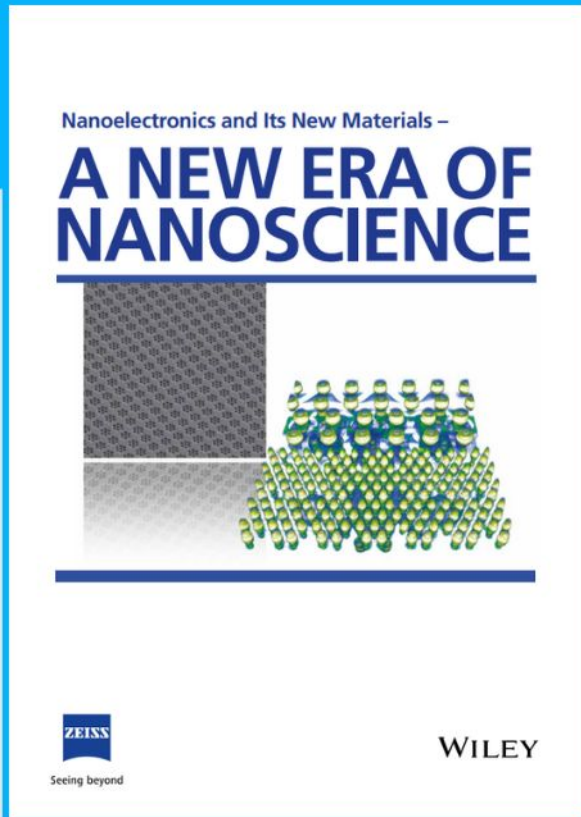




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Bioprinting Neural Systems to Model Central Nervous System Diseases

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To date, pharmaceutical progresses in central nervous system (CNS) diseases are clearly hampered by the lack of suitable disease models. Indeed, animal models do not faithfully represent human neurodegenerative processes and human *in vitro* 2D cell culture systems cannot recapitulate the *in vivo* complexity of neural systems. The search for valuable models of neurodegenerative diseases has recently been revived by the addition of 3D culture that allows to re-create the *in vivo* microenvironment including the interactions among different neural cell types and the surrounding extracellular matrix (ECM) components. In this review, the new challenges in the field of CNS diseases *in vitro* 3D modeling are discussed, focusing on the implementation of bioprinting approaches enabling positional control on the generation of the 3D microenvironments. The focus is specifically on the choice of the optimal materials to simulate the ECM brain compartment and the biofabrication technologies needed to shape the cellular components within a microenvironment that significantly represents brain biochemical and biophysical parameters.

specifically strong limitations in the *in vivo* study of the human brain. On the other side, limited source of human neural cells used to be the main obstacle for *in vitro* studies. For this reason animal models have been extensively explored as alternate to elucidate mechanisms of human nervous diseases,^[1] but they cannot answer the questions that are related to the specific individual variability, sensitivity, and complexity of the human brain.^[2] Therefore, besides animal models, we strongly need to employ *in vitro* models of human brain tissue that could substantially help the development of new therapeutic tools. To this aim biomaterial engineering, biofabrication and cell reprogramming technologies can help to design a new set of bioinspired systems that will be used in the near future to model all human brain physiology, pathology, and pharmacology.

3D culture has emerged as a new tool in the field of cell biology and physiology to study cell–cell and cell–extracellular microenvironment interactions in a more *in vivo* like situation. Cells behave rather differently in a 3D system than in a traditional 2D system in terms of morphology, viability, proliferation, differentiation, and gene expression profile that result to be closer to the *in vivo* situation.^[3] Moreover, *in vitro* 3D models serve as a platform in a more convenient, economic and high-throughput way to investigate toxicology and drug

1. Introduction

1.1. State of the Art of Neural In Vitro Models

Central nervous system (CNS) diseases, such as Parkinson's disease (PD) and Alzheimer's disease (AD), involve progressive dysfunction of distinct neuronal populations. Notwithstanding the growing amount of studies on CNS diseases, our understanding of brain functioning is still largely unsatisfactory and we have

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discovery.^[1a,4] The use of spheroid- or organoid-based methods has given rise to sophisticated in vitro tissue models such as brain,^[4f,5] which present, to certain extent, either key structures or key functions that closely resemble neural tissues. Spheroid- or organoid-based neural tissue models have the advantage to exploit internally intrinsic signaling and cues to drive spontaneous differentiation of embryonic stem cells (ESCs)/induced pluripotent stem cells (iPSCs)/neural stem cells (NSCs)/neural progenitor cells (NPCs) into a mixed population of neurons and glia.^[6] In addition, cells within a self-assembled structure secrete their own extracellular matrix (ECM), which closely resembles the desired matrix stiffness and composition of the in vivo microenvironment.^[7] Notably, it has recently been shown that brain organoids can also be polarized giving rise to neural phenotypes that resemble specific CNS territories.^[8] Limitations of the brain organoid system include reproducibility; indeed, the consistency of these models is poorly controlled in terms of size and cell viability. Moreover, most of the spheroid- or organoid-based models fail to grow beyond 2–3 mm and longer than a few months since necrosis is always found in the core area due to insufficient waste and nutrients exchange.^[9] An additional vasculature induction may allow reaching bigger sizes and longer cultivation times.^[10]

1.2. Challenges in Neural In Vitro Modeling

Notwithstanding the recent progresses in the development of neural 3D models, many hurdles are still hindering their implementation in the biomedical field. Indeed, CNS likely bears the most complex tissue architecture of our body and spontaneous growth in 3D matrix leads to the generation of tissue-like structures that can only recapitulate the early neurodevelopment stages that are therefore not helpful to model most neurodegenerative diseases. Therefore, in order to model complex neural structures (i.e., neuronal pathways), we need to have precise positional control of the neural cells and of the microenvironment complexity.^[11] Bioprinting emerges as a powerful tool to impose positional control in 3D neural models. Indeed, this technology can bring together biomaterials, bioactive factors, and cells to fabricate 3D cellular structures that mimic in vivo neural architecture characteristics.^[12] Bioink development has a pivotal role in the progress of the bioprinting field.^[13] Currently, for neural tissue engineering, natural hydrogels such as Matrigel and collagen still perform best in forming 3D cellular networks while keeping the highest cell viability.^[14] However, the batch-to-batch inconsistencies of natural hydrogels and their not-xeno-free condition limit their use in standardized protocols.^[15]

A possible alternative is based on synthetic hydrogels that can be modified to support neural cell survival and maintenance by finely tuning mechanical and chemical properties.^[16] In addition to functional bioinks, bioprinting allows spatial control of printed biomaterials as well as cells. Therefore, printing brain-like structures, which comprise different neural cell types in different layers, has become a potential approach to generate complex neural tissues.^[17] Moreover, with bioprinting techniques, a gradient of either mechanical or biochemical properties can easily be achieved.^[18] This feature of bioprinting greatly favors research in the context of neuron axon outgrowth guidance and neural



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circuits modeling. So far, several bioprinting techniques have been used for neural tissue modeling and a variety of neural tissue-like models have been constructed.^[19]

In this review, we will summarize the field of in vitro neural tissue engineering; current status of bioprinting building blocks and technologies; existing drawbacks of bioprinting and possible solutions; and potential future applications that could lead to the generation of complex neural in vitro models for biomedical applications (**Figure 1**).

2. Bioprinting Technologies for Neural Cells

Herein, we summarize the concept of bioprinting, categorize each technique based on their signature printing modality as well as their main dis-/advantages (**Table 1**), and give an outlook on the latest advances of the past 5 years in creating neural models. For further reading on the topic of biofabrication including bioprinting, we refer the reader to several

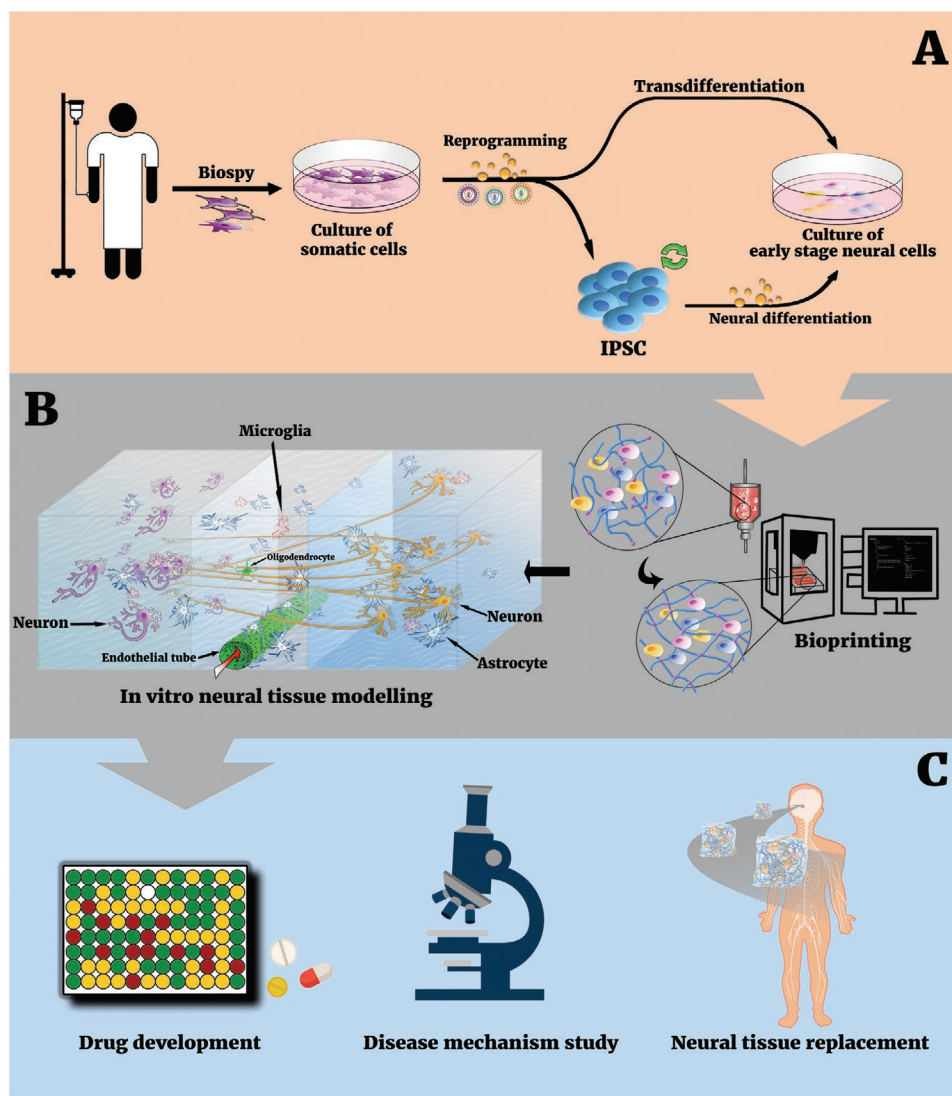


Figure 1. Schematic representation of personalized in vitro bioprinted neural tissue models. A) Derivation of neural cells from somatic cells of an individual through direct cell reprogramming or induced pluripotent stem cell (iPSC) approach. B) Bioprinting with patient-specific neural cells to generate neural tissue models conveying features of neuronal circuit and blood–brain barrier formation. C) Intended applications with bioprinted neural tissue models.

recent reviews focusing specifically on biofabrication and bioprinting,^[20] their application in in vitro models,^[21] and on the challenges toward the bioprinting of functional living tissues.^[22]

Conceptually the process of bioprinting encompasses the controlled and automated spatial deposition of a cell-enriched suspension or hydrogel formulation often referred to as the “bioink.”^[23] These printable materials are used to generate 3D biological objects starting from a customizable CAD design or from a medical image.^[24] Dedicated softwares convert the information in the CAD design into the instructions for the printer, which dispenses or patterns the bioink in a layer-by-layer fashion.^[25]

2.1. Nozzle-Based Techniques

The signature of this family of techniques is the directed deposition from a nozzle of cell suspensions, cell aggregates, or

hydrogels, either as cell-free biomaterial inks or including living cells (Figure 2). When hydrogels with low mechanical properties are used, which is a typical situation the field of neural tissue engineering, these printed structures are stabilized via crosslinking mechanisms that depend on the chemistry of the ink (i.e., ionic, pH, light- or enzymatically induced). The brain is the softest tissue in the human body,^[50] which makes neural cells extraordinary sensitive to mechanical stimuli and their environment.^[26,51] Hence, optimizing the microenvironment is an ongoing quest especially in tailoring the hydrogel composition.^[16] Particularly, stability and shape fidelity post printing are daunting challenges when using bioinks suitable for neuronal patterning, which often should display marked viscoelasticity and low stiffness. In neural tissue engineering, elastic moduli in the range 0.1–50 kPa have shown effects on neurite sprouting, morphology as well as differentiation, and preserving neuronal stemness,^[51b] whereas the

Table 1. Comprehensive overview of common bioprinting techniques and its applicability for neural modeling from the last 5 years.

| Printing modalities | Reported printed features width | Cell type | Viability readout | Advantages and salient achievements | Limitations and challenges |
|---------------------------------------|---|---|--|---|--|
| Nozzle-based | | | | | |
| Inkjet | 50–80 μm ^[26] | Rat (adult) glial and retinal cells ^[26] | Trypan blue: $\approx 69\%$ (postprinting) ^[26] | <ul style="list-style-type: none"> Delivery rate: $\approx 7 \text{ mm}^3 \text{ min}^{-1}$^[27] Fabrication rate: 1–10k droplets s^{-1}^[28] Bioink viscosity range: 2–20 mPa s^[29] Compatible with other biofabrication strategies involving extrusion of thermoplastics^[30] | <ul style="list-style-type: none"> Limited structural integrity and shape fidelity^[27,31] Reported cell numbers varied per deposited drop^[26] Low cell densities ($< 1 \times 10^6$; 2100 cells mm^{-1} (physiological) vs 20 cells mm^{-1} (printed))^[30] |
| | $\approx 300 \mu\text{m}$ ^[30] | P2–4 rat primary retinal ganglion cells (RGCs) ^[30] | Calcein AM/SYTOX: 1.2-fold increased survivability (printed vs nonprinted) if growth medium was added to the bioink formulation (postprinting) ^[30] | | |
| Extrusion-based | 200 μm ^[32] | hiPSC-derived spinal progenitor cells (sNPCs) and oligodendrocyte progenitor cells (OPCs) ^[32] | Calcein AM/EthD: 5, 15, 30 min of exposure to nonhumidified environment: $\approx 98\%$, $\approx 45\%$, $\approx 0\%$ ^[32] | <ul style="list-style-type: none"> Wide range of viscosity (30 mPa s to 60 kPa s)^[33] Achieved in situ reprogramming and differentiation^[34] High cell densities (8×10^7 cells mL^{-1})^[34a] Shown formation of intercellular connections in neuronal networks^[35] High density of cell aggregates are printable, microfluidic-chip enabled complex prints^[36] Easy accessibility: RepRap hardware^[37] Embedding bioprinting strategies (e.g., FRESH)^[38] | <ul style="list-style-type: none"> Poor structural integrity and shape fidelity in low-viscosity inks, and potential cell damage from shear forces while extruding^[27,31] Slow printing speed may lead to gel dehydration thus requiring strategies to control the printing environment^[32] |
| | $\approx 410 \mu\text{m}$ (nozzle diameter) ^[34b] | Adult fibroblasts ^[34b] | VB-48/propidium iodide (PI): $\approx 65\%$ (24 h post printing) CCK-8: $\approx 85\%$ (recovery after day 7) ^[34b] | | |
| | $\approx 200 \mu\text{m}$ (nozzle diameter) ^[34a] | hiPSC ^[34a] | PrestoBlue: proliferation over 9 days of culture ^[34a] | | |
| | $\approx 100 \mu\text{m}$ (nozzle- ϕ) ^[35] | hiPSC-derived neural precursors or cells (NPCs) ^[35] | Calcein AM/EthD: $\approx 80\%$ (postprinting) ^[35] | | |
| | $\approx 400 \mu\text{m}$ (anticipated by aggregate size) ^[36] | hiPSC-derived neural aggregates ^[36] | Calcein AM/EthD: $\approx 95\%$ (day 10), $\approx 65\%$ (day 15) Guava ViaCount (FACS): $\approx 90\%$ (day 6) ^[36] | | |
| Optic-based | | | | | |
| Laser-induced forward transfer (LIFT) | $\approx 200 \mu\text{m}$ ^[39] | E15 rat primary dorsal root ganglia (DRG) ^[39] | Live-Dye /PI: $\approx 85\%$ (24 h post printing) ^[39] | <ul style="list-style-type: none"> Successful prints performed with hyaluronic acid and Matrigel^[40] Neurite growths reported^[39] Proven in situ differentiation of bioprinted cells^[34] | <ul style="list-style-type: none"> Low cell density: ≈ 80 cells per drop^[39] Limited manufacturer diversity might affect device accessibility^[41] |
| | Not assessed ^[40] | hiPSC ^[40] | Trypan Blue: $\approx 82\%$ (2–3 h post printing) ^[40] | | |
| Stereolithography (SLA) | $\approx 190 \mu\text{m}$ ^[42] | Mouse NSCs (NE-4C) ^[42] | Calcein AM/PI: $\approx 100\text{--}70\%$, 40–120 mW laser power) ^[42] | <ul style="list-style-type: none"> $\approx 5 \mu\text{m}$ micrometer-scale resolution achievable^[43] Custom devices available^[44] Combined with 3D printing using PCL fibers^[42] Used with conductive graphene-loaded bioinks^[45] | <ul style="list-style-type: none"> Potential cell damage due to UV light exposure Laser output affects cell survival^[42] Potential toxicity from photosensitive resins and initiators^[27,31] |
| | $\approx 1 \text{ k} \mu\text{m}$ ^[45] | Mouse NSCs ^[45] | CCK-8: Proliferation over 5 days of culture ^[45] | | |
| Digital light processing (DLP) | 50–100 μm ^[46] | No current report on the bioprinting of cells of neuronal lineage | | <ul style="list-style-type: none"> Delivery rate: $\approx 20 \text{ mm}^3 \text{ min}^{-1}$^[27] High Resolutions $< 100 \mu\text{m}$ achievable^[46] Easily accessible: Commercial projectors^[46a] Biocompatible polyethylene glycol diacrylate (PEGDA) and Gel-MA resins already available^[47] | <ul style="list-style-type: none"> Potential cell damage due to UV light exposure Potential toxicity from photosensitive resins and initiators^[27,31] |
| Two-photon polymerization (2PP) | $< 1 \mu\text{m}$ ^[48] | | | <ul style="list-style-type: none"> Highest lateral resolution $\approx 100 \text{ nm}$^[48b] Suitable for nano- and micropatterning potential | <ul style="list-style-type: none"> Costly laser-based equipment^[48b] Potential toxicity from initiators^[49] |

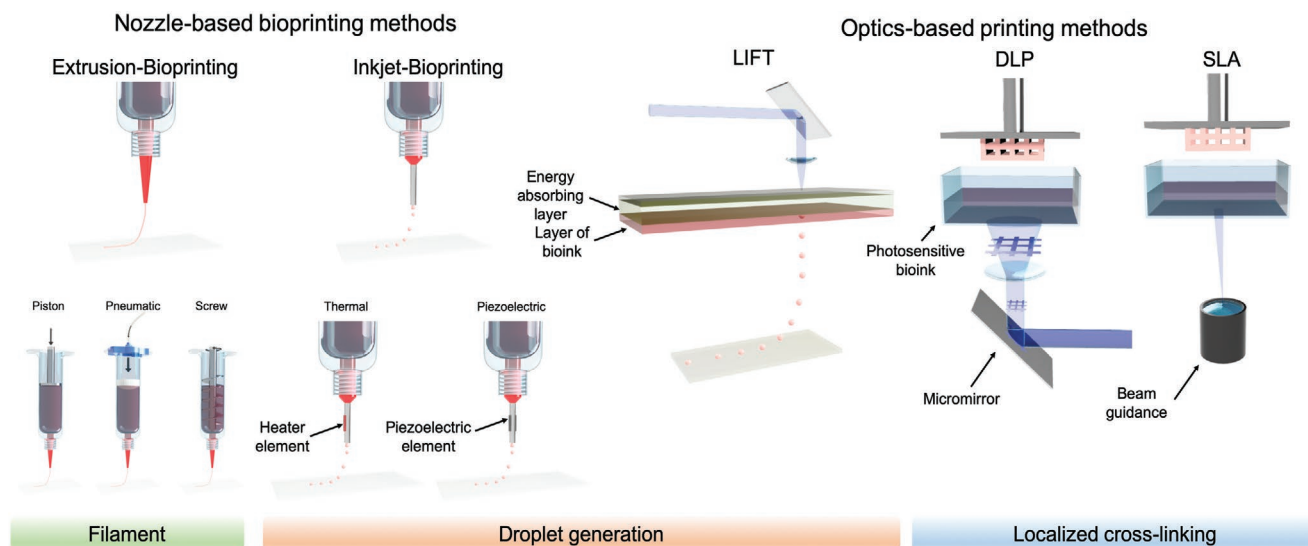


Figure 2. Common bioprinting techniques can be categorized into nozzle-based or optical-based printing methods. Nozzle-driven printing can generate either filaments or droplets, based on the modality of dispensing of the material. Filament geometries are produced by mechanical forces (applied via piston, pneumatic devices, or screw-driven extruders) while droplet geometries are obtained by localized heating or pressure increase within a nozzle. Another drop-on-demand technique (laser-induced forward transfer, LIFT) relies on a pulsed laser transferring energy in the form of heat to a water-based bioink, propelling it in the form of a droplet onto a moving collector plate. Additionally, optical bioprinting methods can be based on localized crosslinking of a photosensitive, cell-laden resin, either by means of laser scanning (SLA) or digital light projection (DLP) driven by a micromirror array.

native compressive stiffness of brain tissue has been reported to vary regionally from 0.3 to 2.7 kPa.^[52] Printing with hydrogels displaying similar properties may result in undesired deformation of the constructs postprinting. Additionally, even the printing process can result in decreased cell viability due to shear force during extrusion from the nozzle^[27,53] or dehydration.^[32] To overcome these challenges, viable strategies are the use of spheroids,^[54a] more robust cell types, or stem cells that can be differentiated into neurons postprinting,^[40] even via *in situ* reprogramming.^[34b] Likewise, cell viability can also be improved by accurately designing shear-thinning bioinks, as well as bioinks that display low-shear flow profiles,^[44] or via the selection of printing methods that permit to easily pattern low-viscosity inks for soft tissue engineering.^[38,55]

Inks with low viscosity in the range of 2–20 mPa s have been reported to be processed via inkjet bioprinting.^[29] This method relies on the formation of bioink droplets which are collected onto a plate. The formation of droplets is either controlled by an electrical-controlled thermal expansion or pressure-inducing piezoelectric crystal located in the tip of the nozzle.^[56] Since the method permits to process prevalently low-viscous materials and low cell concentrations to prevent nozzle clogging, it features an excellent viability despite the short exposure of cells to potentially harmful high temperatures or pressure while forming the droplet. Furthermore, the high speed of fabrication is beneficial for a high survivability of the cells.^[16,27] Besides early efforts of facilitating inkjet printing for hippocampal, cortical, and motor neuron bioprinting,^[57] only a few researchers have picked up the method for neural applications. Recent reports also described the successful printing of several adult rat CNS-derived cells.^[26] Although the viability of the printed retinal ganglion neurons and glia decreased to 69% after printing, the cells preserved their phenotype and showed

neurite outgrowth, a promising initial step for the establishment of neural constructs.

The most commonly used nozzle-based printing method is extrusion-based bioprinting. This technique builds their constructs by using a continuous filament or strand of cell-laden polymers. Inside a reservoir, the flow of the material is induced either by a turning screw, a piston, or on pneumatic actuation.^[27,52,56]

In contrast to droplet-based approaches, extrusion bioprinting permits us to work with higher-viscosity materials,^[13] facilitating printing with high shape fidelity. Paired with a wide range of materials, precise control of crosslinking mechanisms, and tuning of the rheological properties of the ink, this method has emerged as a dominant method of choice when it comes to bioprinting, also thanks to its accessible hardware and the presence of several open source, low-cost bioprinting devices.^[37,38] The main drawbacks of this technology are related to its low fabrication speed combined with an inverse relationship between resolution and cell viability.^[27]

The opportunities and limitations of extrusion bioprinting for neural application were showcased by Joung et al.^[32] In the pursuit of treating patients suffering from spinal cord injury with a functional 3D-bioprinted transplantable model, the authors struggled with a limited printing time window. Bioprinting under air exposure dried out the neural progenitor cells within 30 min, resulting in a 100% death rate. Hence, for optimal viability outcome, the constructs had to be printed within 15 min and subsequently submerged in medium.^[32]

In light of such limitations, latest advances in using “embedded” extrusion bioprinting may push the boundaries for neural bioprinting.^[37,38,55] In such a scenario, the bioink is extruded directly into a supportive bath made of a defined hydrogel microstructure, which behaves as a plastic fluid such as gelatine and alginate.^[38,59] This approach prevents the collapse of

3D-printed structure, then allowing us to use bioinks based on collagen and fibrin blends with alginate, which can be processed as a solution and crosslinked in the bath forming gels with an elastic modulus of ≈ 50 kPa.^[38] This approach resulted in an improved cellular viability (i.e., 86% for mouse embryonic stem cells (mESCs)).^[37] Second, embedded bioprinting techniques would avoid potential risks of dehydration that could occur during bioprinting of large constructs, in the case long printing time windows are necessary. It should be noted, however, that such a potential challenge could also be readily overcome in conventional bioprinting setups (i.e., by integrating humidity control features in the printing hardware and environment).

2.2. Optic-Based Techniques

Although nozzle-based techniques are still considered the most common bioprinting techniques, photon- or optical-based bioprinting has steadily been gaining momentum over the past decade as an alternative bioprinting approach. The main feature of these bioprinting approaches is the use of laser or projected light for a controlled deposition of cell-enriched polymers (laser-induced forward transfer (LIFT)) or the spatially selective curing of photosensitive resins by either two-photon effect, a guided single light beam (stereolithography (SLA)), or projection of the entire pattern at once (digital light patterning (DLP)) per layer (Figure 2).^[27]

Analogous to inkjet printing, cell-laden droplets of minute volume can be produced via LIFT.^[60] In this method, a focusing optical system directs a laser beam onto a donor ribbon (glass slide) coated with an energy-absorbing layer (EAL) as well as bioink. The absorbed laser energy will induce the formation of a water vapor bubble at the EAL–bioink interface that will propel a droplet of bioink onto a collecting plate, which moves in the x – y plane.

Recently, Koch et al. showed the potential of LIFT to print human iPSCs (hiPSCs)^[40] in various biomaterials (i.e., hyaluronic acid). While confirming that hiPSCs are sensitive to environmental conditions as well, the authors could exclude that the laser-printing procedure is negatively affecting the pluripotent state or viability. By utilizing a laser beam, the relative size of the droplets can reach the order of the picoliters, and thereby has a higher resolution in contrast to nozzle-based approaches, besides avoiding risks for cell viability that could be given by shear forces. Despite these advantages, the hardware for LIFT has so far been generally expensive, which limits the diffusion of this technology across different research centers.^[60]

Different from LIFT, SLA-based approaches feature a focused laser beam used to directly crosslink photosensitive, hydrogel-based resins^[41] like gelatin methacryloyl (GelMA)^[61] or SilkMA.^[62] Usually these resins crosslink in the spectral range of UV (365 nm) to visible light^[46a] and can encapsulate cells within the printed resin layer, under a photoexposure window that does not harm the embedded cells and their genetic material.^[63] Depending on the printer arrangement, the crosslinked pattern is attached to a movable stage, which subsequently lowers or raises to build objects in a layer-by-layer fashion.^[27]

While the x – y resolution of this approach depends on the laser guidance and beam diameter, the z -resolution relies on

the step size of the platform. Voxel resolution in the range of 5 μm has been achieved with custom-made printers.^[43] In a combined approach with polycaprolactone (PCL) and gelatin-coated electrospun fibers, Lee et al.^[64] printed 3D biomimetic neural tissue equivalents with directed and extended neurite growth from primary cortical neurons.

Among guided laser beam technologies, two-photon polymerization (2PP) printing has not been reported for bioprinting of neurons yet, to the best of our knowledge. On one hand, given that neural cells, particularly primary neurons, are particularly sensitive to environmental stress, photoinitiation-based toxicity could be a possible limitation. Some initiators used in two-photon bioprinting have been shown to be potentially internalized by cells and have resulted in cytotoxicity upon light exposure. This challenge can be solved by designing macromolecular initiators that cannot easily overcome the cell membrane,^[49] and such systems could potentially be beneficial also in the bioprinting of structures embedding neurons. Furthermore, this technology may find applications in neuron printing in the coming future, given the unique advantages that could be provided by the ability of 2PP to resolve features in the order of tens of nanometers.^[42] Harnessing such potential, and considering the known ability of neurons to respond to nanoscale elements also in 3D,^[65] may thus help introducing nano- and micropatterned geometrical features in the proximity of printed hydrogels embedding cells that could be used to guide neurite outgrowth network formation.^[65] Importantly, topographical features around the cells (e.g., to guide axonal alignment or cell migration) could be produced either using the two-photon process not only for printing but also for spatially controlled subtractive manufacturing. A proof of concept of this possibility was showcased by Lunzer et al.^[66] In this study, the fabrication of horseshoe-shaped microchannels with ≈ 20 μm diameter in close proximity of adipose-derived mesenchymal stem cells embedded within a hyaluronic-acid-based hydrogel was reported. By modulating the extent of degradation via altering the laser power (30–100 mW), differences in the ability of the cells to invade the produced microchannels were observed.

In order to overcome the need of a guided and focused laser beam, the bioprinting field has moved toward the projection of dynamic photomasks or patterns onto resins.^[61,67] Core component of this DLP approach is the use of digital mirror devices (DMDs), which most standard projectors feature. In principle, the DMD is an array of micromirrors, which are controlled by a computer to either reflect or deflect the light toward the projection plane. Each mirror thereby represents a single pixel in the projected pattern. Combined with a platform moving in the z -direction, similarly to what is used in SLA bioprinting, the layers are crosslinked by exposure of adequate light energy.

In contrast to SLA or 2PP, projecting entire patterns at once gains the advantage of speed on the cost of losing x – y resolution, depending on the mirror density of the DMD. Industry-grade DMDs reach resolutions in the range of < 50 μm , whereas more cost-efficient DMDs range up to 100–200 μm .^[61,67] In combination with easy obtainable light emitting diodes (LEDs) or bulbs with fixed wavelength emission, DLP bioprinting does not necessarily need to involve laser light.

To date, only a limited amount of studies applied this technique in neural tissue engineering, even though open source

DLP devices^[67] and DLP-ready and biocompatible polyvinyl alcohol–methyl acrylate^[61]/polyethylene glycol (PEG)^[47] + GelMA bioink formulations have been proposed.

Similar to 2PP, scientists have rather used DLP in the context of fabricating scaffolds for neural cell seeding approaches. In particular, PEG- and GelMA-based inks were recently used by Koffler et al.^[47] to create a biomimetic scaffold for spinal cord injury and subsequent seeding of NPCs. Upon engrafting the construct in vivo for 6 months in rats, partially to fully recover of spinal cord functionality and motile function of the animals was observed.^[47]

2.3. Bioassembly Strategies

Other biological fields continue to move into the direction of creating multicellular systems like organoids which match the critical need for a cellular-mimetic microenvironment. Arguably a fusion of organoid technology together with creating reproducible conditions and complex 3D architectures using 3D bioprinting and patterning technologies will lead the field toward a future of multicellular and geometrical complex neural systems. Right on the edge of culturing 3D organoids or spheroids in a regular dish, the idea of patterning assembled cellular blocks envisions to create complex cellular geometries without the need of long-lasting scaffolds or hydrogels therefore deemed as “scaffold-free” approaches.^[27] In comparison with the above-mentioned bioprinting techniques, bioassembly methods facilitate clustering of blocks with high cell density that can closely recapitulate the in vivo physiological conditions. The idea behind this approach ranges from precisely positioning spheroids in needle arrays^[68] to infusing compact nonfused spheroid baths with sacrificial ink.^[69] In the latter case, dense cell-made slurries ($\approx 10^8$ cells mL⁻¹) display a viscoplastic fluid behavior, and can act as a support medium within which an extrusion bioprinter can deposit a sacrificial ink consisting of gelatine. Once heated to 37 °C the sacrificial ink can be liquefied, leaving vascular-like channels within the dense cell assembly that can be used to actively perfuse nutrients.^[69] Among several cell-based structures, this method was applied also to print vessels within assembled cerebral organoids, potentially offering new opportunities to increase the size of in vitro brain models. Preformed assembly blocks with high neuron content have also been produced in the form of fibrous units produced by coaxial extrusion,^[70] elongated single neuron cultures in micromolds produced by soft lithography,^[71] or more complex cell patterns produced within millimeter-sized molds.^[72] These basic elements are assembled together into selected geometries via micromanipulation or solely by cell self-assembly, forming larger electrophysiologically functional networks that, although used so far only for in vivo transplantation,^[73] may offer a powerful tool for in vitro disease studies.

2.4. Field-Based Cell Assembly and Bioprinting Strategies

While bioassembly approaches take advantage of the self-organization capacity of cells and organoids, other techniques artificially mediate cell aggregation making use of exogenously

applied force fields based on magnetic, acoustic, and optical manipulation of cells,^[74] materials, or growth factors.

The potential of field-based approaches in neuron assembly was initially demonstrated using an acoustic sorting method termed bioacoustic levitation (BAL), applied to fabricate patterns that resemble the cerebral layering.^[75] HUES54-derived neuronal progenitor cells were mixed within fibrin and subsequently exposed to ultrasonic acoustic standing waves. Independence of the acoustic frequency, the method creates pressure fields, which manipulate the position of the cells within the gel. Within seconds this will lead to a sorting into a layered fashion, which upon culturing over a period of days (7 days) leads to intra- and interlayered neurite elongations.^[75] Magnetic field can potentially also be utilized, provided that magnetic nanoparticles or magnetosensitive ions (i.e., Gd³⁺ in solution) are supplied to a cell suspension.^[76] More recently, the use of optical fields to sculpt in 3D cell-laden hydrogels in a layerless fashion has led to the development of volumetric bioprinting (VBP).^[77] Applying the principles of optical tomography in reverse, this technique casts a series of tomographic projections onto a rotating cell-laden bioink. The cumulative light dose given by the convergence of the projections in specific voxels within the volume results in the polymerization of large cell-laden constructs within seconds.^[77] Such a rapid printing approach could solve current limitations in neuron bioprinting given by the long processing time experienced in extrusion printing by these labile cells.^[32]

3. Bioinks, Biomaterial Inks, and Cell Types for the Biofabrication of Neural Pathways

3D culture environments are a fundamental component for establishing models of multiple pathways and systems within the CNS that accurately capture the complex interactions between different neurons and stromal cell subtypes. As the first step, accurately designed biomaterials are needed to successfully guide cell response and facilitate differentiation into fully mature neurons and to promote their subsequent long-term survival. Biofabrication technologies allow controlling the spatial patterning of multiple cell types and materials through automated processes, including bioprinting and bioassembly.^[53] While cells are fundamental components of any biofabrication strategy, the building blocks in bioprinting are either carrier solutions or materials embedding cells, termed bioinks, or cell-free biomaterial inks, on which living cells are then incorporated post printing.^[78] Despite the high potential of bioprinting in capturing salient features of the 3D architecture of neural systems, the field of bioprinting of brain and CNS structures is comparatively young, compared to that of printing other tissues like skin,^[79] cartilage,^[80] bone,^[81] vascular structures,^[82] and cardiac muscle,^[83] among others. Nonetheless, several notable examples of neural tissue inks have been reported in the literature, highlighting the challenges in identifying suitable biomaterials for bioprinting neurons and NPCs and their supporting milieu. Additionally, important lessons can be derived from neural models and implantable grafts produced through more conventional tissue engineering technologies. Based on such background information, this section will highlight the

Table 2. Materials used as bioink components for the biofabrication of functional constructs capturing CNS functions.

| Main ink components | Printing method | Main findings and neuron functions observed | Ref. |
|---|--------------------------------------|---|-------|
| Cell culture media | Inkjet | Preserved high viability postprinting and ability to form neurite outgrowth comparable to nonprinted cells | [26] |
| Dulbecco's phosphate-buffered saline solution and fibrin | Inkjet | Preserved viability post-printing. Healthy electrophysiological activity, as measured with patch-clamp tests. | [57b] |
| Polyethylene glycol diacrylate (PEGDA) and gelatin methacryloyl (GelMA) | Digital light projection | Cells were seeded onto printed scaffolds, fabricated to mimic the geometry of the rat and human spinal cord Cell-laden scaffolds implanted in rodents showed axonal regeneration and partially restored impaired locomotor functions | [47] |
| Carboxymethyl-chitosan and agarose blend | Extrusion | Printed cells were able to differentiate into both neurons and neuroglia. Formation of synaptic contacts in 3D | [95] |
| RGD-modified gellan gum | Coaxial extrusion | Printing of a multilayered construct, with cells in the first and last layers. Neurons sprout processes through the middle layers, mimicking the brain cortex | [17] |
| GelMA | Extrusion | A compartment with glioblastoma multiforme (GBM) cells was encased in a printed macrophage-laden gel. GBM cells recruit macrophages and trigger their differentiation to tumor-associated macrophages. Co-culture boosts the invasion of GMB in the surrounding gel | [100] |
| Dopamine-functionalized GelMA | Extrusion | Dopamine functionalization did not boost proliferation. Enhanced differentiation of NSCs into maturing neurons | [102] |
| Fibrinogen, RGD–alginate, hyaluronan blend | Extrusion | Alignment of Schwann cells along the main axis of the bioprinted filament, via shear-induced alignment of fibrin nanofibers | [103] |
| gelatin-fibrin blend, Matrigel | Extrusion | Porous channels printed with the supportive ink and filled with Matrigel to form a pattern of alternated segments containing either NPCs or OPCs. Axonal sprouting and NPC maturation along the channel. No observed OPC maturation or axon myelination | [32] |
| Alginate/methyl-cellulose blend (supporting) | | | |
| Decellularized brain ECM | Extrusion | Observed insurgence of chemoradiation and temozolomide resistance in cells within bioprinted cultures | [107] |
| Silk fibroin | Extrusion in suspended nanoclay bath | Contextual differentiation of neuronal cells and myoblasts. Formation of synaptic contact with acetylcholine and glutamic acid stimulation of human myocytes | [104] |

most recent advances in bioinks/biomaterial inks (Table 2) for biofabrication of neural networks, as well as key biomaterial-based platforms and neural cell types that could lead to significant advances once implemented in automated biofabrication processes.

3.1. Low Moduli Matrices, High Shape Fidelity Printing, and the Shape-to-Function Relationship

Among the many classes of biomaterials investigated to act as artificial surrogates of the extracellular matrix, hydrogels are a prevalent choice as bioinks for biofabrication, due to their water-rich environment suitable for cell embedding and for the subsequent postprinting culture. Importantly, hydrogels permit the efficient exchange of nutrients and catabolites to sustain the metabolic demands of neurons. Thanks to their high water content, hydrogels can also be designed to closely resemble the extracellular environment of the brain and its typically low mechanical properties (0.1–2 kPa). Healthy human brain displays shear stiffness values between 2 and 4 kPa,^[84] and such a value can decrease, together with a loss of viscoelasticity, as hallmark of many neurodegenerative conditions, such as AD and PD, multiple sclerosis, and amyotrophic lateral sclerosis diseases.^[85] Additionally, mechanobiological response to matrix stiffness is a powerful regulator of both progenitor and differentiated cells in the nervous system,^[86] and hydrogels with elastic moduli as low as in the

range of 10–100 Pa have been used in vitro to facilitate neuronal cell sprouting.^[87] Hence, using hydrogels able to reproduce such stiffness ranges is important, not only to model the ECM interactions in healthy and pathological conditions, but also to facilitate cell migration, axonal sprouting, and growth, interneuron and neuron–stromal cell interactions. These characteristics are paramount in defining the functionality of brain circuits, and that would otherwise be hampered in stiffer, highly crosslinked hydrogels. Such design requirement renders printing 3D structures with high shape fidelity particularly challenging.^[13,88] Ideal hydrogels for biofabrication should rapidly gelate into shape stable, stiff structures postprinting. However, soft hydrogel structures, beneficial for 3D cell culture, can easily deform postprinting, either under the effect of gravity or flow due to surface tension and poor elastic properties.^[89] Advances in biofabrication have brought forward new methods to tune the rheological properties of soft inks or to modify the printing environment to ensure high shape fidelity (i.e., by printing with microfluidics printheads^[90] or by extrusion in supporting baths,^[55a] among other strategies), and such methods, of interest also for brain tissue bioprinting, have been extensively reviewed elsewhere, together with key strategies to facilitate the maturation of bioprinted constructs into functional tissues.^[22] Finally, although mechanical and rheological properties play a paramount role both in guiding cell fate^[51b,91] and determining printability, ideal hydrogels should present biochemical properties and cell adhesive domains relevant for the desired cell subsets to be cultured, as well as

permitting the transmission of electrical signals across the bio-fabricated neural networks.

3.2. Natural-Origin Hydrogels

Hydrogels derived from extracellular components can readily be recognized by cells and often offer ligands that promote cell attachment, migration, and proliferation. Additionally, they can generally be remodeled and degraded into metabolizable compounds, such as amino acids or carbohydrates. Brain ECM is prevalently composed by proteoglycans and glycosaminoglycans (GAGs) such as chondroitin sulfate and hyaluronan and link proteins, particularly in the perineural nets around neuronal processes and in the interstitial space.^[92] Proteoglycans and GAGs are expressed by glial cells and neurons, and regulate a wide array of phenomena including plasticity and inflammatory processes.^[93] Different from most tissues, the brain ECM has limited collagen content, but basal lamina components including laminins, fibronectin, and collagen type IV are present especially at the interface between the CNS and the blood vessels.^[92] In order to mimic glycan component of GAGs, many natural-origin hydrogels derived from polysaccharides, often used in tissue engineering, such as alginate, chitosan, cellulose, and agarose, have been used as bioink components for patterning neuronal cell-laden 3D constructs.

Differentiation of hESCs into precursors of midbrain dopamine neurons was achieved in 3D cultures in alginate,^[94] a material commonly used as bioink due to its shear-thinning behavior and rapid gelation kinetics in the presence of divalent cations, and tested also as the substrate for bioprinting neurons in the context of peripheral nerve repair.^[94] Blend bioinks composed of carboxymethyl chitosan and agarose were used to print hNSCs and supported cell function, as demonstrated by differentiating the cells in situ into neurons and neuroglia. Culture and maturation of the printed system resulted in the establishment of an interconnected neural network, synaptic activity, spontaneous neural activity, as well as a bicuculline-induced increase in calcium response.^[95] Likewise alginate, carboxymethyl chitosan, and agarose blends were used to print iPSCs and subsequently trigger their neural differentiation. High cell viability and pluripotency maintenance were observed.^[34a] Importantly, cells that were initially dispersed homogeneously into the hydrogel matrix migrated throughout the construct over time and formed interconnected networks of aggregates within 9 days of culture. Gellan gum, a polysaccharide of bacterial origin, which undergoes ionic crosslinking in the presence of cations, was modified to carry the fibronectin-derived integrin ligand arginylglycylaspartic acid and used to encapsulate primary cortical neurons.^[17] Using a coaxial needle, this bioink was printed as core material in the inner bore of the nozzle, while ensheathed in an outer flow of its crosslinker (a solution with cations). Printed filaments were stack in multiple layers to print a layered structure mimicking the brain cortex, in which primary cortical neurons originating from BALB/cArcAusb mice' embryos could project their axons across the different layers.^[17]

Besides polysaccharides, other ECM constituents like collagen, laminin, fibrin, and fibronectin can also be used in

bioinks as their native composition typically presents functional domains and nanoarchitectures that facilitate adhesion, guidance of neural cell morphology, and proliferation.^[18b,96] Collagen has been used extensively as a coating in 2D cultures as well as a scaffold material for the culture of neurons and glial cells in a 3D environment,^[97] with even reports of extended cultures and prolonged survival of differentiated neurons up to 73 days.^[97b] Gelatins, hydrolyzed forms of collagen, are also common alternatives in bioprinting, also due to the ease of production of such material from wastes of the food and leather industries. Given the instability at physiological temperature and rapid degradation of pristine gelatin, crosslinkable versions of this material are required for long-term cell culture. Photocrosslinkable, methacryloyl-modified gelatin (GelMA) rapidly became widespread cell carriers for bioprinting due to their rapid crosslinking kinetics and versatility in obtaining hydrogels with a wide array of mechanical properties,^[98] and recently, attention is being attracted also by thiol-ene crosslinkable gelatin variants.^[99] In the framework of recapitulating in vitro neural systems, GelMA has been used for modeling the micro-environment of brain tumors, specifically how the interplay between tumor-associated macrophages and glioblastoma cells promotes tumor invasiveness into a bioprinted mini brain.^[100] Dopamine-functionalized GelMA was also used to print mouse NSCs as well as human-derived glioblastoma cells.^[101] Although the functionalized hydrogel did not induce any noticeable effect on cell proliferation, an improved differentiation of the neural cells and enhanced expression of the neural markers β -III tubulin (TUBB3) and microtubule-associated protein 2 (MAP2) were observed, together with the formation of an interconnected neural network.^[102] Interestingly, some ECM proteins, such as collagen and fibrin, typically self-organize into micro- and nanoscale fibrillar structures that can facilitate the spreading and elongation of neural processes. In combination with extrusion bioprinting, such materials can be used to hierarchically organize printed cells, guiding their alignment not only by confining them within a printed filament, but also via the (sub)micrometer fibrillary elements that build up the bulk of the hydrogel. This concept was recently exemplified via printing Schwann cells in fibrin.^[103] Controlling the printhead velocity and extrusion pressure, it is even possible to align such ECM fibers along the printing direction, which, in turn, align the printed cells within a preferential direction,^[103] a condition desirable to create defined pathways within engineered neural networks. In addition to native and brain-derived components, ECM proteins of nonmammalian origin such as silk fibroin (SF) have been used as successful bioink component to print neural cell lines.^[104]

Finally, despite such availability of different natural materials to establish fully chemically defined bioinks, complex mixtures of ECM components, such as Matrigel, still remain a preferred culture substrate for neurons. 3D cultures in Matrigel were shown to support long-term survival of neural cells up to 5 months^[105] as well as to permit the culture of multicellular structures like midbrain-mimetic organoids.^[106] Importantly, in a bioprinted gelatin-fibrin structure with combined iPSC-derived cells, ventral NPCs and oligodendrocytes, maturation, and axonal sprouting along printed channels was observed only when Matrigel was added to the bioink, underscoring the

impact of the diverse factors that compose such materials.^[32] However, since Matrigel is derived from the basal membrane of mouse sarcoma, its use results in large batch-to-batch variability and often inconsistencies in experiments performed across different batches, questioning its efficacy and pharmacological relevance as substrate to generate reliable 3D models for studying neurological diseases. Alternatively, CNS decellularized extracellular matrix extracts could also be obtained from human or animal cadaveric donor tissues, reconstituted to form printable hydrogels. Such a type of bioink has recently been proposed to culture glioblastoma cells from human tissue biopsies in order to perform drug efficacy tests to identify the best treatment for each donor, establishing a bioprinted platform for personalized medicine.^[107]

3.3. Synthetic and Hybrid Hydrogels

Given the inherent batch variability within natural origin materials, different types of synthetic, hydrogel-forming polymers have been studied as 3D culture platforms and bioinks for neural tissue engineering. These are often used in combination with biologically derived cues such as ECM-derived proteins, GAGs, growth factors, or biofunctional peptides to form natural/synthetic hybrid systems and to improve their biological performance and promote the bioactivity of the embedded cells. A key advantage of fully chemically defined systems and of highly tunable synthetic materials is the ability to accurately control the physicochemical properties of the hydrogel together with the controlled introduction of bioactive cues to enable the rational design of engineered matrices to guide stem cell differentiation.^[108] Despite these advantages, a limited number of synthetic materials have been used for brain tissue bioprinting, and most reports describe more conventional tissue engineering strategies. Nonetheless, important lessons can be learnt from such studies. Amidst synthetic hydrogels, systems based on polyethylene glycol and its derivatives remain among the most explored. For instance, PEG–hyaluronan hydrogels, modified with matrix metalloproteinases cleavable crosslinkers, have been used to study the response of glioblastoma cell lines to increasing values of stiffness of artificial extracellular matrices,^[109] and star-shaped PEG–heparin hydrogels modified with RGD peptides have successfully been employed to culture primary nerve cells as well as NSCs.^[110] Tyramine-modified polyvinyl alcohol, modified with silk sericin and gelatin to improve its biofunctionality, was used successfully to culture a neuronal cell line (PC12) and support the formation and outgrowth of dendritic processes.^[111] Polyurethane-based hydrogels have been proposed as injectable systems for treating traumatic brain injuries (as tested *in vivo* in a zebrafish model), and the possibility of bioprinting NSCs within such materials was proven.^[112] Specific classes of synthetic polymers have also been studied due to their excellent conductive properties, such as the case of polypyrroles (PPy). Besides excellent electrical properties, PPy have favorable cell and tissue compatibility. However, due to poor solubility and degradation profile, the use of PPy requires their combination with other materials, such as silk fibroin.^[113] Johnson et al. integrated electrical, topographical, and chemical cues into a tissue scaffold in order to promote neuron

regeneration.^[114] A PPy-coated SF (PPy/SF) conductive composite scaffold was fabricated with an electrical conductivity of 1×10^{-5} – 1×10^{-3} S cm⁻¹, nanoscale fibers, and without cytotoxic properties, allowing the culture of Schwann cells.^[115] Synthetic peptides have also been investigated as biomimetic hydrogel-forming materials, as these can be designed in their entirety to embed, together with cells, bioactive moieties or domains able to guide the differentiation and maturation of neural cell subtypes. Few peptide-based bioinks are currently available for bioprinting^[116] although limited work has been performed in the field of neural tissue bioprinting, and most data on peptide-based hydrogels and neurons are based on conventional tissue engineering strategies. Among notable examples, amphiphilic peptides are able to self-assemble into nanofibrillar hydrogel structures upon exposure to physiological temperature. These hydrogels show domains that can capture specific growth factors or even mimic the bioactivity of such potent biomolecules that have been proposed for neural tissue repair.^[117] Another example is Puramatrix, a commercially available synthetic matrix based on the acetyl–(Arg–Ala–Asp–Ala)₄–CONH₂ peptide hydrosol. Under physiological salt conditions, it self-assembles into a 3D hydrogel with a nanometer scale fibrous structure and has been shown to support dorsal root ganglia outgrowth in a 3D culture system combined with a PEG-based hydrogel.^[118]

3.4. Neural Cell Types Used in Bioprinting Approaches

Several cell types have been broadly used for neural tissue printing such as primary cells, immortalized cell lines, and iPSCs (Table 3). Depending on what purpose researchers wish to serve, it is important to consider which cell type should be used.

Primary neural cells can keep their authenticity so that any experiment readouts coming out of these cells would mostly reflect on a real *in vivo* situation. Either adult or embryonic neural cells have been isolated to print.^[17,26] Drawbacks come as, on the one hand, animal dissection is intrinsically laborious, and these cells can only be passaged *in vitro* for limited times, thus hampering the 3D printing process, which always requires large amounts of cells. On the other hand, regarding usage of human neural cells, the cell sources are much more restricted and ethical issues are inevitable.

The use of immortalized neural cell lines addresses the problem that primary cells can be expanded *in vitro* for limited time. Some human neural cell lines such as glioma-/glioblastoma-derived ones are also established to serve human tissue engineering purposes. Such cell lines can be passaged for a number of times while maintaining the ability to differentiate into neurons and glial cells when exposed to differentiation medium.^[119] However, one general problem about immortalized cells is that immortalization to some extent modifies the chromatin leading to a loss of certain functions of the cells.^[120] This could greatly limit the use of immortalized cells when a lot of functional tests are needed during the experiments.

Human ESCs/NSCs/NPCs can be a potential solution to the problems shared by the previous cell types but their availability is still limited. On the other side, the dawn of iPSC technology

Table 3. Main neural cell types used in bioprinting approaches.

| Category | Starting cell type during printing | Terminal neural phenotype obtained | Ref. | |
|---|---|--|---|-------|
| Primary cells | Mouse cortical neurons | Neurons (TUBB3+); Astrocytes (GFAP+) | [17] | |
| | Rat retinal ganglion cells | Neurons (TUBB3+); Glial cells (Vimentin+) | [26] | |
| | Rat hippocampal and cortical cells | Neurons (TUBB3+) | [57b] | |
| | Mouse NSCs | Neurons (TUBB3+) | [102] | |
| | Rat Schwann cells | Schwann cells (S100b+) | [103,115] | |
| | Mouse NSCs | Neurons (TUBB3+); Astrocytes (GFAP+) | [112] | |
| | Rat astrocytes and neurons | Neurons (MAP2+); Astrocytes (GFAP+) | [124] | |
| | Porcine Schwann cells | Schwann cells (S100b+) | [125] | |
| | Rat NSCs | Astrocytes (GFAP+) | [126] | |
| | Mouse NSCs | Labeled with PKH26 dye | [112] | |
| | Rat NPCs | Neurons (MAP2+); Astrocytes (GFAP+); Oligodendrocytes (Olig2+); Schwann cells (S100b+) | [47] | |
| | Rat superior cervical ganglia (SCG) sensory neurons and hippocampal neurons | Neurons (Tau+) | [127] | |
| | Rat NSCs | Neurons (NF-H+); Astrocytes (GFAP+) | [128] | |
| | Established cell lines | Mouse NSC (C17.2) | Morphology under a bright field microscope | [18b] |
| | | Human NPCs (NT2) | Neurons (TUBB3+) | [57b] |
| Mouse NSCs (NE-4C) | | Neurons (TUBB3+) | [64] | |
| Human NSCs (ReNcell CX) | | Neurons (TUBB3+); GABAergic neurons (TUBB3+/GABA+/GAD+); Oligodendrocytes (OLIGO2+); Astrocytes (GFAP+) | [95] | |
| Mouse glioblastoma (GL261) | | Glioblastoma (GFAP+/Chil1+) | [129] | |
| Rat Schwann cells (S16Y); Rat neuronal cell line (PC-12); Human glioblastoma (D54-MG) | | Not mentioned | [101] | |
| Human glioblastoma (U-87 MG) | | Glioblastoma (F-actin+) | [107] | |
| Human NPCs (ReNcell VM) | | Neurons (MAP2+); Astrocytes (GFAP+) | [130] | |
| Mouse neuroblastoma (NG108-15) | | Morphology under a bright field microscope | [125] | |
| Rat neuronal cell line (PC-12) | | Neurons (TUBB3+) | [131,132] | |
| Human glioma (U87) | | Neurons (TUBB3+) | [133] | |
| Mouse NPCs (NE-4C) | | Neurons (TUBB3+); Astrocytes (GFAP+) | [134] | |
| Human neuroblastoma (SH-SY5Y) | | SH-SY5Y (NFH+) | [135] | |
| iPSCs | | hiPSCs | Neurons (TUBB3+); | [40] |
| | | hiPSCs | Ventral midbrain dopaminergic neurons (TUBB3+/TH+/FOXA2+/LMX1A+) | [54a] |
| | | hiPSC-derived organ building blocks (OBBs) | Neurons (TUBB3+) | [69] |
| | | hiPSC-derived spinal neuronal progenitor cells (sNPCs) | Neurons (TUBB3+) | [32] |
| | | miPSC-derived oligodendrocyte progenitor cells (OPCs) | Oligodendrocytes (labeled with enhanced green fluorescent protein or mCherry) | [32] |
| | hiPSCs | Neurons (MAP2+); GABA neurons (GABA+) Astrocytes (GFAP+) | [34a] | |
| | hiPSC-derived neuronal and glial precursor cells | Neurons (MAP2+); Astrocytes (GFAP+) | [35] | |
| | hiPSC-derived NSCs | NSCs (Nestin+/SOX2+/SOX1+/PAX6+) | [135] | |
| | hiPSC-derived neural aggregates | Neurons (TUBB3+) | [36] | |
| | hiPSC-derived NPCs | Spinal cord motor neurons (TUBB3+/ChaT+); Astrocytes (GFAP+) | [136] | |

has made it possible to derive large amounts of human neural cells in vitro.^[121] This has largely bypassed the ethical issues of neural cells obtained from either human embryos or embryonic stem cells. Recent advances in cell reprogramming even allows for direct conversion of somatic cells into neurons or glial cells.^[122] Moreover, with the iPSC approach, patient-specific neural models can be derived, making the development of patient-specific models as well as personalized medicine possible.^[123]

4. Future Directions in Advanced Neural In Vitro Modeling

The electrophysiological activity of neurons and specifically the transmission of electric and biochemical signals across specific neural networks is intimately intertwined with the geometrical and architectural compositions of these networks, which connect different neural subsets. On one hand, biofabrication technologies can, depending on their resolution, enable to pick and place or dispense the required cells, multicellular building blocks, and cell-laden hydrogels that are needed to mimic the in vivo relative positioning and interconnectivity of healthy and diseased neural pathways. On the other hand, events and signals that influence NSC fate decisions or the maturation of differentiated cells are affected by a wide array of physicochemical cues that should be applied synergistically to cell printing and assembly in 3D. Combining knowledge from material science, brain and neural cell biology, and electrophysiology with biofabrication technologies can pave the way to the generation of neuronal systems in vitro that can capture the complexity of their native counterparts.

4.1. Axon and Dendritic Process Guidance

Besides key considerations on cell sources and biomaterials as building blocks, the guidance of cellular processes responsible for the interconnectivity of neural networks is paramount in the generation of functional models. Indeed, neurite-outgrowth factors have demonstrated potential applications in peripheral nerve injuries, providing a basis for their use in 3D neural circuit models.^[137] When building extensive neural networks in vitro, the guidance of axons in an efficient and long-lasting manner will be essential.^[138] On the other hand, since axon length influences sensitivity and is potentially involved in disease progression, ideally, the axons between nuclei should be of the same length as in physiological conditions. As discussed before, many different factors influence cell differentiation and axon protrusion. Different methods have been employed in an attempt to mimic this environment in vitro including those of chemical, physical, and electrical nature.

4.1.1. Chemical Methods

Biochemical cues are usually patterned onto a flat surface using microcontact printing (μ CP). The potential bioactive molecules can be divided into two classes of neurite-outgrowth factors:

ECM constituents and neurotrophins. In a 3D setting, biologically active molecules could be encapsulated and incorporated into a hydrogel before printing. Tang-Schomer showed that the delivery of ECM components promoted axon growth.^[137] Laminin and fibronectin showed increased axon growth in comparison to neurotrophic factors like brain-derived neurotrophic factor, glial cell-derived neurotrophic factor, nerve growth factor, and neurotrophin-3 (1×10^{-6} M). Neurons have shown extreme sensitivity to subtle changes in the gradient steepness and concentration of insulin-like growth factor 1.^[139] These results suggest that cells are highly sensitive to these gradients, but not much research using different factors has been done. For an in-depth review on structural axon guidance, the reader is referred to the study of Seiradake et al.^[140] Another powerful factor in axon guidance is the addition of embedded or tethered biological cues to the permissive 3D scaffold when exogenous differentiation is deemed essential. In the case of dopamine neurons, it has previously been shown that the combination of neurotrophic factors and GAG-based matrices can improve maturation and neurite outgrowth.^[141] Developmental-inspired approaches will be probably be the key for future neural pathway models. Specifically, several axon guidance molecules are known to drive neurite outgrowth during CNS development.^[142] Interestingly, in the case of dopamine neurons, it has already been shown that mouse primary dopamine neurons outgrowth can be guided by a focal source of netrin-1 by interacting with the deleted in colorectal cancer receptor expressed on the dopamine neuron cones.^[143] Therefore, overexpression into target neurons of axon guidance molecules such as netrin-1 could be used to create a gradient to drive neuronal connections in vitro.^[144]

4.1.2. Topography and Mechanical Cues

Different physical restraints like microgrooves and micropillars have been used in axon guidance. Axons and dendrites have shown the ability to climb over microgrooves up to 600 nm high.^[145] Micropillars, however, contributed to proliferation and differentiation in vitro. Neurite length seemed to be the longest on the pillars with the smallest interspacing with a width of 2 μ m.^[146] Parallel and aligned electrospun fibers do not form a physical restraint but provide contact guidance for cell spreading, migration, and axonal growth,^[147] as shown with polylactide electrospun mats implanted in vivo in mice brains.^[148] Such nanofibrous mats closely mimic the topography of acellular nerve matrix, only with larger diameters of microchannels to promote nerve infiltration. In addition, it is possible to introduce nanotopographical elements like grooves and pores onto electrospun fibers, for instance, via the addition of different combinations of solvents into the polymer solution. This approach has been shown to improve cell alignment along the main direction of the fibers via mechanosensing, as shown by the enhanced shuttling of yes-associated protein from the cytoplasm to the nucleus.^[149] In addition, the use of nanofibers made from materials displaying a low elastic modulus, such as fibrin, is preferred when aiming to promote neurite outgrowth.^[150] However, electrospinning does not typically allow for the incorporation of cells^[151] and it has most often been used to form 2D culture surfaces.

Nonetheless, the potential of electrospun nanoscale structures to align cells can be translated to 3D settings. For this purpose, microfragments of electrospun nanofibers with embedded paramagnetic ferric oxide nanoparticles have been suspended together with cells into a fibrin precursor solution.^[152] In such a setup, the application of a magnetic field can induce alignment of the microfibers, which can be stabilized upon gelation of the fibrin and that in turn was shown to guide the alignment of neurons derived from the dorsal root ganglia.^[152] Interestingly, such an improved cell alignment leads to the propagation of calcium signals across the direction of cell alignment. Similar effects can be obtained using magnetoresponsive rod-shaped nanogels.^[153] Depending on the hydrogel of choice, these composite systems can also be used as injectable cell delivery vehicles^[154] and therefore may be readily translated as components for bioprintable inks.^[155]

4.1.3. Electrical Stimulation and Electroconductive Elements

The use of exogenous electric signaling significantly promotes axon growth and creates large-scale axon alignment in 3D.^[156] Neurite outgrowth has been known to naturally orientate toward the negative pole in a direct current electric field.^[157] A different form of electrostimulation is using an alternating current (AC), attracting the growing tips of axons. Axons orient themselves perpendicular to the adjacent electrode.^[137] Axon length reached $1296.1 \pm 49.8 \mu\text{m}$ after 4 days of 2 Hz stimulation.^[137] Although promising, this is still far from biomedical relevant neuronal pathways such as the 4 cm estimated nigrostriatal dopamine axons in the human brain. Indeed, in the CNS, the axons can be far away from the cell bodies, encountering very different kinds of biophysical environments that change over time, making it difficult to mimic such dynamic environment *in vitro*.^[145b,158] Regardless of the method at hand, axonal dispersion and off-target reinnervation remain a challenge.^[159]

To enhance the control over the electrical properties of the engineered neural networks, additive manufacturing technologies also provide the opportunity to fabricate composite structures embedding conductive elements.^[160] Conductive polymers, such as polyaniline, poly(3,4-ethylenedioxythiophene) (PEDOT) and polystyrene sulfonate (PSS) are often combined with hydrogels suitable for cell culture to create 3D substrates for neuronal cell culture.^[161] For instance, oligoaniline-doped chitosan was successfully used to induce the differentiation of olfactory ecto-mesenchymal stem cells toward dopaminergic neurons,^[162] which are key actors in PD. Likewise, the embedding in biocompatible hydrogels of nanoconductive elements, such as graphene,^[163] black phosphorus,^[164] carbon nanotubes,^[165] and nanoclay,^[166] has been reported. Importantly, conductive bioinks for bioprinting have already been proposed and promisingly tested *in vitro* and *in vivo* for skeletal and cardiac muscle cultures.^[166] A possible limitation for the use of nanoconductive materials is linked to the difficulties in their processability. This issue could partially be overcome by using a mix of blended materials such as polyaniline/poly(lactic acid) that showed to be compatible with the generation of electrospun fibers that support neuron outgrowth.^[167] Another challenge in the application of nanoconductive materials is their

limited elasticity that hinders their capacity to mimic *in vivo* mechanical properties. To address this limitation, it is possible to design an elastic material (i.e., PCL) with interspersed blocks of conductive polymers.^[168] Finally, it has to be considered that conductive polymers and nanomaterials could exert cytotoxic effects on neural cells, and such negative effects can depend on the chemistry, size, and geometry of the material, as shown, for instance, in studies involving specific formulations of carbon nanotubes and graphene.^[169] Overall, the combination of the described approaches with the increasing work reported in the literature on injectable conductive hydrogels for neuron embedding pave the way toward the generation of 3D-patterned neural networks with enhanced electric signal transmission.

4.2. Toward Modeling Neural Environment Complexity

Though representing as one of the most promising methods in neural tissue engineering, bioprinting approaches still need to be adapted to implement several aspects that contribute to neural complexity. Glial cells such as astrocytes and microglia are important elements in CNS providing support to neurons and maintaining brain homeostasis, which are crucial to generate a neural tissue model that aims to recreate the neuronal microenvironment. Printing with NSCs or NPCs normally leads to the generation of a mixed population of neurons and glia if no lineage-specific patterning cues are included in the culture medium to drive a certain neural fate.^[32,34a,95,112,124,130] A possible solution to this issue would be a multistep modeling strategy where, in the first place, NSCs/NPCs are printed and differentiated into neurons followed by subsequent printing of astrocytes as well as microglia in the culture system to further support neurons. This approach could allow the combination of different neural types but would not be helpful to also implement different neuronal types that could generate specific neural circuits. For example, a challenging, yet important goal in PD modeling, would be to recreate the *in vitro* nigrostriatal pathway and the neural circuitry of the basal ganglia, in which it is interconnected (**Figure 3**). In order to recreate several interconnected neuronal clusters of dopamine (DA), gamma-aminobutyric acid (GABA), and glutamate neurons, we can conceive to take advantage of cell reprogramming techniques to impose specific neuronal and neural phenotypes by overexpressing key transcription factors (TFs) active during CNS development.^[170] Indeed, using doxycycline-inducible viral vectors we can hypothesize to first bioprint ESCs/iPSCs transduced with different reprogramming TF cocktails to generate different neuronal or neural phenotypes and then to induce the differentiation simultaneously by adding doxycycline to the medium. The combination of bioprinting and cell reprogramming would have the advantage to simplify the problems due to the sensitivity of specific neural cells to bioprinting, allowing a simultaneous assembly of complex neural systems.

Another challenge is to print vasculatures with a size equivalent to brain capillaries. Under same perfusion volume, capillaries have higher efficiency in nutrient and waste exchange.^[171] Different groups have achieved printing vasculatures with diameters as long as hundreds of micrometers.^[172] Building vasculature on a much smaller scale indeed requires higher

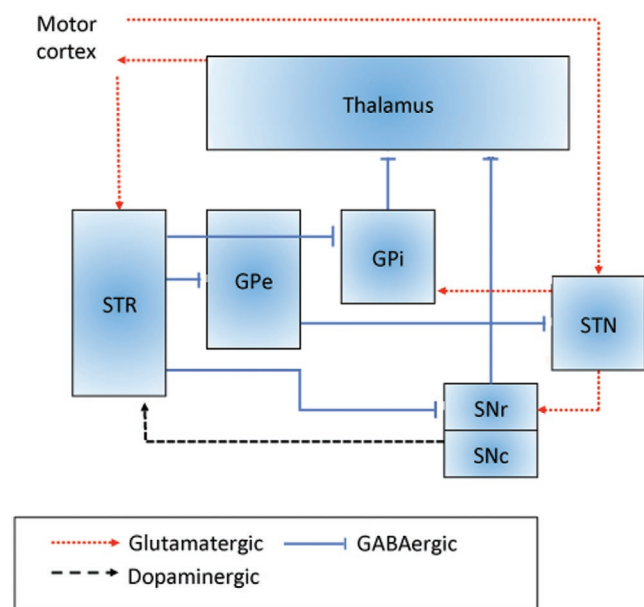


Figure 3. The circuitry of the basal ganglia. The nigrostriatal pathway is the main neuronal pathway degenerated in Parkinson's disease and is composed by midbrain substantia nigra (SN) dopamine neurons that project toward GABA medium spiny neurons in the striatum (STR). The nigrostriatal pathway is itself embedded in a broader circuitry: the basal ganglia. Within basal ganglia, each neuronal cluster can exert excitatory (with glutamate neurons), inhibitory (with GABAergic neurons), or modulatory (with dopamine neurons) action. SNc: substantia nigra pars compacta; SNr: substantia nigra pars reticulata; STN: subthalamic nucleus; GPe: globus pallidus external; and GPi: globus pallidus internal.

resolution of printing processes where the mechanical properties of bioink and the size of the printer nozzle should be finely tuned. Endothelial cells embedded in 3D gel can also result in spontaneous angiogenesis without any intended manipulation.^[173] The generation of capillaries in bioprinting neural tissue models remains a crucial achievement in order to simulate physiological interaction between neural cells and the vascular system.

In addition, finding an universal medium to support both neurons and vasculatures within the printed model is also a hard task. Serum interferes with neuron behavior in many ways; thus, a serum-free environment is favorable for neuron health in the long run, whereas serum is crucial for endothelial cells which form the vasculature.^[174] Besides, other factors such as (vascular endothelial growth factor, fibroblast growth factor, heparin, and hydrocortisone) are also necessary for maintaining vasculature functions, thus raising questions whether these factors are toxic to neural cells. Instead of finding a compromise in the universal medium composition, several studies have tried to feed neural cells and endothelial cells separately using each one's own medium.^[173b,175] A possible approach is to create another channel loaded with a serum-free medium to nourish surrounding neural tissues while keeping the serum-containing medium in the endothelial tubes to support vasculatures.

Finally, another development step that needs to be embedded in advanced in vitro neural models is the possibility of measuring physiological parameters such as the electric activity.

Extracellular electrodes are capable of stimulating and recording nerve fibers without disruption and can therefore provide a more physiologically accurate reading. Multiple electrode methods have been used over time; however, the amount of stimulation and recording sites has always been a challenge. Microelectrode arrays (MEAs) have overcome this problem, giving them a significant advantage over traditional methods. It has mostly been used in describing spatiotemporal dynamics. By bioprinting onto MEAs, different neural cells can be stimulated and recorded at the same time. Samhaber et al. present a new method for precision patterning of neurons on MEAs, utilizing μ CP combined with a custom-made device to position patterns on MEAs with high precision. This technique is called accurate positioning microcontact printing (AP- μ CP). Survival of cells on these arrays depends on the preparations. Close contact between the cells and array has to be established.^[176] A limitation of this method is that nutrient and oxygen diffusion is blocked from one side,^[176] but we clearly need to take into consideration how to implement physiological recordings in the next-generation biofabricated neural systems.

4.3. Final Remarks

Though representing as one of the most promising methods in neural tissue engineering, bioprinting approaches still fall short in several aspects, which remain to be addressed in the coming years.

These aspects include first of all implementation of in vitro axon guidance that is crucial to recreate in vitro neuronal pathways rather than randomly connected neurons. Although first studies show the integration of fiber-guided axonal growth, it still is questionable, if the integration of fibers is in the spatial range of directed neurite growth. Directed neurite growth and thus guided intercellular connectivity would be of particular interest to improve the reliability of drug discovery platforms for diseases where specific neuronal pathways such as the nigrostriatal one in PD are degenerated.

Other key points are the optimization of printing parameters to increase neural cell viability; the identification of bioink compositions, which properly mimic natural brain ECM biomacromolecules to better support the assembly of different neural cells; the incorporation of perfusion systems to achieve long-term cell survival; and the controlled generation of neural circuitry and systems to monitor their electrical activity.

In conclusion, notwithstanding all the described approaches, bioprinting is still a technique within a large portfolio of techniques and cannot per se answer all the needs in the field of in vitro neural modeling. Indeed, it has to be considered that other biological fields continue to move into the direction of creating multicellular systems like organoids matching the critical need for a cellular-defined microenvironment. Moreover, bioprinting technologies further require scalability and creative combination with additional biofabrication methods as well as low-cost hardware and easy accessibility to advance in the field of neural applications. Arguably, a fusion of organoid technology together with creating reproducible 3D architectures and conditions could help bioprinting to move toward the generation of complex multicellular neural systems.

All these action points will be instrumental to move toward reliable in vitro modeling of human neural tissues, therefore helping to limit the usage of improper animal models and at the same time paving the way for the improvement of biomedical research in the field of neurological diseases.

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Conflict of Interest

The authors declare no conflict of interest.

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