Nanofabricating neural networks

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Regina Luttge

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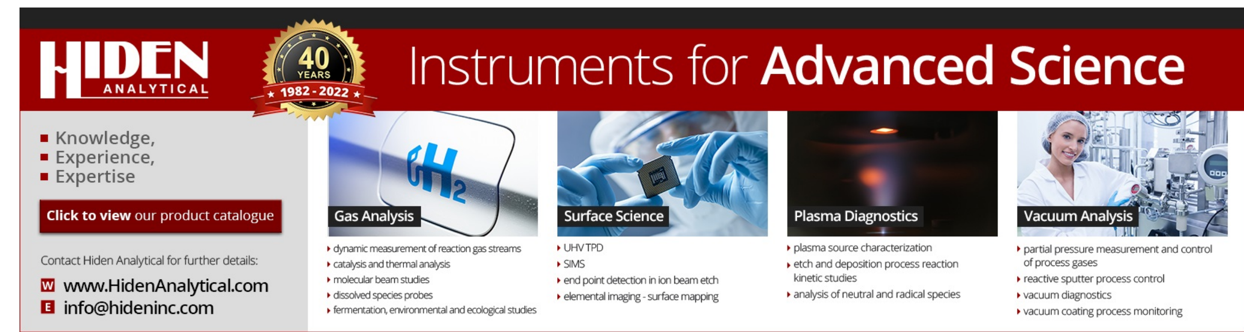

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ABSTRACT

Nanofabrication can help us to emulate natural intelligence. Forward-engineering brain gained enormous momentum but still falls short in human neurodegenerative disease modeling. Here, organ-on-chip (OoC) implementation of tissue culture concepts in microfluidic formats already progressed with the identification of our knowledge gap in toxicology and drug metabolism studies. We believe that the self-organization of stem cells and chip technology is a key to advance such complex *in vitro* tissue models, including models of the human nervous system as envisaged in this review. However, current cultured networks of neurons show limited resemblance with the biological functions in the real nervous system or brain tissues. To take full advantage of scaling in the engineering domain of electron-, ion-, and photon beam technology and nanofabrication methods, more research is needed to meet the requirements of this specific field of chip technology applications. So far, surface topographies, microfluidics, and sensor and actuator integration concepts have all contributed to the patterning and control of neural network formation processes *in vitro*. However, when probing the state of the art for this type of miniaturized three-dimensional tissue models in PubMed, it was realized that there is very little systematic cross-disciplinary research with biomaterials originally formed for tissue engineering purposes translated to on-chip solutions for *in vitro* modeling. Therefore, this review contributes to the formulation of a sound design concept based on the understanding of the existing knowledge and the technical challenges toward finding better treatments and potential cures for devastating neurodegenerative diseases, like Parkinson's disease. Subsequently, an integration strategy based on a modular approach is proposed for nervous system-on-chip (NoC) models that can yield efficient and informative optical and electronic NoC readouts in validating and optimizing these conceptual choices in the innovative process of a fast growing and exciting new OoC industry.

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I. INTRODUCTION

After more than 100 years of neural cultures[1] and the emergence of organ-on-chip (OoC) technology around 2010 pioneered by Huh *et al.*[2] in their lung-on-chip paper, technical developments in nano- and microfabrication methods exploiting manipulations that utilize electron-, ion-, and photon-beam material interactions can now be applied to unravel the workings of the human brain. To this end, we aim to address nanofabricating neural networks to put the human nervous system-on-chip (NoC) firmly onto the map.

In retrospect, this is possible, thanks to the inception of microelectronic chip manufacturing capabilities and the initiation of investigations of chemical analytical techniques for single cells, captured in terms like micrototal analysis systems[3] (μTAS) and lab-on-chip (LoC), which were coined about 30 years ago, driven by exploring nano- and microfluidic phenomena. Since OoCs were introduced to model tissues in a miniaturized culture format, much more research on OoC technology and its applications proceeded. Earlier, these systems were defined as microphysiological systems (MPSs) to distinguish them from the prior developments of

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in-plane microfluidic platforms for cellomics.[4] Since then, microfluidics and many new developments in nano- and microfabrication methods have proven themselves yielding a plethora of miniaturized optical, chemical, and electrical sensors in contributing to the research domains of chemistry, biology, and medicine.

Consequently, the quest to test and improve the clinical relevance of this novel generation of (micro)biomedical devices for culturing tissues and modeling organ functions out-of-plane exploiting microfluidic concepts in the so-called three-dimensional (3D) models, progressed with the identification of our knowledge gap in toxicology and drug metabolism studies.[5,6] Benam *et al.*,[7] for example, emphasized in their review on engineering *in vitro* disease models, the merging of tissue engineering and microfabrication as being beneficial in 2015. More recent attention to OoCs and their exploitation was given in papers by Vunjak-Novakovic *et al.*[8] and Low *et al.*,[9] both highlighting the onset of OoC technology readiness toward human *in vitro* organ models.

While OoCs mature, this review focuses on advances in OoCs to model the physiology of neural networks of the human nervous system, i.e., NoCs. The development of on-chip brain models and their readouts were just recently reviewed by Forro *et al.*[10] More generally, we[11] previously reviewed electrical readouts in 3D cell cultures, and readers may find these references helpful to continue their further studies on the matter. Predictive modeling of complex neurodegenerative diseases of the human nervous system, however, relies on further improvements of these emerging OoC techniques across multiple disciplines beyond sensing capabilities. A need, which was also recognized by Bae *et al.*,[12] who summarized this evolving field of OoC research from the perspective of enabling methods ranging from microfluidic chips to biomaterials for 3D culture and novel types of readouts. This previous review article on neurodegenerative disease modeling proves the potential of these systems in tackling incurable diseases. The success of such advanced tissue models will rely on developments in stem-cell technology and, specifically, the capability of forming cells and tissues from human induced pluripotent stem cells (hiPSCs) from an adult source, as well as nano- and microfabrication methods alike.

To connect and efficiently investigate hiPSC-derived neural functions with multimodal readouts requires new chip designs, nano- and microfabrication methods, materials, packaging, and interconnects for media and reagents exchange in addition to access to a reliable stem-cell source. Designing an NoC platform technology and bringing these elements together is also the objective of our EU funded FET-proactive-CONNECT project.[13]

Beyond short-term cell-on-chip experiments utilizing, e.g., circulating cells in solution and integrated impedance sensors, disease modeling of the nervous system must include long-term culture formats including static and dynamic stimulating input functions to yield mature and complex microtissue assemblies in gaining functional resemblance to the human nervous system *in vivo*.[14] To this end in 2019, Black *et al.*[15] discussed these various engineering efforts as an emerging neurotechnology by defining novel models for a pharmaceutical approach in treating complex diseases such as pain disorders and concluded that there are great perspectives but still many challenges to solve before value can be created.

Henceforward, in this introduction (Sec. I), NoC technology is addressed by three additional sections: strategies in nanofabricating neural networks (Sec. II), advances in Parkinson's disease (PD) modeling (Sec. III), and remaining challenges in this field of research (Sec. IV). Finally, this review will provide an outlook and conclude with the major findings of this review (Sec. V). In more detail, Sec. II covers strategies in nanofabrication neural networks and is organized in three subsections as follows: (Sec. II A) novel properties of soft materials, (Sec. II B) nanotopographic-induced 3D neuro-architecture, and (Sec. II C) concepts of on-chip single neuron detection in 3D-cultured nervous tissues. Consequently, Secs. III and IV summarize the advances for on-chip PD modeling as envisaged in CONNECT[13] and contemplate remaining design challenges, respectively. Finally, Sec. V formulates an NoC design concept proposal as currently investigated in CONNECT, organized in four subsections: (Sec. V A) components, (Sec. V B) integration strategy, and (Sec. V C) manufacturability, as well as (Sec. V D) an overall conclusion to complete our outlook on further NoC technology research.

II. STRATEGIES IN NANOFABRICATING NEURAL NETWORKS

Wang *et al.*[16] stated the needs for multiorgan microphysiological systems (MOMs) as follows: (1) reproducible and readily interpreted results pertinent to drug development, (2) reliable, cost-effective methods to construct integrated MOMs that deliver results of sufficient quality to satisfy the first need, and (3) device formats that enable industrial adoption and are capable of high-throughput measurements for drug screening and mechanistic disease observations.

The above requirements for such systems lead us to three strategic aspects in the design process of building systems referred to as either MOMs or organ-on-chip, respectively, with a neuronal tissue signature: (A) selection of suitable scaffolds, i.e., soft materials, (B) implementation of control in dynamic microenvironments, i.e., nanotopography, and (C) long-term stable sensing, i.e., single neuron detectors for capturing signaling of single neurons within their circuitry. We will discuss the state of the art of these aspects in the following Subsections II A–II C.

A. Novel properties of soft materials

1. Bioreactors and scaffolding materials

We previously contributed to this on-chip technology research field of defining 3D culture formats for neuronal cells by designing and validating a hybrid bioreactor on microelectrode arrays (MEAs)[17] containing a hydrogel as a soft scaffolding material (Fig. 1). Optimization of such culture systems is still ongoing in CONNECT,[13] as also introduced in the outlook (Sec. V) of this review.

Specific emphasis of these types of tissue-engineered constructs is on achieving an *in vivo*-like cell morphology. Figure 2 compares an example of 2D versus 3D cultures of the neuronal model cell line SH-SY5Y on a flat substrate (2D) [Fig. 2 (top panel)] versus suspended cells within Matrigel^{TM} (3D) [Fig. 2 (bottom panel).

The cells' morphology of the example depicted in Fig. 2 has been characterized for neurite length and soma size to provide

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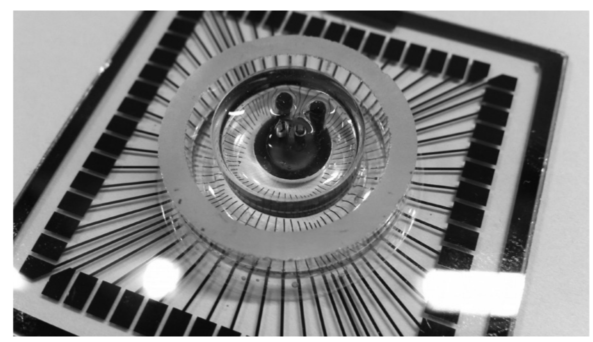

FIG. 1. Hybrid bioreactor atop of commercial microelectrode array. (Reprinted with permission from Schurink and Luttge, J. Vac. Sci. Technol. B **31**, 06F903 (2013). Copyright 2013, American Vacuum Society.)

information on differentiation properties, e.g., outgrowths. Different hydrogels that are all considered for use in nanofabricating neural networks, like Matrigel, Collagen-I, and Puramatrix, perform equally good in lengths of neurites [Fig. 3(a)] and diameters of somas [Fig. 3(b)]; however, control of the performance in such matrices can highly depend on cell number per dispensed volume and is far less trivial to be optimized than anticipated so far in the literature as it can also be anticipated by the large error bars in Fig. 3. On the other hand, in a standardized culture environment, such parameters could be helpful for the development of repetitive scaffolds in NoCs based on locking properties in the utilized soft materials. Hence, our previous work[18] suggests the

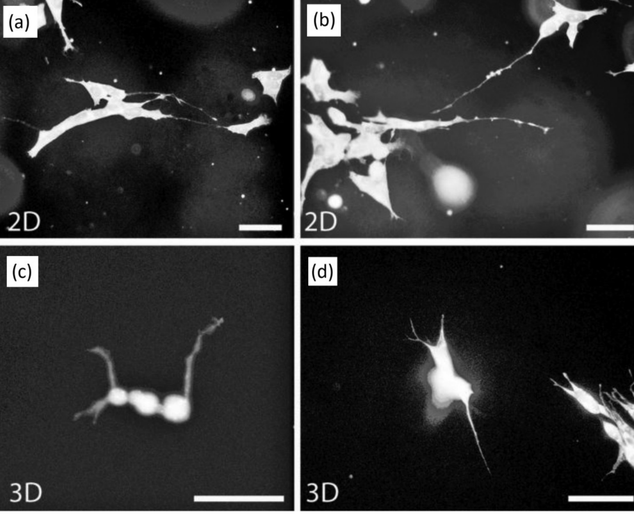

FIG. 2. Epifluorescent images of fixed SH-SY5Y cells for two independent wells comparing fibronectin-coated glass bottom-Ibidi μ-slide (Ibidi GmbH) grown cells as a flat 2D control (a) and (b) with cells in 3D inside Matrigel (c) and (d). Scale bars = 50 μm. (Reprinted with permission from Frimat *et al.*, J. Vac. Sci. Technol. B **33**, 06F902 (2015). Copyright 2015, American Vacuum Society.)

neurite length and soma size as a coupled measure to compare different hydrogels for their performance as a 3D soft scaffolding material. By means of such type of experiment, a large statistically significant deviation from the baseline values for the expected neurite length or soma size would also be an indicator for promoting or hampering connectivity in such networks influencing the network function.

2. Biomarkers

As mentioned in Sec. II A 1, the neurite length or the so-called neurite extension distance per cell can be used as a performance indicator in biologically characterizing material properties (often these indicators are coined biomarkers in this context). To assess acceleration or inhibition of outgrowths forming networks, thanks to adhesion to the microenvironment of a 3D structured biomaterial, measurements must be done under normalized culture conditions. To this end, we assume that increasing values of neurite lengths specify the material as biofriendly. In conclusion, materials assisting neurons to sustain a long extension distance promote healthy states of network formation inside a scaffolding material and neurite length postulates as one of the important biomarkers in neural cultures. Comparing the quantity of outgrowths in 3D by measuring the neurite length directly to 2D cultures as a potential control culture is not possible from an engineering point of view since the viewing conditions interfere with the accuracy of the measurement and such experimental conditions for data collection first must be investigated more carefully as it is also discussed as part of the outlook (Sec. V).

Based on our own experience in such culture formats[17,18] and viewing Wang *et al.*[16] statements more carefully, design parameters and culture formats deserve a critical assessment of defining biomarkers and then specifically those revealing functional performance of constructed neural networks at a circuitry level. Considering recent technical developments such analysis also took place elsewhere, covering long-term biomedical investigations in multiorgans-on-chip,[19] the recapitulation of the complex 3D interactions[20] and providing controlled biophysical cues, and the creation of patient-specific models,[21] e.g., by means of 3D bioprinting.[22] Based on this body of the literature, ideally, OoCs offer insights into dynamic metabolic processes of living cells with high selectivity and sensitivity revealed by biomarkers such as lactate or glucose but in conjunction with parameters such as *p*H and dissolved oxygen in a (semi)continuous and automated multiplexed readout mode for repetitive runs of long-term cultures. Moreover, factors such as cardiotoxicity, neurotoxicity, and hepatotoxicity in drug screening are not satisfied by parameter evaluation at a single time point. In relevant physiological scenarios, cells experience a great many of signals and interact responsively with multiple cell types across the extracellular matrix (ECM) by cytokines and physical factors, hence, a limited number of integrated biomarker sensors will not suffice a full assessment of such effects. Next to the (bio)chemical nature relevant to all OoCs, for NoC cultures, the visualization of the spatiotemporal morphological changes of single neurons are important markers of a healthy versus a disease state of a network, too, due to the extensive connectivity across central or peripheral nervous system components

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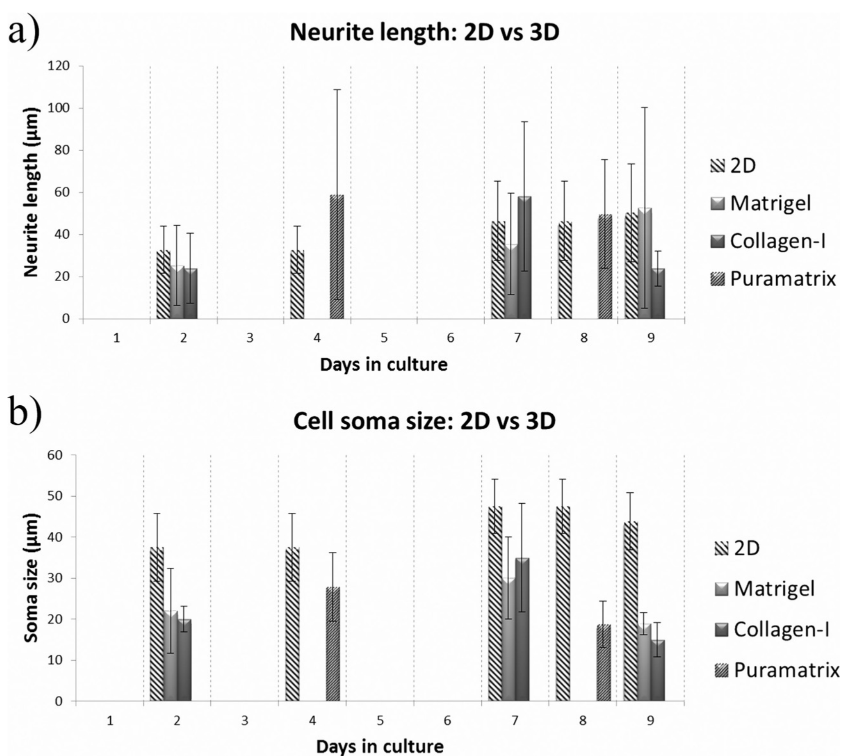

FIG. 3. Comparison of SH-SY5Y differentiated cells on flat surfaces (2D, polystyrene) and inside different biogels (3D = Matrigel, Collagen-I and Puramatrix). (a) Neurite outgrowth length measurements are comparable after 9 days in culture between 2D and 3D cultures, averaging at $50 \pm 23\,\mu m$ for 2D and $40 \pm 27\,\mu m$ for 3D in length (n = 3). (b) SH-SY5Y cell size decreases from $44 \pm 7\,\mu m$ for 2D flat surfaces to $19 \pm 5\,\mu m$ (n = 3) inside 3D biogels after a period of 9 days. (Reprinted with permission from Frimat *et al.*, J. Vac. Sci. Technol. B **33**, 06F902 (2015). Copyright 2015, American Vacuum Society.)

and tissues of other organs of the human body, which at least rudimentarily need to be mimicked in a physiological relevant NoC to reveal meaningful signatures for a range of biomarkers to be registered in such a system.

3. State of the art in nanofabricating neural networks

So far, the selection of papers mentioned above in Secs. I A 1 and I A 2 served as an introduction and only cover a limited scope of the developments in this field since the combined keywords search in PubMed[23] on "*in vitro* AND neural" returned nearly 30 000 hits. Probing this long list of references with the keyword "fabricat*" because fabrication methods are the keen interest of the Electron, Ion, Photon Beam Technology and Nanofabrication (EIPBN) community, we narrowed the selection to 412 publications relevant to this review on nanofabricating neural networks unless otherwise indicated in Secs. II B and II C. Insights are collected and presented then, initially, from an even narrower set of papers by limiting the publications of interest again to the keyword "biomaterial." This selection returned 21 hits serving as a detailed sample of the background literature important to our own research and will be summarized in Sec. II B. Combining the 412 hits with "single neuron," instead, returned three papers, which we review in Sec. II C in order to collect an initial understanding of novel fabrication principles offering advances in biomarker identification for NoC applications on a short- and midterm scale of future technical developments in this branch of research.

Hitherto, works on the implementation of soft materials with top-down modified biomimetic surfaces[24] and eavesdropping into the signaling of neurocircuits upon applying local mechanical stimulation[25] captured our interest. Hence, we further tailored the 21 PubMed hits by the term "soft," leading us to a sample of four intriguing papers.

One of the four reviews is the recent review by Papadimitrou *et al.*,[26] providing an outstanding set of references to fabrication techniques for soft scaffolds as an overview. The other three papers spotlight exciting new developments in soft materials take advantage of the nanoscale. Subsequently, these works are discussed one by one.

First, Yao *et al.*[27] investigated the co-effects of matrix low elasticity and aligned topography on stem-cell neurogenic differentiation and rapid neurite outgrowth. The learnings of this research are (1) a better understanding in providing hybrid biophysical cues to instruct cell behavior *in vitro* and *in vivo*, (2) offering scaffolds with a soft elastic character (elasticity ~ 1 kPa) and a hierarchically linear-ordered structure from the nanoscale to the macroscale. In their example, such properties promoted the neurogenic differentiation of human umbilical cord mesenchymal stem cells (hUMSCs), and (3) matrix elasticity and aligned topography co-effectively induce dorsal root ganglion (DRG) neurons to rapidly project numerous long neurite outgrowths longitudinally along the fibers of the hierarchically aligned fibrillar fibrin hydrogel (AFG) that was fabricated through electrospinning and a concurrent molecular self-assembly process. The authors established a neurite extension distance as a quantitative measure for a comparison of different

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culture environments They concluded the benefits of hierarchical microstructures in a biomaterial by the fact that total neurite extension distance measured after 3 days determined in the absence of neurotrophic factor supplements returned higher values compared to materials that did not contain such hierarchy. However, it does not yet provide proof that hierarchy in the texture of materials only will outperform a material system for neural cultures that adds neurotrophic supplements. Therefore, more research on "dimensional hierarchy" as a design factor is vital.

Second, Zhou *et al.*[28] studied soft conducting polymer (CP) hydrogels cross-linked and doped by tannic acid (TA) for spinal cord injury repair. Their hydrogels exhibit electronic conductivity of 0.05–0.18 S/cm and Young's moduli of 0.3–2.2 kPa. These material's characteristics accelerated differentiation processes of neural stem cells (NSCs) into neurons and, hence, propose an advantage over nonconductive and harder plastic culture ware, like polystyrene (PS) or glass. The material suppressed the development of astrocytes *in vitro* and activated endogenous NSC neurogenesis in the lesion area, resulting in significant recovery of the locomotor function in an *in vivo* model. Using this type of soft scaffold, TA concentration controls the material's mechanical properties. Since stem-cell-derived neuronal cultures can be either driven into a culture setup mainly revealing cortical cells, i.e., neurons, a more balanced mix and, hence, the ratio of neurons to glia cells (e.g., astrocytes) may add as a biomarker or quality factor for healthy state neural networks.

Third, O'Grady *et al.*[29] discussed biofunctionalized hydrogels in their research. Their findings suggest that the addition of N-cadherin extracellular peptide epitope to gelatin methacrylate (GelMA), resulting in a biomaterial termed GelMA-Cad supports the formation of synapses, hence, connected neural networks at the cellular and molecular levels. After photopolymerization, GelMA-Cad forms soft hydrogels (on the order of 2 kPa) that can maintain patterned architectures. These improvements in interactions between the hydrogel mimicking the ECM and the iPSC-derived neurons thanks to biofunctionalization is an interesting approach for three-dimensional *in vitro* models of the human nervous system. Similarly, the concept of soft scaffolds, i.e., hydrogels, for extending a culture into 3D using GelMA has also been already briefly explored by us[30] in addressing mechanotransduction processes in stiff-soft interface layers by stacking such a soft material onto a solid support, like a microscope glass slide.

In conclusion, controlling the structural appearance of scaffolding materials, like introducing a hierarchical texture and spatially adjusted Young's moduli in the soft regime, are beneficial. This argument is supported by the culture results achieved with electrospun aligned fibrillar fibrin, chemically induced cross-linked conducting polymers, and in photopolymerizable hydrogels as examples of such patterned soft materials. These findings provide new directions for the construction and optimization of culture conditions in NoC technology, when utilizing stem cells for human tissue formation *in vitro*.

B. Fabrication of a nanotopography to induce differentiation in cultured neurons

Papers reviewed in this subsection are drawn from the same sample of publications as in Sec. II A in our PubMed search (21 papers) but here combined with the keyword "nano" instead of soft, which returned three hits. One hit was outside of the scope of this review and one of the other two was already mentioned among the novel soft materials, i.e., the conducting polymer-based hydrogel investigated by Zhou *et al.*[28] Again, this narrow sample of publications from the vast scientific literature only probes for an understanding of the inventiveness or novelty in the biomaterials domain and that already proved to be beneficial to nanofabricating neural networks in some way. To evaluate how nanotopography may induce differentiation for specific cells it would be useful to take the technical strategies of fabrication a step further in the development chain of NoC technology.

To widen the outcome of our literature study from above, we run a new search series combining the long list based on the two keywords "*in vitro*" AND "neural" (30 000 hits), subsequently, with the search term "surface topography." This search returned a sample of 13 papers. One of these 13 papers is an excellent review from 2018 in *ChemPhysChem* by Simitzi *et al.*,[31] comprehensively summarizing knowledge related to the specific importance of surface topography in controlling outgrowth and function in neural stem cells *in vitro* as well as the different approaches that incorporate artificial nano- and microscale surface topographical features targeting to recapitulate the *in vivo* NSC niche discontinuities and features. In the review by Simitzi *et al.*,[31] also the paper by Czeisler *et al.*,[32] it has been highlighted already, which focuses on the cell–ECM interactions showing distinct outcomes in different topographical contexts based on observations in real brain tissue. The "surface topography" restricted search also returned a paper by Krivko *et al.*[33] published in 1993. The latter is an interesting study on an early attempt to characterize age-dependent cell membrane surface topography and describes neuronal cell surface and neural processes by their corresponding adhesion protein density at 5 and 12 days in culture and which could act as additional biomarkers. Although historically probably one of the first papers attempting to simulate cell topography by measuring the height profile of cell membrane proteins for the understanding of cell-adhesion interactions, there is no direct information about physically tuning a culture substrate or scaffold surface to model adhesion behavior *in vitro*, hence, we do not go in further details of the content of this paper by Krivko *et al.*[33] describing the naturally occurring lateral patterns of neural cell-adhesion molecules (CAMs) on the surface (cell membrane) of hippocampal cells developing *in vitro*.

Finally, a paper by Zhang *et al.*[34] focuses on the study of dystroglycanopathy (a disorder affecting motor and mental development in children) and is concerned with the characterization of naturally occurring surface topography among other biomedical investigations in their studies and, hence, their work can be also omitted in the context of this review. Nevertheless, Zhang *et al.*[34] make clear that building topography into *in vitro* culture microenvironments should be an essential design parameter of making such a culture instructive for stem-cell-derived progenitor cells to form neurons specifically in predefined locations by design rather than other cell types, like glia cells. To detail such a nature-inspired material design and its potential to form distinguished functional neural circuits by means of selecting one over an alternative fabrication method, we can introduce design features for nanofabricating neural networks as it is reviewed in the following papers.

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First, Miri *et al.*[35] postulate in their paper a method for the neural stem-cell therapy based on nanofibrous scaffolds, which exhibited the proliferation and neural differentiation of stem and progenitor cells. This is a clear trend in this field of research, of which the investigation of poly (L-lactic acid) (PLLA) is an example for this category of materials. Compared to cultures in the standard well-plate format, these randomly orientated 3D PLLA scaffolds have a distinguished topography at different length scales. These scaffolds were assessed against 2D controls at the same cell seeding density. While such a definition of performing a control experiment from the perspective of an engineering approach may need further debate, for now, we can consider 2D cultures in standard 24-well-plates as a gold standard to benchmark cell viability and differentiation. The latter, though, should also take the preparational steps of surface treatments into account, like solution-based coatings of adhesion promotors, e.g., poly(ethyleneimine) as well as CAMs, like laminin prior to seeding cells.

Among many other studies on synthetic polymeric scaffolds for their adhesion ability to cells—a topic too broad to just cite in a few references here—the nanofibrous PLLA scaffold investigated by Miri *et al.*[35] serves us as a recent illustration of this type of study and the controversy of findings, when it comes to cell-adhesion studies on biomaterials in the literature. The authors confirm indeed the adhesion of neural stem- and progenitor cells harvested from the subventricular zone (SVZ-derived NSPCs) from the adult mouse brain after the material was treated with fetal bovine serum (FBS), which facilitates hydrophilicity by absorbing enough trace ECM proteins onto the scaffold's fibers. Unfortunately, we cannot simply conclude that the polymer itself has good cell-adhesion properties for neurons from this observation in general. For instance, as also cited by Miri *et al.*,[35] PLLA has been studied in a previous research paper from 2007 by Bhang *et al.*[36] in a biocompatibility test performed by culturing hippocampal progenitor cells (HiB5) on films of poly(lactic-*co*-glycolic acid) (PLGA), poly (L-lactide-*co*-ϵ-caprolactone) (PLCL), and poly(L-lactic acid) (PLLA) or in the presence of extracts from these polymers. Results of the second study disclosed that a hydrophilic PLGA surface outperforms the hydrophobic surfaces of PLCL and PLLA. This finding suggests that molecular scale surface modification of a culture substrate and the cell type need to be taken carefully into account in evaluating cell viability and differentiation performance.

In general, many different types of polymers can be used in forming a patterned culture scaffold for neurons. From our review, a clear trend is presenting polymer-based electrospun scaffolds for their benefits in nerve regeneration because it appears to accelerate length of neurite outgrowths when an aligned feature with fibers in the submicrometer diameter range is resulting from this fabrication technique. Hence, fibrous 3D materials in scaffold design are an attractive route forward also to be applied in constructing an NoC platform with the drawback that there is limited control on the overall precision of these features by the electrospinning technique and quite a wide distribution in design dimensions (pore size, fiber length, and diameter) have to be taken into account. Overall, one can suggest such features are clearly beneficial. Ziemba *et al.*,[37] for example, found that poly-L-lactic acid-*co*-poly(pentadecalactone) electrospun fibers result in greater neurite outgrowth of chick dorsal root ganglia *in vitro* compared to poly-L-lactic acid fibers. Similarly, Wang *et al.*[38] observed orientated guidance of peripheral nerve regeneration using an aligned texture in their microtube array sheet (MTAS), when preparing conduits based on electrospun PLLA.

Since, the investigated electrospun scaffolds have either an aligned or random fibrous topography, with a mixed matrix of nano- and micrometer diameter fibers that are key in positively influencing differentiation of stem-cell-derived cultures and neurite outgrowths dependent on different blends of the polymeric materials, it would be even better to understand the underlying molecular mechanism of these dimensional features in interaction with cell membrane molecules for synapse formation. Producing a narrowly defined distribution of nanoscale arrayed features with a highly defined geometry throughout a scaffold material (i.e., generating a metamaterial) is possible by lithographic techniques separating such parameters from the functional properties induced by the chemical structure of a scaffolding base material. This has been a direction of cell culture research studying topographical control of cells on surfaces [i.e., often referred to as two-dimensional (2D) cultures] pioneered via in-depth reviews of the state of the art and their own original research more than three decades ago by Curtis and Wilkinson.[39]

Alternatively, next to geometric features and chemical composition, also conductive polymers can be prepared by electrospun composite materials forming instructive nanotopography as demonstrated, for example, by Sadeghi *et al.*[40] They investigated the effect of chitosan on hydrophilicity and bioactivity of electrospun conductive composite scaffold for neural tissue engineering. Also, Rasti *et al.*[41] prepared conductive composite scaffolds by electrospinning yielding hybrid poly(ϵ-caprolactone) (PCL)/gelatin scaffold with the controlled release of triiodothyronine (T3). This trend in material developments may be considered to return many good candidates for 3D *in vitro* modeling by exploiting their nanotopography in addition to soluble factors in inducing or at least enhancing differentiation of neurons in these scaffolds when implemented in NoCs. However, it is still a highly debated topic and information so far is inconclusive on how topographic and electrical effects on synaptic connectivity in neuronal cultures work together, specifically, when the resulting neural network is in a 3D configuration. Hence, the mechanisms to construct relevant and controlled neurocircuitry have not been elucidated.

With a similar aim, like the early research review by Curtis and Wilkinson[39] prompts us to, the remaining five but older papers focus on a change in surface roughness or micropatterned texture with lateral dimensions at the microscale and can assist in further clarification in nanofabricating neural networks by design. Height dimensions of these textured films are in the nanometer to micrometer range. With respect to these five papers returned from our PubMed search, we prepared Table I to provide an overview of these illustrative fabrication concepts being explored not only with respect to the specific findings described in these papers but as examples of the broader scope of technical alternatives, a designer in the OoC technology field can exploit thanks to highly controllable nano- and microfabricated methods.

Hence, these papers are relevant for our cause of defining technical fabrication capabilities of surface topographies that already indicate informative proof of their influence on the architecture of cultured neural networks, which has been also demonstrated in our own work.[42]

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TABLE I. Surface topography affects cultured neurons: techniques and responses.

Technique/reference	Advantages	Disadvantages	Potential impact *in vitro*
Micropatterned hydrogenated amorphous carbon (Ref. 46) - Radiofrequency plasma-enhanced chemical vapor deposition of the 24 nm thick C:H film on glass through a mask	- Material facilitates sensor integration - Single mechanotransducer stimulus - Nanoridge width/spacing of 40/30 μm induced hBM-MSCs to acquire neuronal characteristics in the absence of differentiating agents	- Occurrence of a mechanotransducer effect exerted only by optimal nano/microstructure dimensions - Needs special equipment	Guides mesenchymal stem cells toward neuronal differentiation
Poly(3,4-ethylene dioxythiophene) (PEDOT) coatings on medical electrodes (Ref. 47) - Laser roughening of platinum (Pt) surface occurs during ablation of encapsulation layer - Galvanostatical electrodeposition of PDOT - Use dopants in the polymer for finetuning properties	- Improved the passive stability and chronic stimulation lifetime - Can be used as a postprocessing step on prefabricated micropatterned electrode arrays - No high-tech equipment needed in the deposition process	- Stability strongly dependent on the substrate properties - Did not improve charge injection limit of the material - Procedure used a special laser equipment for roughening as a by-product of the machining process (reproducibility issues could occur)	PEDOT doped with paratoluene sulfonate (PEDOT/pTS) was found to be the most stable conductive polymer (CP) on roughened Pt and presented a surface topography, which encouraged neural cell attachment
Microstructured nerve conduits affect nerve repair (Ref. 48) - Solvent-cast ultrathin poly(ε-caprolactone)/polylactic acid blended films - Rolled up solvent-cast films to form a conduit	- Easy to cast films on the microstructured silicon mold - Patterned films had excellent mechanical properties and were stronger than the natural nerve	- Needs manual labor to form 3D structures (e.g., tubes) - Solvent evaporation rate has a significant effect on the film structure	Degradable polymer conduits may offer an alternative to autografts. Neural cell line (NG108-15), *in vitro* experiments confirmed good cell attachment and proliferation, but biomechanical and *in vitro* studies demonstrated that nerve cell responses are affected by the shape of longitudinal grooves and, particularly, by the angle of the slope of the groove walls
Influence of chitosan concentration on cell viability and proliferation *in vitro* (Ref. 49) - Changing film topography by chitosan concentration - 1% chitosan films showed an AFM profile with higher nanoroughness profile than that observed in 2% films	- Simple, easy, inexpensive film property modification by concentration difference of chitosan in solution - Does not require large equipment - Material with 2% allows 3D scaffolding by alkaline precipitation	- Natural polysaccharide, prone to variations - Pore shape and interpore openings cannot be controlled in the preparation method for 3D - Relatively time-consuming procedure prior to culture - Procedure for coating procedure in well plate cultures are not clearly described	Porous tubes punched out from 2% chitosan hydrogels are mechanically stable enough to be used to fabricate nerve bridges (3D scaffolds)
Textured PMMA to culture Bergmann and cortical radial glia (Ref. 50) - Nanoimprint method - Grooved patters with micropatterns consisted of 2 μm wide lines and spaces, 1 μm deep/tall (coined: Ln2 PMMA)	- Easy to upscale - Efficient and reproducible method - Glia grown on Ln2 PMMA adopt a BRG/CRG phenotype without added growth factors	- Needs access to the nanoimprint process and mold - Not all cell types may favor the specific dimensions	Grooved scaffold induces the dedifferentiation of glial cells into functional radial glia cells. Ln2 PMMA provides an *in vitro* model to study neuron-radial glia interactions

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This limited output on the search term "surface topography," when probing our PubMed long list search of 30 k hits, suggests that modification of the lateral scale in the submicrometer range of textured surface topographies by chip technology has not been fully absorbed by the biomedical sciences community to the same extent that similar but far less accurate nanotopographies made by electrospinning. Of course, many papers on nano- and microscale guidance features, like pillars and grooves, have been published already and were also reviewed elsewhere for their utility in cell cultures,[43,44] driven by the discoveries made in the field of mechanobiology[45] but not up till now dedicated to neural cultures. It would be interesting to start categorizing designed surface topographies by their fabrication method and base materials to subsequently characterize their value-enhancing potential for nervous system models. Such categorizing effort, however, is beyond the scope of this paper, and Table I should be appreciated as kick-off in brainstorming recent advances in biomimetic surfaces and scaffolding structures for models of the nervous system including multiorgan models investigating the innervation of organ tissues.

In conclusion, biomimetic surface topography benefits neural cell response. In established cell culture, protocols mimicry of extracellular matrix components are achieved by coating the surface with a cell-adhesion molecule from solution to achieve topographical cues. From the presented papers in Table I; however, there are some clues that such an additional molecular layer prior to cell seeding could be obsolete for a certain combination of materials, textural shapes and dimensions, media, and cell types. On the other hand, this will also depend on the application of the cultured cells or the intended assays that one wants to utilize an OoC for. Unfortunately, OoCs, and specifically NoCs, are not a one-fits-all approach making the route to standardization, which is important in setting up microenvironments for drug screening and disease modeling to gain statistically relevant results in repetitive cell cultures, quite complex.

C. Single neuron detector

Sensing capabilities in the neuroscience field allow for single cell resolution by patch clamping, which is a gold standard measurement technique in most neuro(electro)-physiology labs.[51] Since in established patch-clamp throughput is very low, there is an increasing interest in single neuron resolution activity measurements and stimulation within connected networks in a high-throughput fashion. The latter requires integrated chip-based sensors and actuator arrays. Hence, we explored the selection of 412 papers referred to in Secs. II A and II B, for research trends in the fabrication of single neuron detectors by adding the keyword "single neuron" to the list of previous keywords "*in vitro*" AND "neural" AND "fabricat*."

Many similar papers may be found using a slightly different keyword selection, but the three papers reviewed next already provide meaningful insights, when considering novel technical routes in nanofabricating neural networks and form a good starting point for further research into this matter. One of the three papers is a very recent review paper, of which we will not relist the studied techniques and devices discussed in there by Bang *et al.*[52] but recommend it for further reading. Here, we focus on the findings of the other two since these explicitly introduced new fabrication directions in this field of work over the last decade.

Cavallo *et al.*[53] explore guidance and 3D confinement to enable local mapping of neuronal signals in utilizing strain-engineered thin film wrinkling as a fabrication strategy, which will also facilitate the fabrication of integrated single neuron detectors. When introduced in 2014, the wrinkling method was not a new technique for micropatterning, still, the authors presented a very original technical proposal on compliant semiconductor scaffolds for organized neural cell cultures. Cavallo and her co-authors formed a crystalline silicon nanomembrane on polydimethylsiloxane (PDMS) that provides a 3D scaffold structure for neurite guidance. The method results a periodic array and helps us to assist in the detailing of neural communication pathways enabled by active sensing and stimulation along the artificial guidance features via devices that can be potentially integrated in silicon.

In the second inventive concept, described by the paper of Jaber *et al.*,[54] a microsystem is designed and fabricated to position neurons inside microwells of a planar MEA by dielectrophoresis (DEP). The connected microwells organize *in vitro* neural networks at the cellular level and aim to study their electrical patterns. With a more recent publication in 2018 by researchers at University of Wisconsin-Madison, Kim *et al.*[55] followed-up on this early concept by Jaber *et al.*[54] in trapping single neurons by DEP and characterized the single neurons in forming neurite outgrowth over the course of 5 days *in vitro*. Most likely, when starting a new search from a different pool of papers or in a different database than PubMed, more publications on single neurons being handled in these or similar types of grid devices correlating single neuron action potential activity to its place and function in a specific neural circuit are expected to be found. Based on the cited literature, ordering and positional cell control in the forms of arrays can assist readout and culture repeatability across experiments and labs for nervous system models. Importantly to notice, there are no studies yet among the 30 k PubMed hits found by combining "*in vitro*" and "neural" that aim specifically on "single neuron" with sufficient sophistication. As a call-to-action, a study setup should be developed to allow us to appropriately mimic the human nervous system's functional circuitry in an engineered environment as an analysis tool of a cultured neural networks' connectivity at high throughput. Linking design parameters to quality of culture data to find performance indicators similarly accurate as it is currently implemented in neuroscience studies within *ex vivo* brain slices or animal models[56] at low throughput would lead us to disruptive technologies in neurodegenerative disease modeling. Section III, subsequently, reflects on such pathways specifically with an eye on Parkinson's disease.

III. ADVANCES IN PARKINSON'S DISEASE MODELING

This section summarizes advances for PD modeling by NoCs. Given our specific research interest in CONNECT,[13] we narrowed our PubMed sample of 412 papers by adding the keyword "Parkinson*." The hits cover a range of seven papers. They show a development that is representative for the research over the last 30 years. At this stage of development in PD therapies, medicine is

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mainly treating symptoms and at best delays the severity of the disease but cannot offer solutions to cure it, yet. Inspired by the 2018 review of Wang *et al.*[16] on MOMs for drug development, we probed here the state of the art on nanofabricating neural networks with a highlight on PD. We realize that there is a risk of duplicating information. Pinpointing, for example, on the work of Choi *et al.*,[21] who described a microdevice platform for an *in vitro* nervous system and its diseases in 2017 and the review of Jadhav *et al.*[57] investigated compartmentalized platforms for neuropharmacological research already in 2016. Hence, we wish to entertain specifically the fabrication concepts behind nanofabricating neural networks to formulate an opinion on how we can synthesize the recent state of the art in innovative NoC solutions to study PD's mechanism-of-actions (MoAs) next.

Investigating MoAs in human cell-based 3D *in vitro* cultures rather than in animal models can open new pathways for treatment modalities and the findings of this literature search also influence our design choices for an NoC approach envisaged to model PD in CONNECT.[13]

In more detail, a first trend in the exploration of 3D scaffolding materials for a PD model can be found in the works on PD treatment of patients that receive stem-cell therapy to replace depleted functional nervous tissue as it is described by Camarata *et al.*[58] in 1992. The authors suggested that the sustainable release of nerve growth factor (NGF) is one of the parameters of importance. Dosing NGF influences the long-term survival of cells in the grafted tissue. They fabricated biodegradable NGF-embedded polymer microspheres. *In vivo* investigations of NGF release thereof demonstrated prolonged graft survival. Furthermore, they assayed neurite outgrowth (similarly as mentioned in Sec. II A 2 that the neurite length can be used as a biomarker) in a dorsal root ganglion tissue culture system. This paper by Camarata *et al.*[58] is co-authored by Turner, who also summarized this type of research two decades later in a review within Krucoff *et al.*[59] in 2019. Various translational neuroscience principles are tailored for the functional restoration of the central nervous system (CNS) by guidance cues. Krucoff *et al.*[59] state the importance of guidance cues in reconnecting neural cells within *in vivo* neural tissues. Hence, their review provides us with an excellent roadmap for our research on nanofabricating neural networks *in vitro*.

The second trend in neuroscaffolding biomaterials is illustrated by the paper of Levenberg *et al.*[60] published in 2005. This paper marks the onset of a fundamentally new era of three-dimensional (3D) engineered polymeric cell culture scaffolds. The authors investigated porous scaffolds for neural cells formed from stem cells. They suggested that cultured hiPSCs provide a treatment for diseases such as Parkinson's disease, spinal cord injury, and glaucoma. Their focus was the study of neurotrophin 3 (NT-3)-induced effects on differentiation. They used a scaffold fabricated from degradable poly(alpha-hydroxy esters), including poly (lactic-*co*-glycolic acid) and poly(L-lactic acid), and observed an increase in numbers of neural structures and staining of nestin and beta(III)tubulin positive cells in retinoic acid (RA)-induced stem-cell-derived neuronal cell cultures, when cultured with both NGF and NT-3 against the control medium. NT-3 also influenced the formation of vascular structures in the engineered tissue but not in the presence of RA. Dosing and time-dependent addition of all-trans retinoic acid (RA) to cultures, also showed enhancement on differentiation and maturation in the works of our CONNECT[13] partner, University of Sheffield, studying RA in enteric nervous systems' (ENS) cell differentiation protocols in the Tsakiridis lab.[61]

The method of preparing the porous 3D culture scaffolds described by Levenberg *et al.*[60] enabled one of the earliest examples of *in vivo*-like brain cell cultures in 3D with generated pores sized between 250 and 500 μm. The authors used a salt-leaching process. With these pore dimensions, it forms the larger length scale of structured biomaterials compared to hydrogel-based scaffolds that form more like a nonwoven mesh of 3D interconnecting physical features on their polymer chains including features down to the nanoscale, and their use has already been reviewed in Sec. II A.

In summary, the concept introduced by this second trend in neuroscaffolding biomaterials needs a set of fine-tuned parameters in handling the neural cells for organoid cultures in PD modeling on chip and these conditions could be very different from the protocol described by Levenberg *et al.*[60]. Luckily, great progress in upscaling accessibility of midbrain organoids[62] representing substantia nigra, i.e., the brain region required for coordinated motor functions particularly affected in PD due to dying dopaminergic cells, has already been made, and adapting suitable neuroscaffolding biomaterials may not be as complex as presented by the salt-leaching method for modeling PD in an NoCs culture format.

A third trend in neuroscaffolding biomaterials, utilizing inorganic thin film semiconductors, originated around the same time as the works by Levenberg *et al.*[60] and demands specific chip technology. The paper by Hassel *et al.*[63] demonstrated an early example of this type of material for neural integration in hybrid electrochemical neural prostheses. Shortly after, Frewin *et al.*[64] published their research on the evaluation of the general biocompatibility levels of single crystal cubic silicon carbide (3C-SiC) and nanocrystalline diamond (NCD) by using *in vitro* techniques. The latter is part of the developments in brain machine interface (BMI) devices that offer a platform potentially leading to therapeutic approaches for people with extreme disabilities, such as amyotrophic lateral sclerosis (ALS) and PD. Their findings are also particularly useful in providing new directions for the development of NoC analysis methods applying these chemically inert semiconductor materials. Next to cell viability, Frewin *et al.*[64] also applied atomic force microscopy to quantify cell morphology on the different substrates along with assessing the substrate's tolerance to lamellipodia extension. Both materials, NCD and 3C-SiC, showed good cell viability for the H4 human neuroglioma but only 3C-SiC was found superior for both H4 and PC12 rat pheochromocytoma cell lines used in their experimental design. Building a knowledge foundation to assess the compatibility of advanced material systems with neuronal cell cultures, like it is published by Frewin *et al.*,[64] is an important milestone. The CONNECT consortium[13] addresses such types of semiconductor materials for their integration of electrical and electrochemical sensors in NoCs. Based on our partners' earlier experiences with this type of materials and sensor platform fabrication methods at Aalto University,[65] these new materials are characterized for their performance in electrochemical analyses of neurotransmitters, their biocompatibility, and patternability.

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Regarding PD modeling, a plethora of research on soft scaffolds, like hydrogels or electrospun matrices, was previously introduced for *in vitro* brain models and is already reflected up on in Sec. II A. Fernandez-Serra *et al.*,[66] for example, showed in their review in 2020 the huge impact of hydrogels in neuroprotection and functional rewiring. Thus, the research of these materials opens up many new routes for NoC technology applied to PD modeling. Next to hydrogels and electrospun matrices, NCD and 3C-SiC-based nanomaterials clearly open fundamentally new possibilities in NoC-based PD modeling.

Finally, we want to complete this section by mentioning Carelli *et al.*,[67] who studied neural precursor cells expanded in a 3D microengineered niche. They employed two-photon laser polymerization within a homemade SZ2080 photoresist to generate microcavities to accommodate stem cells, which they called "nichoids" with a raster-type arrangement of cavities with dimensions of repetitive 90×90 and $30\,\mu$m height onto a circular glass coverslip. They grew neural progenitor cells inside the nichoid for 7 days and thoroughly characterized them prior to implantation in a murine experimental model of PD, in which parkinsonism was induced by the intraperitoneal administration of the neurotoxin MPTP in C57/bl mice. They claim that this way of culturing presents enhanced therapeutic efficacy *in vivo*, which would truly be a breakthrough, if positive clinical outcomes keep up with this promise of *in vivo* modeled enhancement.

In conclusion, there is a commonly recognized unmet need for hiPSC-derived *in vitro* nervous system models in pharmaceutical drug screening and toxicology applications in the literature. The reviewed papers and the therein identified trends in biomaterials point us into new directions for novel treatments of yet uncurable central nervous system disorders, like PD, when utilizing appropriate scaffolding and guidance cues in miniaturized 3D models of PD. Furthermore, the novel neuroscaffolding biomaterials for treatment are also useful in modeling these diseases *in vitro* and may serve as modules or inserts in NoCs. However, the reports also confirm that enabling techniques are not ready to fulfill this urgent need for disease models with higher predictability of success in translational medical research, yet. On the other hand, established or emerging techniques and instruments in the electron-, ion-, and photon beam technology and nanofabrication (EIPBN) research community provide a tremendously rich pool of appropriate knowledge and capabilities in devising better organ model systems, when such solutions indeed origin in chip technology. Hence, Sec. IV presents insights into the remaining challenges in tackling higher functional connectivity, precision, throughput, and reproducibility in devising NoC-based PD models to stimulate further discussion on the topic.

IV. REMAINING CHALLENGES

The technological strategies to engineer physical environments offering an intelligent scaffold as presented in Secs. II A–II C form the basis for building *in vivo*-like neurocircuits, which resemble neurological functional connections of human nervous tissues. As we learned from Sec. III, further research in 3D scaffolds is highly beneficial to the development of tissue grafts as also currently developed for treatment modalities in PD patients. However, soft materials, like hydrogels or electrospun fibrous matrices to study the disease and develop pharma- and electroceuticals by NoCs have not reached their full potential. Neither do lithographic-made nano- and microscale features return the direct clinical value as biomimetic surfaces, yet. The latter are investigated as topographical cues for their influence on neural cell differentiation and aligned connectivity for more than 30 + years when taking some of the earliest 2D culture examples into account without much deeper insights into what makes a good neurodegenerative disease model.

On the contrary, contemporary preclinical technology keeps running cell cultures on far less defined but regulatory confirmed combinations of biopolymer coatings from wet solutions either as precoated supplies or freshly prepared in the culture laboratory, using either glass culture flasks or wells or plastic molded ones of PS, cyclic olefin copolymer (COC), polycarbonate (PC), and polyethylene terephthalate (PET) or polymethylmethacrylate (PMMA), despite the knowledge that these models are insufficient.

A. Gaining spatial and temporal resolution for data collection

Methods for fully integrated, low-cost, single neuron detectors to probe 3D neural dynamics in cultured neural networks with a high spatial and temporal resolution at the tissue functional level are still lacking. To this end, we can take advantage of the learnings from chip fabrication techniques developed for large area displays,[68] mobile telecommunication, integrated circuits for computing and photonics applications as well as control systems technology, i.e., sensors and actuators.[69] What we need next is a thorough description of what the essential requirements are to tackle the remaining challenges in fulfilling technical-robust manufacturing solutions for collecting meaningful data in models of the human nervous system *in vitro*.

As a first important requirement, we need to increase the yield of stem-cell culture in 3D, thoroughly defined by design factors, such as the number of cells, their controlled distribution, potentially their spatial patterning per experiment, etc. Second, there is a requirement for easy to evaluate biomarkers that keep a stable baseline in a system defined as healthy. Although somewhat cumbersome in handling due to manual pipetting, planar integrated microelectrode arrays (MEAs) provide us with an early proof-of-concept of a reusable electrical readout modality in minimalistic NoC microfluidic formats,[70] thanks to the reversible bonding capabilities of polydimethylsiloxane (PDMS) and a route forward in assessing such biomarkers in an effective and efficient manner. Many more examples for microfluidic components integration with single sensors or arrays of repetitive electrical and electrochemical sensors can be found in the literature as illustrated in collections such as themed issues of highly esteemed publishers like the Royal Society of Chemistry in its journal Lab Chip.[71] What else needs a microphysiological integrated system to enable models for the human nervous systems? In fact, we do not know the actual input-output functions yet since stem-cell-derived neural networks are still in their infancy for instructive microenvironments; however, one would like to implement simple geometric features in these wet tissue models to guide the process of data

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collection for high- or at least medium throughput screening under controlled culture conditions, of which we summarize additional challenges next in Sec. IV.

B. Compartmentalized organization

Interestingly, results with forebrain and midbrain-organoids derived from human embryonic stem cells (hESCs) cultured in a connected microtunnel device (MD) have been just presented by Tong *et al.*[72] This concept of compartmentalized organized but modular assembly of different off-chip cultured organoids with the on-chip host microenvironment for connectivity studies and further integration levels with sensors is very promising. However, it is important to validate the design space for cells derived from an adult rather than embryonic cell sources to move toward a route in pharmaceutical screening applications and reach sufficient maturation of such constructs with efficient and informative culture protocols fit for mass manufactured NoC studies.

Foremost, the differentiation of hiPSC into neurons and their complementing extracellular matrices (including glia type cells) is a multiparameter design space problem. Specific cell phenotypes generally occur with a relatively broad statistical variance in cell cycles upon the required large heterogeneity of characteristic features for different types of cells within a single organoid, which is required for informative and disease specific function. To learn more about these needs, researchers at Harvard[73] shared their dynamic modeling in 3D cell culture concept details open access and with an interactive system to collect feedback from users of the information disclosed in the online report for testing soluble compounds on the 3D-cultured tissues already in 2013. The implementation of cell patterning by (bio)chemical cues simply by the exchange of media and time-dependent addition of nerve growth factors (NGF) currently dominates the generation of biological constructs resembling neural tissues in a dish. Although much progress has been made for well-plate generated brain organoids, thanks to the development of detailed culture protocols for hiPSC-derived cells agglomerating into self-organized 3D structures. When putting hiPSCs in geometric confinement of a 384-well-plate reservoir, for example, research at Kushner and co-workers' lab[74] (who are also a partner in CONNECT)[13] demonstrated the most stunning results on functional complexity of brain organoids forming a layered cortical system upon confinement of stem-cell-derived cultures in space by self-organization. A phenomenon that is still currently being characterized also elsewhere.[75] Overall, these aspects detail the challenges occurring in the need to deviate from the established workflow when implementing OoCs to be reflected upon in Sec. IV C in more detail.

C. Workflows

Utilization of manual pipetting workflows for OoC-implemented culture processes makes it difficult to test notable merits of the specific design of a physical microenvironment, like a nanotopography, independently from variations of other biochemical cues. To narrow down the variability, culture environments must offer control over the culture conditions ideally in a (semi)automated fashion and by considering advantages of microfluidic confinement and transport phenomena underlying physical scaling laws in such downscaled culture ware, like in the nervous system model in the example by Tong *et al.*,[72] of which the next challenge is up-numbering these culture systems serving the unmet need in neurodegenerative disease modeling in the pharmaceutical industry by cost-effective means of manufacturing.

For an NoC aiming to resemble the gut-brain axis as in the CONNECT project,[13] overall, researchers made already good progress in reducing size variations and yield in numbering up of floating brain organoid production at least for a genetic origin of PD and disease modeling can now take place in comparing cells and cell networks of healthy versus brain organoids with a PD signature off-chip.

Subsequently, by inserting floating brain organoids directly into platforms facilitating microscale physiological culture conditions, screening throughput and efficacy in the experimental study design can be increased but on the sacrifice that small errors in volume dispensing can have enormous effects on the collected information. Molding techniques in chip fabrication of the culture reservoirs or interconnecting features and other high-volume manufacturing techniques, like large-area display technology,[68] can reduce the variations in the production of these physical microenvironments for NoCs. Yet, variations in the fluidic cell or organoid handling to load and refresh an NoC still form major bottlenecks. Microfluidics can standardize these liquid handling steps, however, also introduces new variabilities since stem cells are shear force sensitive and can change their cell identity upon receiving mechanical cues. Thus, the microfluidic chip layout and routing of interconnecting constructs can also influence the outcomes from cultures chip-to-chip and need critical consideration in an application-driven NoC design cycle.

Interchip variations within experiments from the same cell batch and chip-culture variations batch-to-batch are not well understood due to a lack of standardization and limited fabrication capacity in the research labs constructing these integrated microfluidic NoCs only at low numbers, which is insufficient for statistical relevant biological research. Therefore, standardization and subsequently upscaling of production capacity is important for making a significant breakthrough in this field of research and development. Besides, simply ensuring cell survival of such delicate cells over several weeks as needed for human iPSC-derived neural networks in engineered microenvironments is not trivial because physical boundaries in the chip environment can introduce unforeseen zones of depleted medium and growth factors or accumulated cell waste (by)products.

In addition, highly specialized neural cell culture-based techniques, like commercially available cortical cell source systems and protocols, do not directly match with the new culture formats on chip using a range of materials rather than one single plastic. Neither do these homogenic systems yet resemble the range of cellular processes needed to study interactive processes like variations in action potential signaling across layers of different cultured neurocircuits in diseases due to missing or highly reduced extracellular matrix components and the absence of whole cells that normally regulate molecular processes such as immunoactive cells, etcetera.

D. Cocultures

Besides the aforementioned chip-based challenges, the coculture of cells and control of cell-cell interactions need to be considered in validating NoCs for complex disease models such as PD.

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Cell biologists and experts from the technical disciplines must work hand-in-hand in one development hub, like in CONNECT,[13] to overcome these challenges before high-volume scale production for advanced, integrated microfluidic chips can kick in to make these model systems affordable in industrial pharmaceutical screening applications and toxicology.

Despite the many challenges, researchers working on the interface between biology and engineering made already significant progress in showcasing central nervous systems cells cocultured with other cells delivering great many interesting functionalities, for example, such as demonstrated for models of the neurovasculature unit[76] or the blood-brain barrier,[77,78] In this respect, hiPSC sources are ready for their commercial distribution to start addressing the deficiencies in the robustness of chip-based protocols and providing effective culture conditions for disease or even patient-specific models, including coatings and soluble factors in form of molecules for cell priming, next. To close this gap of workflows in 3D co-cultures within an established cell culture lab infrastructure toward the application of on-chip culture protocols should take advantage of the entire chip fabrication toolbox including chip design modeling prior to production. Progress in this manner will eventually allow us to set up controlled culture conditions and perform efficient, high-quality testing even for complex tissues such as the nervous system *in vitro* instead of using unnecessary numbers of animals in preclinical studies.

E. Main challenges in NoC-PD modeling

To summarize, the three most important challenges in NoC-PD modeling are (1) implementation of compartmentalized, dynamic 3D models must support long-term culture settings, (2) liquid handling for loading and refreshing chips must be simple and standardized, hence tailoring of culture conditions need to be made fit for models of a specific disease in a modular fashion on transferrable NoC platforms, and (3) lack of ideation toward cost-effective pluggable strategies for information-rich data harvesting within these novel on-chip 3D culture formats to seek for either low-cost or reusable integrated sensor array and advanced readout modalities at the single neuron level. The latter must also implement the latest mathematical algorithm currently emerging in the field of Artificial Intelligence for experimental efficiency in harvesting meaningful data from such novel systems.

V. OUTLOOK AND CONCLUSIONS

We reviewed the state of the art on nanofabricating neural networks. As an outlook, here, we formulated a proposal for a design concept that allows us to connect cultured nervous system tissues in a platform that will enable the detailed study of PD by NoCs as it is envisaged in the CONNECT[13] project as well as other devastating nervous system diseases. To meet the core remaining challenges as highlighted in Sec. IV E, this proposal covers (Sec. IV A) components, (Sec. IV B) integration strategies, and (Sec. IV C) manufacturability prior to (Sec. IV D) final conclusions.

A. Components

As the main component of an NoC system, we propose to use the compartmentalized reservoirs of a microtunnel device[79] for connected arrangements of CNS brain organoids, with ENS organoids representing the gut-brain axis. The axonal neural processes of the organoids in the compartments will, respectively, enter the microtunnels. Their trajectory and the microfluidic handling protocol on the chip can be standardized from culture-to-culture as well as lab-to-lab by a simple but very specific geometric confinement for the establishment of neurolinks. When incorporating more than two reservoirs in a MD, various cell identities can be expressed in the tissues cultured in the different reservoirs, thanks to the addition of different nerve growth factors in a diffusion-controlled fashion, i.e., compounds in the medium of one reservoir cannot traverse easily in a microtunnel-restricted flow configuration to another reservoir with a different medium. Figure 4 depicts a schematic drawing of such MD configuration, recently developed in CONNECT [Fig. 4(a)] and a realized MD chip [Fig. 4(b)] that has been tested by completing the polydimethylsiloxane (PDMS) gasket sealed to a microscope coverslip with the simple neuronal model cell line SH-SY5Y for a proof-of-principle as earlier presented by us.[79] Here, such a simple test culture shows that within the confined geometry of the microtunnels, exchange of growth factors can be highly limited by diffusion since the cells lined up in the tunnels do clearly show immunofluorescence staining with F-actin in green and cell nuclei in blue but relatively little neuron-specific staining β-tubulin III in red, indicating relatively few extensions that act as a biomarker for limited differentiation potential inside of the tunnels, whereas such neurospecific stain (β-tubulin III) can be clearly seen for the cells in the reservoirs [Fig. 4(c)].

Since it is our aim to provide an NoC resembling the gut-brain axis for studying molecular trafficking in PD, at least two reservoirs acting like the wells in a microwell plate need to be connected within an MD. These reservoirs should then also be geometrically defined by similar dimensions as in the 384-well-plate but with volumes not larger than a few tens of microliters and, hence, a lower height of the chip layout compared to the existing well-plate standard will allow us to access these cells from the top by established neurophysiology *in vitro* tools, e.g., patch-clamp techniques. In on-chip microwells, we can then directly exploit the newest culture protocols being currently developed and characterized for brain organoids in a 384-well-plate format[74,75] but with the additional benefit that we can physically connect these reservoirs by a functional neurolink through the neural processes that the organoids will form upon differentiation and maturation themselves in these compartmentalized microfluidic configurations like it is demonstrated by the survival, differentiation and elongated outgrowth of the SH-SY5Y cells in the microtunnel device [Fig. 4(c)]. Utilizing the microtunnel approach, several unique microfluidic chip layouts can be realized without significant changes to the cell seeding or culture conditions with each of these layouts, thanks to the reservoir diameters matching earlier studies performed on the 384-well-plate standard. Among potential other benefits, our radial configurations of the microtunnel device[79] could be helpful twofold in allowing us (1) to connect two (or more) reservoirs in a variety of neurocircuit configurations with systematically varying distances, L1 and L2, as well as different angular distributions (α, β) of such microtunnels inside the chip layout [Fig. 4(a)]. This simple layout guides the total number of

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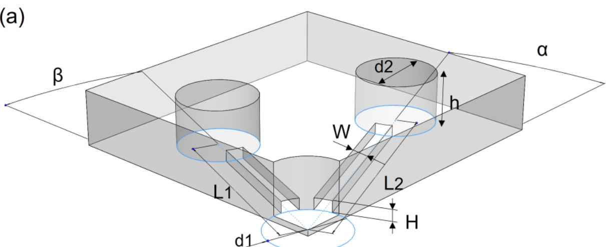

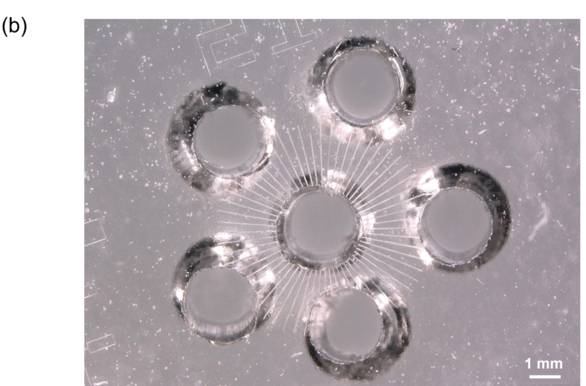

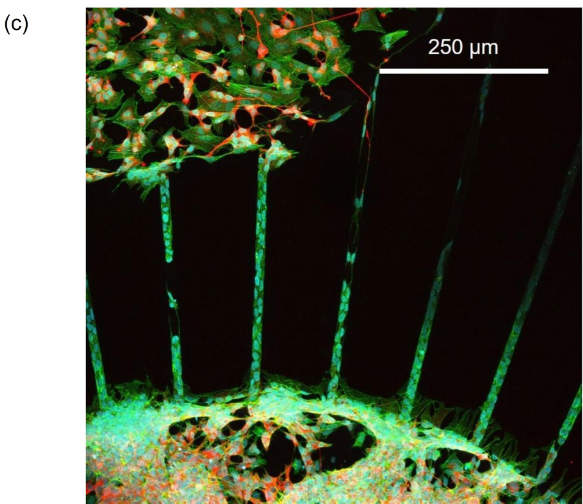

FIG. 4. Schematic drawing of one quarter of a microtunnel device chip layout with respective dimensional input parameters, like well diameters (d1) and (d2) and height (h), microtunnel lengths (L1) and (L2), width (W), and height (H). Furthermore, the microtunnels can be angularly distributed evenly or at demarked positions upon user-defined design inputs (α, β) (a). Realized polydimethylsiloxane microtunnel device (MD) (image courtesy: R. Sabahi Kaviani, Eindhoven University of Technology, 2020) (b) and immunostained SH-SY5Y cells cultured inside such MD (image courtesy: A. J. Bastiaens, Eindhoven University of Technology, 2020) (c).

connective lines simply by punching reservoirs through a user-defined and customized stencil at different positions in the replica-molded microfluidic PDMS gasket using a biopsy tool for the fundamental investigation of the neural communication among organoids of either the same or different tissue identities and (2) to explore the relation of connection length on the signal processing of a CNS organoid, when inserted into a central reservoir being arranged with several peripheral reservoirs in a spiral-type layout, whereas multiple peripheral reservoirs contain, for example, enteric nervous system (ENS) organoids, which should demonstrate no statistical significant variations in their biological construction else then introduced by the neurolink to the central reservoir.

Since brain organoid cultures clearly present radial outgrowth as shown by several works, including researchers of CONNECT,[13] we can take advantage of this symmetry in an NoC design proposal utilizing the radial microtunnel device. A standardized punching layout with several peripheral reservoirs arranged around a central reservoir, whereas then all the microtunnels have the same length, could be helpful in observing retrograde axonal trafficking in PD in a repetitive fashion per connected peripheral reservoir, enabling some level of a statistical analysis of the experiment within a single chip. Once OoC researchers from different laboratories use this NoC layout in a standardized manner, their results can be compared and collected into a database, which can become an organoid-based nervous system atlas for cellular connectivity.

With these various arrangements, we will be able to confine and fine-tune axonal growth dependent on the selected reservoir punching scheme devised to the preferences of a user. The reservoir punching scheme overlays a standardized radial or, alternatively, a linear microtunnel mold configuration, which can be realized by mass-fabricated microfluidic components of the chip in a cost-effective replica molding step or for research purpose by applying well-established polydimethylsiloxane as a material in soft-lithography as in our own example.[79]

B. Integration strategy

The microtunnel geometry also confines the neurolinks as described in Sec. V A to regions-of-interest (RoIs) for standardization in information retrieval. We can either use optical or electrical spectrometric imaging techniques in the vicinity of these RoIs. Since the proposed microtunnel device is a single replica molded optically transparent layer of a defined height, it can facilitate both high-resolution microscopy for optical and neuroelectrophysiological probing of the cultures in the open top reservoirs.

For both, microfluidics as a stand-alone disposable and a sensor-integrated NoC, a practical way forward to reduce variations and feasibility in upscaling is supported by a modular approach. The semifinished microtunnel devices can be fabricated as a standardized component in a foundry service for many different user applications. Punching and assembly may then take place in the user's labs. To add value as a complementary product for further tailoring culture conditions on-chip to a user's need, scaffolding materials can be then prepared as inserts by, e.g., dispensing hydrogels directly or pick-and-place matrices as plug-ins made either by somewhat more complex techniques via salt leaching, electrospinning, or any other layer deposition technique already known in the art, e.g., spin-coating or 3D-printing. Consequently, a list of specifications, i.e., requirements, preferences, constraints (RPCs), for an NoC design must meet culture handling workflows of lab workers

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already familiar with high-throughput analytical methods and robotic liquid handling.

Advanced optical cell probing, for example, in conjunction with controlled axon damage in such a novel chip format is important to our CONNECT[13] partner KU Leuven and an NoC design concept must meet their requirements. In Vanden Berghe and co-worker's lab, the microtunnel-based NoC can assist in the development of label-free optical readouts to perform molecular trafficking and nerve innervation studies.[80] By controlled laser dissection on the chip, molecular traffic can be locally interrupted from one compartment to another compartment and regenerative neural plasticity processes can be observed in a variety of biological arrangements but all based on the same modular NoC platform technology.

Next, in the design process, we need to focus on an integration strategy, which supports the assemble of microtunnel devices to an advanced sensor array. If this step must take place in the biological laboratory of a user also as a postprocessing step, it requires self-alignment with the sensor array with tight control on the positional accuracy, a fast, clean, and sterile assembly/disassembly procedure and also being leak-tight during microfluidic filling experiments as well as an arrangement of the instrumental setup being capable of tapping into the richness of retrieved information of such sensor implementation schemes in an automated fashion at high bandwidths. A pluggable modular approach between a reusable impedance-based sensor array and a disposable microfluidic NoC component could keep the development cost for such high-end measurement electronics manageable on the development path until a clinical value of the system is confirmed.

In the outlook, in next generation microfluidic nervous system-on-chip, systems could be supported and tailored for their culture conditions by a scaffolding plate in a similar fashion as it has been theoretically suggested by us[81] as a single neuron detector readout by means of a microsieve as a disposable and pluggable culture plate. Previously, in our group, Frimat *et al.*[82] demonstrated these types of microsieve-scaffolded neural networks by seeding SH-SY5Y cells via passive flows in parallel to the highly organized cell capturing sites that are easy to monitor either by an electrical sensor array or standard epifluorescent microscopy as a monolayer but still preserve some level of three-dimensionality of the cells in the RoIs. Hence, this type of a modular designed NoC system could further serve standardization and functionalization opportunities. Either way, fully integrated, reversible bonded or pluggable, the sensors should be capable in offering a technical strategy to probe the interior neurocircuitry of a 3D-cultured neural network in a dynamic, long-term culture setting utilizing the neurons in the single neuron detector arrangement as a living but positionally known transducer of the readout technique of models, resembling the complex communication occurring in connected 3D neural networks *in vivo*. An example, of such a fully integrated chip readout technology based on silicon micromachining has been presented by us previously in Schurink *et al.*[83]

To meet the RPCs in integrative strategies for such measurements *in vitro*, further investigations on long-term cultures performed on standard microelectrode arrays in microfluidic-assisted 3D arrangements, like our microbioreactor concept,[84] 3D needlelike electrode arrays,[85] and meshlike integrated systems,[86] as well as many more inspirations for the design of such electrodes taken from integration efforts shown for *in vivo* applications of these types of neuroprobes[87] can be helpful.

Once a miniaturized electrical and electrochemical integrated sensor array is selected, it can be aligned with microtunnels similarly as a photomask to a silicon wafer. Many of such chip assembly strategies exist and do not form a knowledge barrier. Microfluidic devices, however, generally have a large footprint that currently drives the cost per chip, when full integration is desired. The latter hampers NoC market introduction, despite potentially providing access to a highly enriched dataset compared to the current state of the art of oversimplified 2D *in vitro* systems and readouts.

As mentioned in Sec. III, researchers also fabricated electrochemical sensor arrays by micro- and nanofabrication using typical semiconductor substrates.[65] Such sensor array plates as well as commercially available microelectrode arrays for capacitive-coupled measurements of electrogenic cells are still difficult to reuse despite reversible bonding of a microfluidic chip component, like it is possible for our microtunnel[79] or microbioreactor devices.[84] Therefore, so far, such sensors are realized as a needle-type device, which could be used also as a complementor to our NoC hardware similarly as the pipette in a patch-clamp setup. However, when taking the requirements for dynamics studies in long-term cultures as a requirement into account, this concept may need further refinement.

Potentially, the work presented by Cavallo *et al.*[53] can positively influence this direction of advanced device integration in terms of cost by a strain-engineered thin-film semiconductor layer integration concept on soft substrates, like PDMS, in future NoC developments. However, to take full advantage of existing nano- and microfabrication methods as established in the art of the electron-, ion-, and photon beam technology and nanofabrication community for integration strategies in NoCs, further research is needed.

C. Manufacturability

An NoC design concept for nanofabricating neural networks must consider the manufacturability of the system. As presented in Secs. V A and V B. We propose to choose a modular approach for integration and allow some level of assembly of the NoC system at the user's premises. Demonstration of such modules of an NoC system offers a high potential for connecting also other chip types for nervous system's tissue culture with a standardized microtunnel device,[79] for example, by bonding it to a plastic microsieve substrate,[88] which can potentially act as a disposable interface also to a reusable impedance-based sensor array as proposed by Demircan Yalçin and Luttge.[81] The modular system approach is complemented with soft material scaffolds as inserts placed inside the reservoirs hosting the cells or organoids as an off-the-shelf component or freshly prepared in the biological laboratory. Liquid dispensed soft material inserts and culture conditions, as presented, for example, by Akcay and Luttge,[30] are comparable with the size of wells in a 384-well-plate format, and the wells on chip are of about the same size and can simply be realized by punching for the fundamental research studies required in this development phase. A set of nine individual wells of a 384-well plate format could then

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be connected in a sort of unit cell for our novel CONNECT[13] integrated NoC for PD that is currently developed by the consortium. This type of modular chip technology features several benefits in the manufacturability of NoCs for research. When mapping the 384-well-plate layout for the reservoirs to a 24-well-plate of a MEA sensor array format, it will allow us to optimize long-term single organoid culture conditions first in a cost-effective manner in a standard low-tech well-plate prior to carrying out the connectivity study design in a high-tech integrated NoC. Herein, the standardized microfluidic component introduced in Sec. V A controls shear and diffusion processes and is easy to handle for manual alignment and reversible bonding to already available electronic readout systems at the user's lab. Techniques and methods to support such manual assembly strategies at an end-user's lab need to be still critically investigated and researched and are not yet at a high enough technology readiness level to be produced at high yields.

D. Final conclusions

We reviewed the state of the art of enabling technologies to advance 3D culture models for complex *in vitro* disease modeling as it is needed specifically in neurodegenerative diseases, like Parkinson's disease. Our innovative design proposal of the CONNECT project in putting the human gut-brain axis on chip partially implements these findings. Thanks to numerous advances in nano- and microfabrication, microfluidics, scaffold materials, and human stem-cell-derived organoid technology, a great many different user-defined NoC modules can be combined into one and the same NoC system if designed modular. These NoC systems then offer a broad range of new tools to be used in fulfilling the technical challenges toward finding better treatments and potentially cures for devastating neurodegenerative diseases. Subsequently, an integration strategy based on a modular approach also yields efficient and informative optical and electronic NoC readouts in validating and optimizing these conceptual choices in the innovative process of a fast growing and exciting new OoC industry.

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AUTHOR DECLARATIONS

Conflict of Interest

The author has no conflicts to disclose.

DATA AVAILABILITY

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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