all had similar modulus as brain tissues (0.5 kPa to 1 kPa), exhibited better neural differentiation in comparison to other groups. This systematic study provided instructive guidance for engineering 3D neural tissue environment.

3.2.1.2. Mimicking neuronal networks formation

Tang-schomer *et al.* assembled a cerebrum-like tissue using a highly porous layered silk protein-based scaffold that was coated with polylysine as the “grey matter”. They then combined the structure with collagen gel to fill up the central zone of the silk scaffold in order to mimic the “axon-only white matter” [19] (Figure 3 (i)). Through optimizing various parameters including cell seeding density, matrix components, and stiffness, they were able to maintain viable and functional macroscopic neural tissue blocks (millimeter scale) for 9 weeks. The silk-collagen scaffold possessed similar mechanical property as that of mouse and rat brains. As a result, robust axonal development and network interconnectivity were detected in the silk-collagen group. In contrast, neurons were randomly dispersed in pure collagen group. The results demonstrated the critical effects of matrix mechanical stiffness on neural network formation. The scaffold was further utilized to simulate TBI with a weight-drop method. Notably, by monitoring the post-injury neurochemical changes, electrophysiological activities and cellular damages, these cortical-like tissues resulted in network hyperactivity that was reminiscent of the *in vivo* brain in a model of TBI. This study provided a straightforward model to emulate the neural network formation and the key features of brain cortex. Further incorporation of glia cells could be done to improve the physiological relevance and provide deeper insights into neural-glia cell interactions.

Figure 3. Engineering-based models provide better control over matrix composition and organization. (i) Modular design of engineering scaffolds (A) to epitomize brain cortical tissues with selected biomaterials (collagen and silk) and tissue growth condition within the matrix. Scaffold with compliant mechanical properties yielded neural network formation (B,C) (β -tubulin in green and MAP2 in red) [19] (reproduced with permission). (ii). Engineering method (3D patterning technique using PDMS microchambers) to organize collagen fibers (A) and direct neurite elongation (B). C. Left; The fluorescent image at 14 DIV indicated the network formation (MAP2 in green, dopamine neuron marker TH in red, and Hoechst 33258 nuclear staining in blue). Right: zoom in picture shows a merge of the neurite area [120] (reproduced with permission).

3.2.1.3. Modelling neurological diseases

Functional materials with/without extrinsic factors have been found to be promising in recapitulating the key features of neurological diseases [13, 19, 22, 92, 113, 116, 121]. Zhang *et al.* differentiated human neuroepithelial stem cells within PuraMatrix (PM) (laminin and a peptide that self assembles into nanofibers) and examined the cellular responses to A β treatment, as a 3D model of AD [13]. Interestingly, they specifically observed p21-activated kinase-mediated sensing of A β oligomers in their 3D models but not in conventional 2D cultures. The authors attributed this better *in vivo*- like response to the synergistic effects of the compliant hydrogel and selective biochemical cues that were utilized in the model. Also modeling AD, Choi *et al.* employed thick Matrigel as the embedding medium to culture human neural stem cells (NSCs) to overexpress AD mutations [22]. Remarkably, this 3D Matrigel culture system allowed them to document the first robust A β plaque formation and subsequent tau tangles *in vitro*, which has not been shown in conventional 2D cultures. Taken together, these studies provided compelling evidence to support the pivotal role of ECM in shaping physiologically accurate cellular behaviors.

Cell biology-based models depend on cells spontaneous organization, while engineering-based models dedicate great efforts into piecing together the necessary components to mimic the tissue microenvironment with increased complexity and fidelity [98, 107, 110, 122-128]. By incorporating well-defined cellular components and necessary chemical and physical cues that govern cell fate, these 3D engineering-inspired models are capable of presenting the cells with a more consistent and controllable microenvironment.

3.2.2. Microfluidic technology-based models

Different from other 3D culture models, the key feature of microfluidic platforms is the manipulation of fluid flow to define the culture environment. Perfusion flow, co-culture of cells, spatial control over cell patterning, ECM/material organization and soluble factor gradients generation can be achieved via microfluidic platforms [129, 130]. Apart from these added values of microfluidic devices, they are typically fabricated by lithography with great repeatability, high resolution and excellent optical clarity [131]. As such, these structures have been increasingly employed in studies that focus on mimicking the cortical structure [110], directing neurite growth [106], modelling brain environment such as the blood-brain barrier (BBB) [132] and recapitulating the predominant features of neurological diseases [54, 66, 133, 134].

3.2.2.1. Generating biomimetic gradients

Gradients of ECM components, mechanical stiffness and signaling factors have great impact on biological processes [30, 135], such as axon guidance [136], embryogenesis [137], chemotaxis [138]. Therefore, involvement of gradation is expected to maximize the authenticity of the *in vitro* models [139]. By manipulating the flow parameters, microfluidic systems provide intricate spatial control over cells, matrices and biochemical cues [104, 110]. Pedron *et al.* generated a 3D model that contained spatially graded hydrogel (a mixture of hyaluronic acid (HA) and gelatin methacrylate (gelMA)) and U87 cells to model the heterogeneous nature of glioma microenvironment [104]. The device was composed of two parts, a microfluidic diffusive mixer and a connected mold. Gradients in cell density, matrix composition and structure were then generated by injecting precursor suspensions of HA and gelMA with varied cell density. This method provided an effective path to form gradients in matrices and cell density across biomaterials and further investigate the effects of these parameters in directing cell fate.

Figure 4. (i). Microfluidic platform (A) to mimic neural circuit formation (B), visualized with Calcein AM and Hoechst. scale bar: 200 μ m. and to facilitate synapses formation (C) (Neuronal cells stained with synaptophysin in red, PSD95 in green at day 12) [106] (reproduced with permission). (ii). Microfluidic platform for modelling Alzheimer's disease. The image showed the comparison of the three-dimensional brain-on-a-chip with or without the presence of an interstitial level of flow. The interstitial flow enhanced the synapse formation (synapsin IIa in red), the differentiation of NPCs (Nestin in green) into neurons (β -III tubulin in red) and the protein aggregation when treated with A β (thioflavin S in green). scale bar: 100 μ m [66] (reproduced with permission).

3.2.2.2. Mimicking neuronal networks formation

Bang *et al.* generated a 3D microfluidic model of a simplified neuronal circuit with primary rat cortical neurons [106]. The device was filled with Matrigel and fluid flow was delivered to align the Matrigel structure along the flow direction during gelation (Figure 4 (i)). Consequently, neurites grew into the Matrigel at an average speed of $254.2 \pm 6.5 \mu\text{m/day}$, resulting in approximately 1500 μm long axon bundles being formed after 6 days *in vitro* (DIV). The authors delineated a quantitative model that described the kinetics of long axon bundles formation and elongation along the direction of the flow, suggesting an instructive role of the orientation of ECM on axonal guidance and fasciculation.

3.2.2.3. Modelling neurological diseases

Other than neurite growth, microfluidic technologies can also be integrated with developmental biology to study neurodegenerative diseases [66, 133]. By incorporating spheroid models on a microfluidic device, Park *et al.* exploited

fluid flow to emulate the interstitial flow of cerebro-spinal fluid to investigate the AD environment [66]. A microfluidic chip with 50 cylindrical concave microwells (diameter, 600 μm ; height, 400 μm) was fabricated for spheroid (prenatal rat cortical neurons) formation, and an interstitial flow was applied simultaneously (Figure 4 (ii)). In order to model AD, the spheroids were treated with A β . The results demonstrated that the neural network formation in neurospheroids was significantly reinforced by fluidic flow. More strikingly, greater infiltration of A β to the central zone of the neurospheroid was observed under dynamic culture conditions. In addition, cell viability was greatly reduced as compared to static cultures, and the results were consistent with the previously reported neurotoxic effects of A β . Taken together, the integration of spheroids and microfluidic technology yielded a 3D *in vitro* model with more relevant physiological outcomes.

Although not many studies on 3D neural tissues via microfluidic technology have been reported, the above examples revealed the potential of microfluidics for constructing 3D *in vitro* models that bear higher fidelity and possibility of control over culture conditions of the designed work. Despite the great potential, several limitations still remained. The introduced fluid flow needs to be well controlled. Otherwise, the bubbles that are generated during the fluid flow process may compromise cell viability [140]. Additionally, although the intricate design of the fluid flow is facilely manipulated *in vitro*, it may be difficult to introduce to *in vivo* or clinical applications.

4. Advancement of bioprinted neural tissue models

Bioprinting is an offshoot of 3D printing technology. It incorporates biomaterials, cells and bioactive molecules to construct living tissues with superior spatial precision. Driven by the hypothesis that precise cell arrangement can give rise to physiologically relevant cues and subsequently generate functional tissues, bioprinting has been broadly investigated to create heterogeneous tissue models [141]. In contrast to the traditional method of two-step scaffold printing followed by cell seeding, bioprinting deposits bioink that contains both materials and cells simultaneously. Consequently, tissues are constructed in a single step. Cell biology-based models require professional skills to deal with the cells, while bioprinting works in an organized and automatic manner [142-146]. This enables the generation of heterogeneous tissue models with great consistency, which greatly benefits downstream high throughput drug testing and clinical applications.

4.1. Printing techniques

The major platforms for bioprinting include droplet-, extrusion- and laser-based printers (Figure 5) [147-150]. According to different driving forces, droplet-based printing can be categorized into inkjet printing (thermal, piezoelectric, electro hydrodynamic jetting and electro static bioprinting), acoustic droplet ejection, micro-valve printing [151-156]. Typically, droplet-based printing is applicable for materials with low viscosities ranging from 3.5 to 70 mPa•s, while extrusion-based bioprinting is able to print materials within a broader range of higher viscosities (30 mPa•s - 6×10^7 mPa•s) (as shown in Table 4) [157, 158]. In addition, extrusion-based bioprinting also involves lower cost and is a relatively simpler method. The material extrusion process can be either pneumatically or mechanically driven. On the other hand, laser-based bioprinting, although highly expensive, is the most accurate and precise method. It is capable of manipulating individual cell and arranging cells into precise patterns. For a detailed review of these bioprinting techniques, please refer to [150, 159, 160].

4.2. Bioink and printing strategies

Despite the extensive materials that have been investigated for 3D neural cell culture, some of them cannot be easily manipulated by printing. Currently available materials are mainly alginate [161-163], Poly(ethylene glycol) diacrylate (PEGDA) [164], fibrin [165], chitosan [166-169], hyaluronic acid [170], silk fibroin [171-173], gelatin [174-176], agarose [177], methycellulose [178, 179], collagen [180, 181], etc. Several detailed reviews have illustrated the properties of these available bioinks from various perspectives [160, 182, 183]. Different from the traditional cell encapsulation process, in which cells are flooded with a bulk of materials or biochemical signals, bioprinting requires the bioinks to have proper gelation speed which matches the printing speed [184]. In addition, for materials with low viscosity, z direction stacking is often impeded. To address this issue, several strategies have been applied to modify and optimize bioink printability and structure fidelity. Here we draw on a few specific examples to elaborate on some of the most commonly used strategies.

Table 4. Bioprinting techniques

Bioprinting techniques	Types		Printing resolution	Material viscosity	Capability	Advantages	Disadvantages	Ref
Droplet-based	Inkjet printing	Piezoelectric inkjet printing	10 ~ 200 μm	3.5 ~ 70 mPa·s	<ul style="list-style-type: none">Drop-on-demand printingContinuous filament deposition	<ul style="list-style-type: none">High resolution	<ul style="list-style-type: none">Cell sedimentation clogging	[185-188]
		Thermo inkjet printing						[165, 189]
		Electro hydrodynamic jetting						[190, 191]
		Electro static bioprinting						[192]
	Acoustic droplet ejection		5 ~ 300 μm			<ul style="list-style-type: none">High resolution	<ul style="list-style-type: none">Limited material choices	[152, 193]
	Microvalve-based printing		100 ~ 500 μm			<ul style="list-style-type: none">Handy to control and easy to pattern biologicsBroad choice of materials	<ul style="list-style-type: none">Cell sedimentationLow resolution	[194]
Extrusion-based	Mechanical-driven		50 ~ 500 μm	30 mPa·s to > 6 × 10 ⁷ mPa·s	<ul style="list-style-type: none">Continuous filament deposition	<ul style="list-style-type: none">EconomicalHigh cell density (spheroid)Broad choice of materials	<ul style="list-style-type: none">Low resolution	[195]
	Pneumatic-driven							[196]
Laser-assisted	Laser guided direct writing		~ 10 μm	1 ~ 300 mPa·s	<ul style="list-style-type: none">Single cell manipulation	<ul style="list-style-type: none">High resolutionNozzle-free printingEase of handlingBroad choice of materials	<ul style="list-style-type: none">ExpensivePossible to transfer cytotoxic materials	[197, 198]

Figure 5. Bioprinting techniques (i) extrusion-based printing; (ii) droplet-based printing (inkjet printing) ; (iii) laser-based printing

Possible strategy to improve the printability of materials is to crosslink the material partially prior to printing. For instance, the inclusion of a small amount of calcium ions could increase the viscosity of alginate, allowing alginate to be more printable [162, 199]. However, this may in turn compromise cell viability due to the increased osmotic pressure. Therefore, the ratio of crosslinker should be carefully managed. Another notable study was conducted by Zhou *et al* [200], they exploited microbial transglutaminase (MTGase) as catalyst to partially crosslink GelMA. Evidenced by the rheological study, GelMA with MTGase treatment exhibited increased viscosity, therefore, better printability than pure GelMA. For UV sensitive materials such as GelMA, hyaluronic acid (HA) methacrylate, PEGDA, partially crosslinking using UV source have been frequently used as well [164, 201, 202].

Figure 6. Printing strategies.(i).nanosilicates enhanced GelMA printing (reproduced with permission from Ref. [203]). (ii).Support bath-based FRESH printing method (reproduced with permission from Ref. [204]) (iii) Complex printed structures printed with in co-axial nozzle (reproduced with permission from Ref.[205]). (iv). Co-axial nozzle printing of Low viscosity material deposition (reproduced with permission from Ref. [206];

Another widely utilized method to improve the printability is blending, which leverages on the unique properties that are offered by multiple materials [146, 205]. For instance, pluronic is known to be thermoresponsive and undergoes gelation at 37°C. By mixing pluronic with alginate [146], the blended hydrogel exhibits greatly enhanced shape fidelity in comparison to pure alginate-based constructs. The bioprinted structure was initially stabilized by the pluronic component and then further fixed by the addition of calcium ions, which crosslinked alginate to offer additional strength. Additionally, nanomaterials have been frequently utilized as rheological modifiers [199, 203, 207-209]. For instance, pure 5% GelMA demands tight temperature control for bioprinting, while with the addition of nanosilicates (0.5% ~ 2%), the mixture exhibited increased viscosity and reinforced mechanical property (Figure 6 (i)) [203].

Printing in a removable bath or using sacrificial materials as supports also enhances printability and shape fidelity [210-214]. In this method, these fugitive materials and inks should be compatible with the bioink and cells, and must be easily removable without cytotoxic byproducts. An innovative method, called FRESH, was recently introduced by Hinton *et al* [204]. Standing for freeform reversible embedding of suspended hydrogels, FRESH utilized a thermoreversible bath (composed of gelatin microparticles) as an extrinsic support to allow internal printing of structures with increased complexity and flexibility (Figure 6 (ii)). The authors demonstrated the efficacy of the support bath by printing fibrin, collagen and Matrigel with complex features such as femurs, hearts and brains with a resolution of 200 µm. The results highlight the potential use for building soft neural tissues with anatomical structures.

On the other hand, printing with co-axial nozzle has also garnered great attention recently [164, 205, 206, 215-218]. Gelation occurs when solutions like alginate and fibrinogen are exposed to their respective crosslinkers. Therefore, simultaneous deposition of the solution and crosslinker through a co-axial nozzle results in instant gel formation at the tip of the needle. By utilizing different configurations in the inner and outer nozzles, crosslinking of materials with low viscosity [206], hollow tubes [164], and core-shell filaments of multiple materials could be achieved (Figure 6 (iii) (iv)) [219].

Other strategies such as imposing temperature control or printing microsphere-embedded gels also showed some promising results on printability improvement and structure stabilization [174, 220].

4.3. Application of bioprinted 3D neural tissues

In the following section, we focused on the applications of bioprinting of neural tissues and discussed the advantages and potential benefits in comparison to the above mentioned models. We identified the major roadblocks in neural tissue modeling, and proposed how such barriers might be overcome in the future (as shown in Table 5).

4.3.1. Multicellular construct

The synergistic effects of compression, tension and shear force, which are generated by various parameters such as materials viscosity, dispensing pressure and nozzle diameter, are inevitable during the bioprinting process. Hence, many initial studies have focused on evaluating the retention of cell viability and functionality after bioprinting. Cell survival rate is one of the most basic assays to evaluate the bioprinting process. An early study by Xu *et al.* investigated the effects of thermal inkjet printing on neural cell viability [221]. Specifically, bioink containing rat embryonic motor neurons in PBS solution was deposited onto soy agar and collagen gel in a pre-defined ring pattern. The results indicated that neural cells are printable. In a later study, Xu *et al.* investigated whether the inkjet printed neuronal cells maintained their functionality [189]. Specifically, cells from a neural crest-derived neuroblastoma line and from embryonic rat brain were ejected to a pre-defined single layer of collagen hydrogel by inkjet printing. The printed cells showed 75% viability after 8 days of culture and exhibited functional electrophysiological properties. Thereafter, the group constructed multilayered neural sheets with fibrin gel, and correspondingly, neurite outgrowth was observed after 12-day of *in vitro* culture. Taken together, these results demonstrated the feasibility of printing neural cells by inkjet printing and suggested the potential of constructing neural tissues using inkjet printing technology.

Mathematical models have also been established to calculate shear stresses that are generated during the extrusion-based and droplet-based printing processes [222, 223], which indicated the synergistic effects of hydrogel viscosity, printing pressure and nozzle diameter on the printing process (e.g. shear stress, printing resolution). Generally speaking, larger nozzle diameters, lower hydrogel viscosity and lower printing pressure would help to prevent cell damage from shear stress during printing.

A major advantage of bioprinting over traditional cell culture methods is the automatic and precise cell arrangement [224, 225]. Recent studies have demonstrated bioprinting of more than one cell type into defined patterns in an attempt to mimic the heterogeneous nature of tissues [186, 188, 226, 227]. Lee *et al.* presented the direct inkjet

printing of 3D multilayered collagen gel with well patterned rat embryonic astrocytes and neurons (Figure 7 (i)) [226]. In this research, sodium bicarbonate solution was applied to collagen as nebulized aerosols to stabilize the multilayered scaffold. Both of the cell types showed similar viability as the control group (manual culture), indicating that cell viability was not greatly influenced by the printing process. Importantly, the patterned neurons showed neurite outgrowth and neural connectivity in three dimensions. In a separate study, Lorber *et al.* used piezoelectric inkjet printing to print adult rat retinal ganglion cells (RGC) and retinal glia, including astrocytes and Muller glia [186]. The effects of the printing process on RGC/glia cell viability and neurite growth were characterized. The results demonstrated that as compared to manual 2D culture, the printed structures showed similar cell numbers and neurite growth, revealing the great potential to construct neural tissues through inkjet printing.

Figure 7. Bioprinting for neural applications. (i) Bioprinted patterns with multiple cell types (neurons and astrocytes). A. Schematics of single-layer and multilayer patterning B. 3D multilayered patterning of the cells in six layers of collagen. C. Cocultured neurons and astrocytes in a single-layer collagen scaffold. (reproduced with permission from Ref. [226]). (ii). The image shows the differentiation ability of bioprinted iPSCs into neural lineage 40 d postprinting including 30–37 d of neural induction and differentiation. (reproduced with permission from Ref.[196]). (iii). Bioprinting of human NSC to construct neural tissues. NSC (stained with SOX2) are differentiated in situ to GABAergic neuron (C: GABA in green and GAD in purple) and supporting glial cells: astrocytes (GFAP in purple) and oligodendrocytes (OLIGO2 in green). (reproduced with permission from Ref.[195]). (iv) Printed brain-like layered structure with neurons in the top and bottom layer, cell-free in the middle layer. A.

colored printed construct with RGD-GG. B. Confocal microscope images of neurons in different layers after 5 days of culture. (reproduced with permission from Ref.[228]).

Although cells that were utilized in the above mentioned studies were all from animal sources, these animal cell-based experiments have generated promising results and allowed further extension to human cells. However, human cell-based studies are scarcely found. One notable research about printing human cells was carried out by Gu *et al.* Specifically, they reported the direct printing of a 3D porous grid structure which incorporated human neural stem cells with a novel bioink. This bioink, which was composed of alginate, agarose and carboxymethyl-chitosan (CMC) supported the proliferation and differentiation of the neural stem cells (Figure 7 (iii)) [196]. Correspondingly, the printed structures were permissive to functional maturation of the differentiated neurons, as they formed synaptic connections and established spontaneous network activities. Nevertheless, bioprinting studies using human cells are limited. This may be primarily due to the lack of accessibility to human neural tissues. However, recent advances in iPSCs technology and neural differentiation protocols offer a potential solution to this problem. The bioprinting process has been proven to be friendly to hESCs and hiPSCs, as demonstrated by several recent studies [174, 229, 230]. Specifically, Alan *et al.* examined both viability and pluripotency of hiPSCs and hESCs after valve-based bioprinting. All hPSCs exhibited an overall viability of over 70% and showed negligible difference as compared to non-printed cells. In addition, when the cells were subsequently exposed to hepatic differentiation media after printing, they became nuclear factor 4 alpha positive and secreted albumin. The results indicated the successful differentiation of hPSCs into hepatocytes. Taken together, the study also suggested the possibility of differentiating cells into neural lineages by using the appropriate differentiation conditions.

4.3.2. 3D structure

The ability to deposit cells and materials at a desired place by bioprinting enabled the construction of anatomical structures, such as ear [210], skin [21], and brain-like constructs [228]. Lozano *et al.* generated a peptide-modified gellan gum (RGD-GG)-based brain-like layered structure by extrusion-based bioprinting [228]. CaCl_2 was used to crosslink the hydrogel and stabilize the multi-layer structure. The results revealed a highly porous structure of the RGD-GG which offers excellent nutrient and oxygen supply and showed more positive effects on cell proliferation and network formation as compared to purified GG [228]. Leveraging on this, they established a three-layer construct that incorporated primary cortical neurons to mimic the brain cortical structure (as shown in Figure 7 (iv)). The

structure included both cellular layers (top and bottom) which had a cell population of 1×10^6 respectively, and an acellular layer in the middle. Over a 5-day culture period, extensive axonal arborization penetrated into the acellular layers, indicating that the bioink was favorable to neurite growth through different layers. These results highlighted the possible control over cells and ECM organization using bioprinting.

In a separate study conducted by Hsieh *et al*, lattice structures were printed with synthesized thermoresponsive biodegradable PU (polyurethane) hydrogels in order to construct a 3D environment for NSCs to study neurological diseases[231]. The PU was crosslinked spontaneously at 37°C, without any toxic crosslinker. The results showed that 25% PU based on poly(ϵ -caprolactone) diol(PCL diol) and poly(D,L-lactide) (PLLA diol) was optimal for promoting cell proliferation and differentiation . Furthermore, the group evaluated the efficacy of their construct by implanting a 3D printed 25% PU construct into adult zebrafish with TBI. After 6 days, animals showed good recovery according to the rescue rate during swimming. Hence, the results demonstrated that PU is an appropriate carrier of NSC and suggested that bioprinting could be a promising alternative for neural transplantation and regeneration. However, PU has a low elastic modulus of ~ 680 Pa. Hence, the lattice structure collapsed after building 8 layers due to insufficient mechanical integrity which needs to be further optimized.

4.3.3. Incorporation of biochemical gradients

Bioprinting enables precise patterning of biochemical cues in a well-organized manner to direct cell fate [165, 232]. Lee *et al*. investigated bioprinting of hydrogels with time-dependent release of growth factors (GF) [165]. Specifically, the authors first patterned neural stem cells in a rectangular shape ($L \times W = 3 \times 2$ mm²) onto a collagen gel substrate. Subsequently, VEGF enriched fibrinogen was printed next to the cells, followed by the printing of thrombin at the same position to crosslink fibrinogen. After that, a layer of collagen gel was printed on top to enclose both the cells and the crosslinked VEGF-containing fibrin gel to prevent VEGF from rapidly diffusing into the surrounding media. Thereafter, the authors monitored cell proliferation, morphology and migration towards the VEGF-releasing fibrin gel. The results showed that cells actively migrated towards the VEGF-containing fibrin gel and exhibited a more complex morphology, observations that are consistent with the known chemottractant role of VEGF. Remarkably, cell migration was sustained over 3 days, indicating that the soluble GF was effectively confined within the fibrin matrices and presumably exerted its function through a time-dependent release mechanism. However, a detailed time course measurement to quantify VEGF is still required to confirm this hypothesis. In sum, this simple

yet elegant study not only demonstrated the feasibility of printing different types of hydrogels (pH-dependent collagen and enzymatically-crosslinked fibrin), but more importantly, provided a prototypic example of engineered neural constructs with controlled release of GFs. By precise patterning of signaling factors, bioprinting can be utilized to induce desired cellular responses and direct stem cell fate.

Table 5. Overview of bioprinted *in vitro* neural models.

Key Features	Printing method	Cell type	Cell viability	Cell density	Printing parameters	Material	Structure	Structure integrity/ crosslinking method	Size	Mechanical property	Physiological study	Ref
3D tissue structure	Thermal Inkjet Printing	Primary hippocampal and cortical cells, NT2 neuronal precursor cells	Hippocampal cells: $74.2 \pm 6.3\%$	2×10^6 cells/ml	Resolution: $85 \mu\text{m}$	Cell suspension single layer: collagen as substrate neural sheet: fibrin gel as substrate	Single layer/ multilayer neural sheets	Fibrinogen crosslinked by thrombin	$25 \text{ mm} \times 5 \text{ mm} \times 1 \text{ mm}$	Tensile modulus $2.92 \pm 0.82 \text{ MPa}$	Electro-physiological	[189]
	Extrusion-based printing	Cortical neurons	$73 \pm 8\%$	1×10^6 cells/ml	Nozzle: inner diameter 0.2 mm and outer diameter 1 mm	RGD-GG	Three discrete layers (the middle layer without cells)	CaCl_2	1 cm in height	N.A.	---	[228]
	Extrusion-based printing	NSC	$> 50\%$ at 24 h $> 100\%$ at 72 h	4×10^6 cells/ml	Nozzle d: $250 \mu\text{m}$	Polyurethane	Layer by layer lattice (max 8 layers)	Thermo-responsive	$1.5 \text{ cm} \times 1.5 \text{ cm} \times 0.15 \text{ cm}$	$\sim 680 \text{ Pa}$	Zebrafish <i>in vivo</i> experiment	[231]
	Extrusion-based printing	hNSC	$\sim 73\%$	1×10^7 cells/ml	Nozzle d: $200 \mu\text{m}$	Alginate, carboxymethyl chitosan, agarose	Lattice structure	CaCl_2	$10 \text{ cm} \times 10 \text{ cm} \times 10 \text{ cm}$	Compression modulus: 7.5 kPa , Indentation modulus: 4.75 kPa	---	[195]
		iPSC	Negligible death	8×10^7 cells/ml								[196]
Multicellular construct	Thermal Inkjet Printing	Astrocytes and neurons	Neuron: $78.6 \pm 0.6\%$ Astrocyte: $78.1 \pm 10.0\%$	neuron: 3×10^6 cells/ml astrocyte: 1×10^6 cells/ml	Resolution neuron: $150 \mu\text{m}$ astrocyte: $300 \mu\text{m}$	Collagen	Multilayer (8 layers)	Sodium bicarbonate solution	ring pattern : $d = 3 \text{ mm}$ cross pattern: 6 mm long orthogonal lines	N.A.	---	[226]
	Piezoelectric Inkjet Printing	RGC, Glia	Glia: $69 \pm 12.2 \%$ RGC: $69 \pm 5.3 \%$	---	Retinal: $50 \mu\text{m}$ nozzle glial cells: $80 \mu\text{m}$ nozzle	Cell suspension (culture medium)	---	---	---	N.A.	---	[186]

	Piezoelectric Inkjet Printing	NG108 neuronal cells and SC	NG108: 86%–96% SCs: 89% ~ 92%	2×10^5 cells/ml	Nozzle d : 60 μ m	Cell suspension (culture medium)	Single layer (printed into 12 well plate)	---	---	N.A.	---	[188]
Incorporation of biochemical gradients	Thermal Inkjet Printing	Murine NSC line C17.2 (signaling factor: VEGF)	$92.89 \pm 2.32\%$	1×10^6 cells/ml	Resolution Collagen: 500 μ m, Cell : 700 μ m	Collagen, fibrin	Double-layered 3D collagen scaffold	Thrombin	14×14mm ² 50um per layer	N.A.	---	[165]
	Inkjet printing	Signaling factor: FGF2, CNTF, FBS (primary fetal NSC seeding)	---	---	---	Poly-acrylamide-based hydrogels as a substrate	---	---	---	N.A.	---	[232]
Luminal structure	Extrusion-based printing	BMSC SC	---	---	Nozzle d : 500 μ m	Agarose (sacrificial material)	Triple-lumen tube	Spontaneous <i>in situ</i> gel	1 cm long, each lumen d = 500	N.A.	Electro-physiological, <i>in vivo</i> experiment with rat	[142, 177]
Bioprinting of stem cells	Extrusion-based printing	mESCs	82% ~ 90% depend on nozzle diameter	3.3×10^6 cells/ml	Nozzle d : 160, 260, 410, 510 μ m	Gelatin/alginate	Grid structure	Nozzle temperature control (5°C), CaCl ₂	8 mm × 8 mm and height of 1mm, thread: 728.2 ± 24.9 μ m gap: 424.3 ± 17.8 μ m	N.A.	---	[174]
	Cell printing integrated with hanging drop method	mESCs	99%	0.1×10^6 cells/ml, 0.5×10^6 cells/ml, 1×10^6 cells/ml	Droplet size 1, 4, 10, and 20 μ l	Cell suspension (culture medium)	Single layer of droplets	---	Droplet size 1, 4, 10, and 20 μ l ,	N.A.	---	[230]
	Valve-based printing process	hiPSCs, hESCs	hESCs: >80% hiPSCs: >60%	1×10^6 cells/ml	Internal diameter of 101.6 μ m	RGD-coupled sodium alginate	Rings, 40 layers	CaCl ₂	Ring structures (d approx. 4 mm)	1.5% w/v Sodium Alginatecrosslinked with 600 mM (6%) 10–20 kPa	/	[194]

FGF2: fibroblast growth factor-2
CNTF: ciliary neurotrophic factor
FBS: fetal bovine serum

5. Challenges and future prospects

To mirror the physiological environment of native tissue, it is crucial to understand how cells behave in the extremely dynamic microenvironment. Three-dimensional *in vitro* culture systems including cell-based models and engineering-based models have made progress in understanding the developmental events of neural tissue. However, insufficient control over cells and signaling molecules leads to deficiencies in capturing cell-cell and cell-ECM interactions. Bridging engineering, material science, and biology, bioprinting is uniquely poised to harness the strengths of each field and exploit the collective efforts to revolutionize biological tissue engineering. While spectacular advances have been achieved in several tissues such as the skins, bones, ears and blood vessels, bioprinting of neural tissues is still at its infancy.

A critical challenge in constructing tissues and organs *in vitro* lies in presenting the cells in a precise and spatially controlled fashion. Technically speaking, through the layer-by-layer fabrication process, bioprinting holds great potential in replicating tissues and organs *in vitro*. However, as seen in Table 5, limited bioprinted neural tissue models are available now. In addition, studies are largely confined to cell morphology assessments, and barely extend to the evaluation of physiological functions of cells. Limitations lie in several aspects as follows:

5.1. Technical issues

Neurons are very sensitive to the surrounding microenvironment, and thus the shear stresses generated during printing cause major concerns regarding cell viability and deformation. As demonstrated in [233, 234], shear stresses are mainly affected by printing pressure, nozzle tip diameter and material viscosity. By reducing material viscosity, increasing nozzle size and selecting the minimum ideal printing pressure, shear stresses can be mitigated; however, this may, in turn, compromise printing resolution. Hence, developing a strategy to balance shear stress and printing resolution is critical.

5.2. Multiple cell types

Although several studies have co-printed multiple cell types, rather limited neural cell types (mainly rodent cortical neurons, Schwann cells, or NSCs) have been analyzed. Other cell types that are present in the nervous system, such as oligodendrocytes, microglia, astrocytes and pericytes, should be considered in order to present a

full repertoire that is reminiscent of the actual nervous system. This poses challenges to cell co-culture and additionally, to the development of suitable materials that can better support the viability and functional integrity of various cell types.

Stem cells have the intrinsic potential to differentiate into various functional cells, and spatially organize themselves through cell-cell and cell-ECM interactions. Therefore, in contrast to co-printing multiple terminal cell types, bioprinting of stem cells with the appropriate differentiation cues may allow the construction of artificial neural tissues with more complex cellular configuration and a better recapitulation of the native tissue microenvironment [232, 235, 236]. The ability to derive hiPSCs from somatic cells of any individual further facilitates the development of personalized neural tissues models.

5.3. *Cell/printing-permissive bioink formulation*

3D printing technology has allowed the generation of many sophisticated structures with fine features out of metals and polymers [237, 238]. However, when extended to bioprinting with mixtures of cells and biomaterials, 3D printing is less developed. One of the major stumbling block is the more stringent requirements for material selection. Balancing cell supportive property and printability appears to be arduous. Bioprinable materials account for only a small portion of biomaterials used for neural tissue regeneration. In addition, the fundamental requirement for a faithful 3D *in vitro* model is to present the cells with the appropriate mechanical support. In particular, it is often difficult to find a printable material that mimics the mechanical behavior of neural tissues and yet, still be able to avoid structural collapse after several layers of material deposition during 3D bioprinting. Hence, printing neural tissues can be technically challenging. Inspirations could be drawn from those existing printing strategies and bioink formulations. Specifically, hydrogels with dynamic mechanical property is of great interest. Bioinks transitioned from stiff to soft yield highly desirable property that is initially bioprintable and subsequently cell-responsive [80, 84].

5.4. *Signaling factors gradients*

Given that most, if not all, organs and tissues are formed via morphogen gradients during organogenesis, such factors are important and should be considered within printed tissues to shape the right 3D spatial orientation of cells. Since organoid models are greatly dependent on the signaling factors, exquisite control over these biochemical

cues could ameliorate the highly variable structures. Hence, bioprinted organoids could potentially yield 3D tissue models with improved consistency and desirable organization.

5.5. *Vascularization*

Vascularization of 3D tissue structures remains one of the greatest challenges for tissue engineering because failure in proper vascularization results in tissue necrosis [239]. To date, there is no available bioprinted neural tissue with vascularization yet, nevertheless, inspirations can be drawn from other relevant works. A pioneering study by Sakaguchi *et al.* successfully fabricated *in vitro* vascularized tissue surrogates through the collaborative efforts of perfusion flow and collagen-based microchannels [240]. Similar designs have been adapted for bioprinted tissue constructs. By directly printing sacrificial materials (e.g. agarose, Pluronic, gelatin bath) or through co-axial printing [164, 206, 212, 217, 241], bioprinting enables the integration of vascular networks into thick tissues. These built-in channels may act as vasculature and furthermore, may be perfused with endothelial cells to facilitate the formation of life-sustaining vascular networks [211, 241].

6. **Summary**

The increasing awareness of combinatorial effects of cells and ECM has promoted the development of 3D culture systems. Three-dimensional tissue models provide more physiologically relevant environment and hold enormous potential to unravel the enigma of neural tissue development. Nevertheless, modelling the nervous system presents great challenges due to the diverse cellular structures within which various neural cells dwell and the extreme complexity of the connectome that forms among the cells.

The past decades have witnessed rapid development and increasing applications of 3D culture models, including spheroids, organoids, engineering-based models and microfluidics, each with its own merits and limitations, as reviewed in this paper. They are all heading towards recapitulating the complex nature of native tissues, defining the key instructive components and constructing a biomimetic environment.

While organoids provide useful insights to the development details of tissues, engineering-inspired scaffold models provide better control over the reproducibility in creating tissues with desirable structures and increased complexity. On the other hand, microfluidic platforms offer dynamic culture conditions which may be more relevant

to the native environment. However, none of these methods are perfect in covering all aspects and offer limited control over the resulting tissue models. The emerging bioprinting method offers tight spatial regulation over materials, cells and signalling factors. However, despite its great potential, there are several limitations that need to be addressed. In particular, further development on bioink formulations is required in order to expand the limited list of printable materials. In addition, more robust cell technology and a better understanding of molecular gradients in native tissue are required. We envision that, in the near future, innovative biomaterials and engineering methodologies will continue to enhance 3D bioprinting capabilities. Together with advances in other disciplines such as cell aggregates, scaffolds and microfluidics, it may be possible to achieve an integrated *in vitro* system that better recapitulates the physiological behaviour and functionality of neural tissues.

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