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Engineering organoids as cerebral disease models

Alexander Geidies*, Marija LJ Medar* and Hannes M Beyer

Cerebral organoids pioneered in replicating complex brain tissue architectures *in vitro,* offering a vast potential for human disease modeling. They enable the *in vitro* study of human physiological and pathophysiological mechanisms of various neurological diseases and disorders. The trajectory of technological advancements in brain organoid generation and engineering over the past decade indicates that the technology might, in the future, mature into indispensable solutions at the horizon of personalized and regenerative medicine. In this review, we highlight recent advances in the engineering of brain organoids as disease models and discuss some of the challenges and opportunities for future research in this rapidly evolving field.

Address
Institute of Synthetic Biology, Heinrich-Heine University Düsseldorf, Düsseldorf 40225, Germany

Corresponding author:
Beyer, Hannes M (hannes.beyer@uni-duesseldorf.de)
* These authors contributed equally.

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Introduction

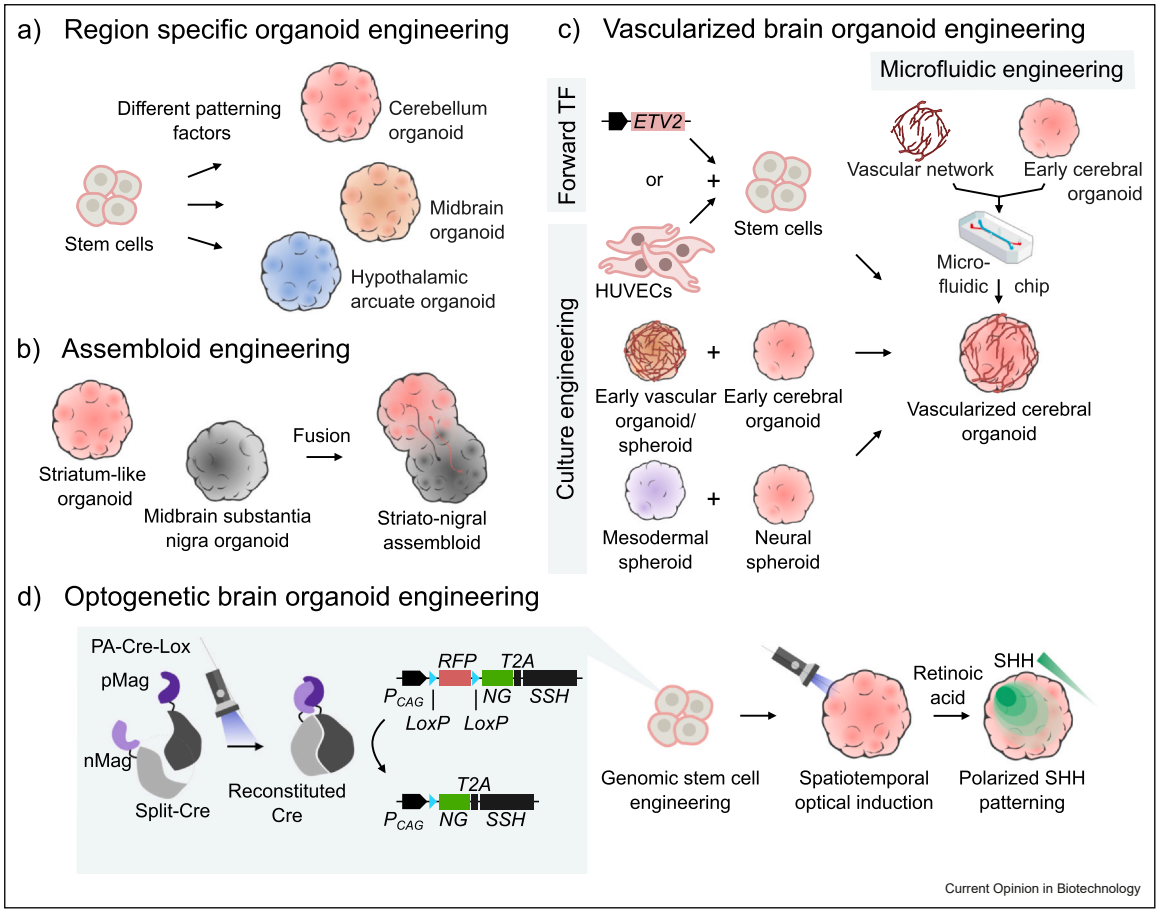
The complexity of the human brain represents a major challenge in our aims to disentangle the structure and function of the organ and gather a growing understanding of neurological processes and diseases. In the past, experimental model systems, such as 2D cell cultures and animal models, have significantly contributed to the current knowledge base; however, they reflect the maturity and functional networks present in the human brain only to a limited extent. To overcome these bottlenecks, scientists aim to reconstruct human 3D brain tissue and make organs *in vitro*, thereby gaining a strong structural resemblance to actual brain tissue compatible with invasive investigations and with higher clinical relevance than animal models [1]. These cerebral organoids (COs) derive from human stem cells and are meticulously formed by initiating spontaneous or guided cell differentiation programs. Brain organoid technologies bear a rising hope in modeling specific structural and functional aspects of the human brain, enabling the study of fundamental processes and diseases with predictive potential for personalized therapy development [2].

A Nobel Prize–winning method developed by Shinya Yamanaka, known as cell re-programming, gives rise to induced pluripotent stem cells (iPSCs) formed from somatic cells [3]. The impact of this technique laid the foundation for personalized therapy and *in vitro* disease modeling including brain organoid protocols, later extending to tissue models of other organs [2]. Transitioning naturally sourced genetic defects into an organoid model system represents a breakthrough technique that opens new possibilities to study diseases *in vitro*, compatible with invasive methods, biobanking, and high-throughput drug screening approaches [4]. Ever since the first brain organoids were reported, significant progress and protocol variations have been developed, among other achievements. Today, brain organoids serve as vital models capable of simulating brain development and physiology and offer profound insights into the pathophysiological mechanisms underlying a range of neurological diseases [1,2]. The ability to grow human tissue in the lab compensates for the low availability of human tissue for research purposes and depicts a rather accurate transition from the model to the human being. In this review, we focus on advances in engineering brain organoids as disease models and discuss challenges and perspectives for future research in this field.

Development and engineering of brain organoids

CO engineering approaches develop at a rapid pace. A decade after the first reports on brain organoid protocols appeared [1], the current literature witnesses a plethora of strategies that mature stem cell–derived spontaneously formed neuronal tissue into advanced brain-like *in vitro* model systems with high clinical relevance [2,5]. Although the first brain organoids represented a conceptual revolution for the study of the brain under laboratory conditions, early and current organoid protocols still entail severe limitations. The main challenges encompass both structural and biological constraints, notably the lack of an aging signature, the need for angiogenesis, the process of myelination, and the involvement of functional immunocompetent microglia [6].

Figure 1



Schematic overview of various brain organoid engineering strategies. **(a)** Guided differentiation of stem cells into different region-specific brain organoids [7,8]. **(b)** Chimeric brain assembloid tissue engineering with the example of engineering a striato-nigral tissue [9]. **(c)** Strategies for engineering vascularized brain organoids. Forward-programming: ETS variant transcription factor 2 (ETV2) expression [22,54]; culture engineering: stem cells co-cultured with human umbilical vein endothelial cells (HUVECs) [55]; fusion of vascular tissues and early COs [56,57] or generation of vascularized COs by tissue assembly engineering of neural and mesodermal spheroids [21]; engineering neurovascular organoids in a 3D-printed microfluidic chip by using *in vitro* formed vascular networks and COs [16]. **(d)** Application of optogenetic sonic hedgehog (SHH) gene-expression control for spatiotemporal patterning of organoid tissue with light [24]. LoxP, Cre recombinase target sequence; NG, NeonGreen; T2A, Thosea asigna virus 2A peptide.

An important leap forward in maturing the structural architecture of COs was made by modeling regional brain identities. Here, precise developmental signals guide the maturation of organoid tissues to differentiate into rather homogeneous cellular identities representing specific brain regions (Figure 1a), such as the cerebellum, midbrain, and hypothalamus [7,8]. Access to region-specific brain *in vitro* tissues therefore enables the subsequent study of diseases relevant to particular brain areas [8].

Another significant engineering concept makes use of fusing organoids into assembloid structures (Figure 1b), achieved by first initiating the formation of region-specific tissues such as striatum (Str) and substantia nigra (SN) organoids, followed by controlled tissue-fusion [9]. This approach enables modeling the various interactions between brain regions, crucial for the study of diseases that disrupt these connections, such as the Timothy syndrome [9,10]. Assembloid technologies have further aided in understanding how different parts of the brain communicate [9].

Engineering organoid-based disease model systems strongly relies on extracellular signal mediators during tissue formation and maturation. Here, biomaterials serve as extracellular matrices (ECMs) equipped with instructive biochemical or mechanical signals that also provide support and allow tissue expansion in three dimensions. Material architectures can encode instructive cues to enhance the translational potential of organoid tissues by providing tunable properties to influence cell fates and tissue functions, and aid in establishing reproducible manufacturing

routines [11]. Cell surface mechanosensory and adhesion molecules sense various cell-generated and material-derived forces, convert them into biochemical signals, and contribute to organogenesis [11,12]. For example, it has been shown that the stiffness of ECMs impacts cell proliferation, migration, differentiation, damage response, and regeneration, among other essential processes during 3D organoid tissue formation. In the brain, such biomechanical properties further influence cortical growth and surface folding, as well as the formation of outer and inner subventricular zones [11]. It has also been acknowledged that brain stiffness is associated with neuronal disorders [13]. Therefore, biomaterial design constitutes an important discipline relevant to refining organoid development protocols. While poorly defined, decellularized natural ECMs with little tunability find widespread application in organoid tissue design, synthetic biomaterials bear the potential to rationally steer tissue formation processes, for example, by engineering material-genetic interfaces and stimulus-responsive materials with tunable properties that could be applied to organoids [14]. Additionally, microfluidic technologies offer control of the extracellular milieu and compartmentalization of cells and signaling factors to regulate the 3D environment, resulting in a technology known as 'organoid-on-a-chip'. Among other examples, this technology helps standardize organoid size using physical constraints and supports the development of vascular networks [15]. Salmon et al. used spatial interactions between organoid and vasculature enabled by a custom-designed 3D-printed microfluidic chip allowing a sequential and developmentally matched co-culture system [16] (Figure 1c). The controlled compartmentalization in organoids-on-chips also enables real-time assessments of neuronal electrical activity and to monitor metabolic changes using embedded sensors, such as an electrochemical biosensor capable of detecting glutamate release from COs [17,18]. Furthermore, biosensors have been developed to monitor neuronal activity within organoids, which is crucial for assessing neuropathological disorders as well as evaluating pharmaceutical treatments and drug screens [18].

The lack of vascularization in organoids limits growth, maturation, and long-term survival due to restricted passive diffusion of nutrients and gases, calling for cellular tissue engineering solutions [19]. In particular, the concept of stem cell forward-programming using suitable transcription factors into endothelial cells has furnished brain organoids with vascularization despite the fact that both tissues derive from different germ layers (Figure 1c) [20–22]. These innovations not only advance organoids as a model for studying neurological diseases but also as chassis for testing defined pharmacological treatments, including the effects of drugs that affect the blood–brain barrier [19].

The high degree of structural complexity across the three-dimensional space of the brain calls for methodological approaches that enable investigations in *in vitro* model systems, for example, the spatial distribution of neural activity. Here, optogenetic technologies open an innovative path forward. The generation of organoids from iPSCs pairs well with genomic stem cell engineering to implant optogenetic genes that can be invoked at any desired differentiation state. The *in vitro* cultivation environments support spatiotemporal optical induction with superior tissue penetration compared with animal models [23]. In addition, the tissue compatibility with live imaging enables real-time monitoring of the optogenetic regulation effects, among other spatial assessments [24]. The integration of the light-sensitive channelrhodopsin-2 (ChR2) into brain organoids permits the dynamic control of specific neuronal populations through light activation [23,25]. Legnini et al. used an optogenetic gene-switch approach relying on a photoactivatable Cre–Lox system to locally induce the expression of the morphogen sonic hedgehog (SHH) in a neurodevelopmental organoid model to guide tissue patterning with optical stimuli (Figure 1d), a concept also compatible with assembloid tissue organizers [26]. During brain development, the SHH morphogen specifies distinct cellular fates along the dorsoventral axis, validated in the *in vitro* model through spatial and single-cell transcriptomic analysis [24]. Spatial transcriptomics and spatial proteomics assessments offer detailed insight into the spatial organization of gene expression and protein distribution, for example, to enable the mapping of cellular and regional patterns [27].

Along the timeline, as the first brain organoid protocols appeared, CRISPR-Cas9 technology co-evolved simultaneously [28]. The integration of CRISPR-derived technologies — including base- and prime-editing derivatives — into organoid cultures enables the precise engineering of target genes, for instance, to manufacture isogenic controls matching personalized model systems or study mutations in the validated background of a deeply characterized cell line [1,29,30]. In addition, CRISPR Activation (CRISPRa) and Interference (CRISPRi) serve as regulatory handles to engineer stem cell and organoid models by enabling targeted promoter control of endogenous genes. CRISPR technologies have already manifested as indispensable elements in specific organoid engineering tasks, [30] for example, to model human diseases, explore cellular functions, and unlock unprecedented opportunities in patient-specific neurological research and therapeutic development [2,28].

Modeling of neurodegenerative diseases

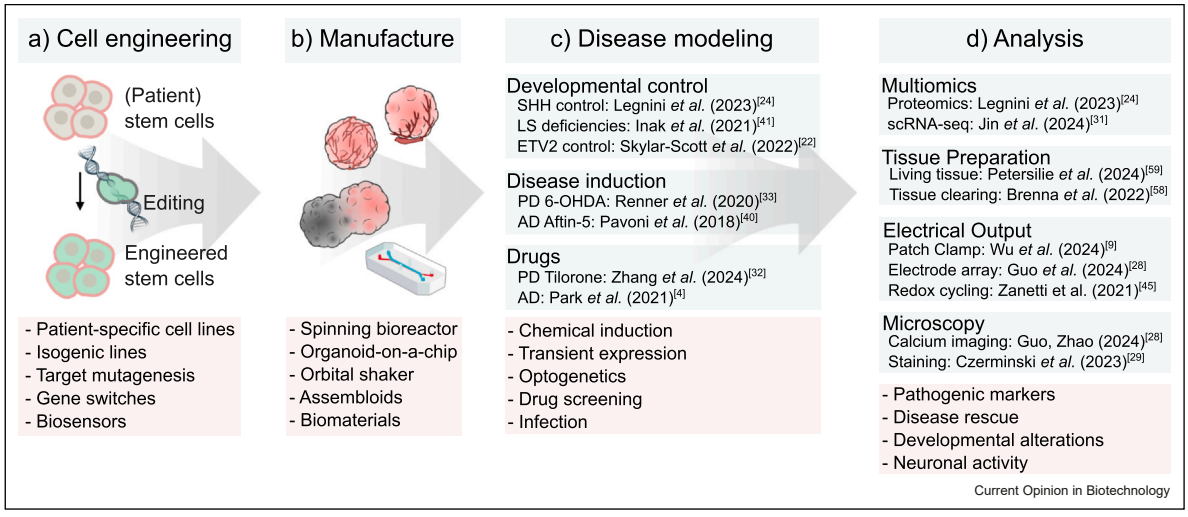
Currently, two major engineering strategies for organoid-based model systems relevant to genetic cerebral pathologies exist. The first approach sources naturally occurring, disease-associated mutations from the sequence space identified from clinical samples to derive organoid models, either through stem cell explanation or re-programming of somatic cells of patients, suitable, for

example, to establish personalized therapies [29]. Alternatively, biological engineering efforts can re-model the genetic background of a disease into a healthy stem cell genotype [2,30].

Trigger-inducible systems that initiate processes, including the onset of a disease, stand at the forefront of stem cell and cellular organoid engineering strategies. Such systems can initiate disease patterns at specific experimental stages, serving as representative models for neurodegenerative diseases like Parkinson's disease (PD) [31,32]. Traditionally, PD pathology has been induced via the application of neurotoxins such as 6-hydroxydopamine (6-OHDA) or 1-methyl-4-phenylpyridinium (MPP+); these chemicals were also used for the simulation of PD in human midbrain organoids (hMOs) [33] (Figure 2). The ability to induce a PD-related phenotype at a desired time point in hMOs has been addressed recently by the use of an optogenetic method [34,35]. Kim et al. fused α-synuclein, whose aggregation initiates the formation of Lewy bodies (LBs), typically associated with PD, to the plant photoreceptor CRY2 [35]. Blue light induces protein oligomerization of CRY2, thereby initiating α-synuclein aggregation and LB formation. The engineered model system served drug identification applications, by assaying the protective properties of a compound library in preventing neuron-loss upon the optogenetic induction.

Alzheimer's disease (AD) has been modeled in COs with great success [36,37]. AD-typical pathogenic traits reflected by COs include increased levels of phosphorylated microtubule-associated tau protein (p-Tau) and amyloid-β (Aβ) aggregates, transcriptomic alterations reminiscent of observations in post-mortem AD samples, and reduced neuronal activity [38,39]. AD can be induced in COs by the addition of small molecules such as Aftin-5, increasing Aβ expression and accumulation in the medium [40]. The overexpression of pathogenic aβ cleavage peptides $A\beta_{40}$ and $A\beta_{42}$ provides a useful approach for the rapid induction of AD pathogenesis in organoid models; however, it does not necessarily resemble natural conditions under which AD develops. Therefore, Vanova et al. employed healthy i3N-iPSCs and CRISPR-engineered them with the presenilin $PSEN1^{A246E}$ mutation. This affects amyloid precursor protein processing and leads to altered $A\beta_{42}/A\beta_{total}$ and $A\beta_{42}/A\beta_{40}$ ratios, thus inducing AD pathogenicity, enabling the study of familial AD under endogenous, constitutive expression conditions [36]. Reports on the successful modeling of neurodegenerative diseases like AD or PD encouraged organoid modeling attempts of lesser-known diseases, for example, Leigh syndrome, which affects mitochondrial metabolism in neural tissue [41]. Engineering these diseases, however, requires sophisticated gene editing methods compatible with mitochondrial genes, which remains challenging with present technologies. Owing to this challenge is the lack of methodologies that would enable the import of CRISPR-associated ribonucleoproteins, such as the BE3 base editor, into mitochondria, limiting the available toolkits to such based on TALENs, previously applied in mitochondria

Figure 2



Workflow of engineered organoid manufacturing. **(a)** Patient-derived stem cells are used as is or genetically engineered, for instance, to carry a functional gene or an introduced mutation, for example, to generate pathogenic isogenic cell lines for generating disease model organoids. **(b)** Organoid model tissues are manufactured from (engineered) stem cells using an advanced repertoire of methods, including assembloids, organoid-on-a-chip, *etc.* **(c)** Organoids are used in appropriate experiments, such as disease induction, therapy development, or developmental control with the aim to model diseases of interest 4,22,24,32,33,40,41. **(d)** Organoids are compatible with a range of functional and analytical methods yielding qualitative and quantitative data [9,24,28,29,31,45,58,59].

[42]. One such example is mitoBE, a base editor, which was successfully used for the genetic editing of mitochondrial (mt)DNA [43].

The continuous progress in modeling neurodegenerative diseases in COs enables increasing possibilities for high-throughput *in vitro* drug screening applications. Initial studies with a focus on drug treatments for AD were reported by Park et al. [4]. Moreover, approaches of hMOs transplantation into PD mice models, which showed restoration of motor functions, highlight the applicability of the organoid model for regenerative medicine [44]. In this example, high-throughput transcriptomic and fluorescence analysis showed a reduction in p-TAU and Aβ aggregation in spontaneous AD-associated COs upon drug application. Furthermore, a redox biosensor based on 3-mercaptopropionic acid (3-MPA) has been utilized for the study of PD in hMOs [45]. This biosensor specifically recognizes dopamine (DA), released from dopaminergic neurons, providing information about neuronal activity and neurotransmitter release. However, genetically encoded biosensors, for example, for Wnt signaling, will represent promising components to derive future-engineered model tissues with intrinsic sensory capabilities [46].

Modeling of neurodevelopmental disorders

Neurodevelopmental diseases often affect the early stages of brain development. Current organoid model systems suffer from a limited degree of maturation but can well mimic early developmental conditions, emphasizing the suitability of the organoid model for application in these disease areas. One prominent example of neurodevelopmental diseases is Huntington's disease [47], also known for its neurodegenerative properties in multiple brain regions, for example, Str and SN in the human brain and impaired neuronal maturation in the early stages of brain development. To observe projections of pathological traits in the circumjacent tissues, a recent approach resorted to the assembloid approach to engineer a striato-nigral tissue (Figure 1b) [9]. In this tissue fusion model, the authors could observe neurite extension protruding from the striatal organoid into the substantia nigra organoid along with altered electrochemical signaling projection assayed with optogenetic field potential recording and calcium imaging (Figure 2).

Limitations of organoid models

Despite several reports on successfully engineered CO disease models, the technology remains currently in its infancy with a list of limitations. Organoids generally mimic the early embryonic stages of organ development; thus, age-dependent effects are not directly accessible in current model systems. While the cellular composition of COs resembles the human brain, certain cell types, like astrocytes, only appear at around 80 days [48]. The full cellular composition of a mature brain remains yet to be modeled with sufficient detail, for example, the lack of microglia cells or vasculature, which do not originate from the ectoderm, require sophisticated engineering strategies to develop mature cerebral *in vitro* models (Figure 2). The sample heterogeneity across batches and individual specimens remains another significant limitation of CO-derived models [49]. The stochastic differences can affect data acquisition and quantitative analysis. The source of these variations is versatile. Thus, strategies to overcome the heterogeneity have to be found at all stages of CO generation. The initial step, that is, the embryoid body (EB) formation, requires particular attention. Differences in EB size and overall cell quality can strongly impact neural induction and nutrient availability and, thus, propagate tissue heterogeneities. To enhance the viability in stem cell cultures and optimize EB formation, the Rho-associated protein kinase (ROCK) inhibitor Y-27632 is commonly used. Lately, a more potent cocktail of four small molecules has been identified, representing a significant advancement in stem cell technologies [50]. Additionally, batch-to-batch variability of ECMs limits the comparability of COs, as variations in overall protein concentration, structural proteins, growth factors, and proteoglycans severely influence cell proliferation, nutrient diffusion, and cell viability [49].

Future perspectives in brain organoid engineering

The recent incorporation of artificial intelligence strategies into biological research extends to the fields of organoid engineering and disease modeling. Employed as a methodological asset in image analysis and high-throughput screening, the technology will likely advance organoid analysis in interpreting or predicting responses to pharmacological treatments [51]. The term 'organoid intelligence' refers to an emerging multidisciplinary field aiming to develop biological computing using brain organoids and brain–machine interface technologies as a powerful and energy-efficient alternative to classical data processing units [52]. In addition, advanced organoid control techniques, for example, spatiotemporal optogenetic strategies illustrated above, still require robust engineering and use protocols to advance the application of COs. While complex developmental features, including forebrain-associated primitive sensory structures resembling cortical-optic vesicles, have been observed in COs [53], the field requires reliable control handles to precisely regulate the underlying molecular events in order to build sophisticated model tissues.

In summary, the possibility of manufacturing cerebral model tissues, for example, from re-programmed patient cells, opens up immense potential for the study of the

human brain and associated diseases *in vitro*, where biological samples remain extremely scarce. The steady advance of engineering techniques across various fronts of organoid biology, ranging from manufacturing conditions and ECMs to cellular engineering and the implementation of synthetic molecular machines, entails exciting future prospects for the field and its relevance in biomedical applications.

CRediT authorship contribution statement

Alexander Geidies: Visualization, Writing – original draft, Writing – review & editing. **Marija LJ Medar:** Visualization, Writing – original draft, Writing – review & editing. **Hannes M Beyer:** Conceptualization, Formal analysis, Funding acquisition, Project administration, Supervision, Visualization, Writing – review & editing. Alexander Geidies and Marija LJ Medar contributed equally.

Data Availability

No data were used for the research described in the article.

Declaration of Competing Interest

The authors have no conflict of interest related to this publication.

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- •• of outstanding interest

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The processing of COs for data generation varies depending on their actual application. This study shows an interesting approach to CO slicing, allowing long-term cultivation followed by diverse analytical techniques.