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Brain organoids and the study of neurodevelopment

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Abstract

Brain organoids are three-dimensional self-assembled structures composed of hundreds of thousands to millions of cells that resemble the cellular organization, transcriptional and epigenetic signature of a developing human brain. Advancements using brain organoids have been made to elucidate the genetic basis of certain neurodevelopmental disorders, such as microcephaly and autism; and to investigate the impact of environmental factors to the brain, such as during Zika infection. It remains to be explored how far brain organoids can functionally mature and process external information. An improved brain organoid model might reproduce important aspects of the human brain in a more reproducible and high-throughput fashion. This novel and complementary approach in the neuroscience toolbox opens perspectives to understand the fundamental features of the human neurodevelopment, with implications to personalize therapeutic opportunities for neurological disorders.

Keywords

brain organoids; pluripotent stem cells; neurodevelopment; disease modeling

A self-organized brain structure in a dish

From a small cluster of embryonic **pluripotent stem cells** (see Glossary), followed by multiple cell divisions, a fascinating and complex system emerges: the human brain. For years, the early stages of human brain development were challenging to study and manipulate. With limited access to live experimental opportunities *in utero*, scientists have relied upon non-invasive indirect techniques to learn about the formation of the human brain,

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Conflict of Interests

Dr. Muotri is a co-founder and has an equity interest in TISMOO, a company dedicated to genetic analysis and brain organoid modeling focusing on therapeutic applications customized for autism spectrum disorder and other neurological disorders with genetic origins. The terms of this arrangement have been reviewed and approved by the University of California San Diego per its conflict of interest policies.

such as ultrasound imaging, abortus material, and animal models. Our knowledge of human neurodevelopment is deficient, with limited information on the essential functional steps of its creation, especially in pathological conditions. In this Opinion article, we argue that human brain organoid technology can fill some of the gaps in our lack of detail about brain development, helping to make a significant impact on our understanding of the assembly of human brain circuitry.

Initial efforts to generate human brain cells *in vitro* involved the use of pluripotent stem cells. Past efforts then focused on finding the right timing and growth factors needed to coax the stem cells into the different subtypes of neurons and glia located in the human brain [1]. Most of these protocols use the transition stage of neural progenitor cells (NPCs) in a conventional tissue culture plate in two-dimensions. However, cells growing in the traditional two-dimensional petri dish environment have limited opportunities to create organized network structures [2, 3]. For some stem cell scientists, the ability to re-create a complex system would be a step-by-step incremental process, in which we need to learn first how to generate all the different types of cells individually using complicated protocols and then connect these cells in three dimensions. However, the genetic background of stem cells instructs the differentiation program to allow the generation of a self-assembling structure upon a transient pharmacological induction [4, 5]. We have learned that when allowed to float freely in a solution or embedded in some matrix in tissue culture flasks, human pluripotent stem cells will self-organize into brain-like spheres, or organoids[6, 7]. These brain organoids are millimeters long and formed by hundreds of thousands to millions of cells, that resemble the organization, developmental timing [5, 8] and transcriptional epigenetic signature of a primitive human fetal brain [6, 9-11]. In the past years, scientists have fine-tuned this strategy to produce two types of brain organoids: patterned or primed, where the cells are guided towards specific brain regions; or non-patterned, allowing for different brain regions to emerge in a single unit [7, 12]. Patterned brain organoids can be induced using a specific set of growth factors, guiding the cells to particular fate decisions, such as forebrain or midbrain [5, 13, 14]. One group were able to balance cellular programming and stochastic conditions by using patterning inductors to guide iPSC differentiation [15]. For instance, forebrain organoids could be generated by traditional dual inhibition of TGFB (transforming growth factor beta)/BMP (bone morphogenetic proteins) pathways and GSK-3 (glycogen synthase kinase 3) [4, 16, 17]. The generation of other brain areas *in vitro* followed similar protocols, however with different patterning factors: SHH (sonic hedgehog) and FGF8 (fibroblast growth factor 8) for midbrain induction and WNT-3A and SHH for hypothalamus formation [8, 15, 18-24].

Poking the brain for answers

Many emerging technologies, such as **patch-clamp recording** and **calcium imaging**, could be used to interrogate the link between genetic programs and function, defining underlying pathophysiology mechanisms or furthering our understanding of neurodevelopment. As a result of the immense growth of sequencing technologies, genomes can be studied at a single-cell level. Early brain diversification can be modeled by breaking the organoid into its parts and correlating cellular trajectories during maturation [9, 23, 25]. In this context, single cell transcriptional profiles of brain organoids are similar to developing human cortex at the

second trimester of gestation [9], followed by a generation of a broad diversity of cells [26]. A single cell gene expression profile of more than 80,000 cells from 6-month-old organoids was analyzed, supporting the cellular diversity necessary for the formation of complex circuitry in the brain[26]. While these studies provided a comprehensive map of the organoid cellular variety, they highlighted the reproducibility limitations and experimental variability.

Human brain organoids can be genetically edited via genome editing technology, such as **Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)** [27-32] or **Transcription activator-like effector nucleases (TALEN)** [33-35], allowing precise editing of a target DNA site or correcting mutations in the patient-derived stem cells. Potential off-target effects can be predicted and minimized with optimized experimental design and controls [36]. The genomic engineering of iPSC lines represents a significant advancement in the establishment of a robust causal link model and phenotypic homogeneity in a disease context. A more reliable disease model leads to robust stem cell-based assays required for preclinical studies to identify and to evaluate candidate drugs in screening platforms for therapy.

The convergence of many cutting-edge techniques will possibly be used to generate custom circuits that could be manipulated with minimal invasiveness. The precise control and measure of neuronal activity over an extended period can be precisely manipulated by introducing light-activated channels into the cell (for more information see review [37]). Channelrhodopsin ChR2 is a subfamily of retinylidene proteins that function as light-gated ion channels and enable light to control electrical excitability. The blue-light sensitive ChR2 and the yellow light-activated chloride pump halorhodopsin together allow multiple color **optical activation** and neural activity silencing. With **optogenetics** and **virus-mediated circuit tracing** tools, it will be possible to activate the synaptic transmission at a single-cell level inside organoids[38, 39]. This level of synaptic control could be used to study the molecular events during the induction of synaptic plasticity and to evaluate the impact of such activation in gene expression profile, synaptogenesis, and network formation. The number of studies using optogenetic probes in iPSC models is relatively limited, but they hold promise for exploring new phenotypes and pathways to understand the etiology of neurodevelopmental disorders[40, 41]. Altogether, brain organoid models when combined with genome editing, gene expression studies at a single-cell level, optogenetics, and other cell-based assays have great potential to push the field synergistically and open unprecedented investigation paths.

Brain organoids as a neurodevelopmental model

Human brain organoids have already contributed to the discovery of novel biological information on the early stages of neurodevelopment and disease progression, and to the ability to manipulate these processes *in vitro*. Perhaps the most striking example of the use of this technique was to show causation between the circulating Zika virus and the outbreak of microcephalic cases in Brazil [42, 43] (Figure 1). By exposing human iPSC-derived brain organoids to an isolated Brazilian Zika strain from an affected febrile proband, it was possible to show how the virus was attracted to neural progenitor cells, due to a set of exclusively expressed membrane receptors [44]. Infection of NPC with Zika virus leads to

cell death and defects in the cortical plate by reducing specific populations of cortical neurons in different layers of the organoid and mouse brain [44]. The organoid model was then used to show that previously approved drugs could prevent the infection [45] or inhibit Zika viral replication by blocking vertical transmission [46]. Other groups have also showed the impact of non-Brazilian Zika strains on human brain organoids [24, 47, 48], although the relevance of these observations to the Brazilian outbreak is debatable [49]. These results were only achieved due to the use of the organoid model to recapitulate cortical layering Zika-induced phenotypes *in vitro*, cytoarchitecture organization that is not observed in a monolayer neuronal culture.

Brain organoids have also contributed to advances in human disease modeling of monogenetic disorders [23] as well as polygenic idiopathic conditions, such as ASDs [50]. Using a previously selected cohort of severe idiopathic ASD individuals with macrocephaly (increased head/brain size) as the endophenotype revealed a significant increase in neural progenitor cell division in patient-derived iPSC-derived brain organoids [50]. Notably, the correlation between iPSC-derived neural progenitor cell division and brain volume was independently validated, indicating the robustness of this association identified in brain organoids to such heterogenous genetic conditions [51]. Brain organoids can also be created from different brain regions. Using an **assembly method** of the dorsal and ventral forebrain, it is possible now to recapitulate the migration of interneurons that happens during human brain neurodevelopment [52, 53]. Using brain organoids, the cellular phenotypes of Timothy syndrome, a rare neurodevelopmental disorder caused by mutations in the $Ca_V1.2$ calcium channel gene, were modeled. By generating organoids from iPSCs derived from patients and comparing them to controls, abnormal migration of interneurons was revealed for the first time [23]. Recently, Karzbrun and coworkers reported a Matrigel-chip approach that enables development of folded human brain organoids. The organoid-on-chip technology allows long term live imaging and provide a novel system to investigate the mechanism of wrinkling of early human brain development [54].

Microcephaly, caused by autosomal-recessive mutations in several genes, was an early target of brain organoid disease modeling [6, 55]. Gabriel and coworkers investigated the impact of centrosomal protein (CPAP) in the regulation of cell-cycle and the generation of neural progenitor cells. The authors proposed that the reduction of progenitor's pool is associated with a premature switching from symmetric to asymmetric division due to defects in centrosome biogenesis, Moreover, their findings point to a important role of cilia function on the neurogenesis and brain size regulation [56]. Another classic example of microcephalic disease modeling using brain organoids was Aicardi Goutieres Syndrome (AGS) type I, caused by mutations in the Threeprime Repair Exonuclease I, *TREX1* gene [57]. AGS is characterized by a dramatic neuronal loss, leading to a life-long disability condition [58]. The lack of robust animal models has blocked the understanding of the pathology and the development of potential treatments [59]. Using **isogenic** and patient-derived iPSCs, it was possible to dissect the contribution of different cell types to AGS. When AGS iPSCs were differentiated into brain organoids, massive cell death was observed, mimicking the neurodegeneration and microcephaly seen during patient neurodevelopment *in utero* [60]. An apparent inflammatory reactivity was found in a homogeneous culture of

astrocytes and validated in brain organoids. The neurotoxicity was further induced through increased secretion of type I interferon, a pro-inflammatory cytokine [60]. When investigating the potential causes of the inflammation, accumulation of L1 retrotransposons was noted in the cytoplasm puncta. L1s are repetitive sequences on the human genome that can autonomously **retrotranspose** using reverse transcriptase during neurogenesis [61, 62]. HIV reverse transcriptase inhibitors and interferon blockers could reduce not only the inflammatory response but also rescued neuronal death and brain organoid size in AGS brain organoids [60]. However, it remains to be determined whether organoids might be used to model the complex neuronal activity that accompany conditions such as schizophrenia, epilepsy, and ASD.

Experimental limitations and alternatives

Despite its potential, brain organoid technology has some significant limitatios. Models are imperfect representations of the reality and the best models are the ones closer to the real situation. All *in vitro* systems are artifacts by nature because cells living in a dish are far from the conditions in the organism. Thus, it is unlikely that the organoids are growing in the optimized culture conditions similar to those found in the human brain *in utero*. Most of the protocols are based on empirical experimentation or rodent brain literature, and thus, several factors are likely to be missing or over-represented. Therefore, using such non-optimized conditions might lead to interpretation artifacts of what happens *in vivo*, during human neural development. For example, the lack of optimization may lead to abnormal neural maturation, failing to produce more organized brain structures. This field and the entire stem cell community would benefit from careful optimization of culture conditions using mathematical models, such as **Design of Experiments** (DOE). Markers of pluripotency and cell growth were used as experimental readouts for the optimization of interactions among the several factors *in silico* and adjusted *in vitro* to improve the maintenance and passage of iPSCs [63]. Therefore, by following such systematic optimization in neuronal culture, not only could we achieve better results regarding differentiation efficiency and reproducibility, but it might also be possible to appropriately age cerebral organoids with selected growth factors and media conditions to model even late-onset disorders, such as Alzheimer's or Parkinson's disease. This statistical strategy allows us to select the most efficient combination and concentration of specific growth factors required for cell maintenance and proliferation (such as FGF and EGF, epidermal growth factor), and differentiation/ specification (such as GDNF; glia-derived neurotrophic factor, BDNF; brain-derived neurotrophic factor, and SHH) for different protocols of brain organoids[15, 26].

Second, the human brain contains billions of cells and is not only composed of neurons[64]. Brain organoids in a dish have only a fraction of the different cellular subtypes expected in the brain, such as endothelial cells, blood, and microglia. For instance, immune cells called microglia are also linked with several neurological conditions. Microglia have a different embryonic origin than the neurons and astrocytes, and consequently, they cannot be produced by the same differentiation protocols for brain organoids [65]. Still, it is possible to produce microglia in parallel and infuse them into the organoids later on [66, 67]. Interestingly, human iPSC-derived microglia actively migrate and extend processes resembling early microglia development when co-cultured in brain organoids [67].

Blood vessels are not a feature of brain organoids, and the lack of vascularization presumably leads to an abnormal distribution of nutrients to the cells, especially to the inner center of the organoids where necrosis is frequently noted [68]. Transplantation of brain organoids into a mouse brain for the animal's blood vessels to naturally vascularize the organoid has been done, but creates new problems: such as **xeno-contamination** [69], size restriction (the growth of the human organoid is restricted by the animal skull size) and lack of throughput (requiring timeconsuming procedures that limited the ability to run hundreds of experiments at the same time) [40, 70]. Alternatively, adding human endothelial cells to give rise to blood vessels that self-organize into an intricate vascular system, might be a solution [70].

Lastly, and perhaps most conceptually important, we have not yet characterized these organoids at the functional level. Millions of synaptic connections and neural networks emerge from the human brain and are shaped during neurodevelopment. It is unknown whether the neurons in organoids are making the proper plastic connections to create circuitry as found among cortical layers in a mature brain or are randomly connecting to each other. Recent evidence suggests that long-term maturation of brain organoids could lead to the emergence of an active neural network, with oscillatory activity such as brain waves observed in an encephalography [71]. A complex circuitry emerges early in development in humans, and it is currently unclear whether the network is shaped exclusively by biological programming prenatally or is guided by environmental activity-dependent cues [72, 73]. The inability to electrophysiologically interrogate the human embryonic brain makes such questions difficult to answer without extrapolations from non-human model systems.

Concluding Remarks

If technical and ethical concerns (see Box 2 and Outstanding questions) could be overcome, an eventual goal in this arena might be the reconstruction of human brain regions or, more ambitiously, the entire human brain. Along the way, brain organoids could yield a multitude of discoveries about the healthy development of the human brain and for personalized medicine. In the future, doctors may use patient-derived brain organoids as a diagnostic tool or to test various drugs and doses before writing out a prescription. However, long before this, we need to learn how to translate the observations from brain organoids in the lab to the actual human brain. Structural malformations, such as microcephaly or macrocephaly, are the obvious starting points because the comparison is visible. For progress to happen in disorders and conditions that affect neural networks, we need brain organoids with sophisticated electrical activity to compare with EEGs from patients (Figure 2). Epilepsy and ASD might be an easy target to validate this idea since an EEG signature might be easier to identify in these conditions due to the early appearance of specific biomarker patterns that can be correlated with the severity of the patients' disorder [74, 75]. The incorporation of bioengineering techniques, such as vascularized fluidic systems to encourage organoid growth and prevent necrosis and multi-electrode arrays system to allow longterm electrophysiological recordings, might bring us one step closer to this futuristic scenario.

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Glossary

Assembly method
a controlled technique for fusion of organoids from different brain regions to recapitulate the saltatory migration of interneurons in vitro

Brain waves or Neural oscillations
Repetitive and synchronized electrical activity generated from neurons communicating with each other. The activity of a large group of neurons can be registered in an electroencephalogram using sensors to detect variations in the local field potential

Calcium imaging
imaging technique designed to measure the amount of calcium on isolated cells. In neurosciences, calcium transients in neurons are frequently associated with functionality

Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)
CRISPR/Cas system is used to create targeted breaks in the DNA, allowing a custom editing of the genome. Originally, CRISPR system confers resistance for prokaryotes by detecting and cutting exogenous DNA elements. In eukaryotes, this system was optimized as a powerful tool for genome editing

Design of Experiments
a mathematical technique that can be used to determine the optimal set of conditions across many different changeable parameters

Electroencephalography
method to record electrical brain activity in a noninvasive fashion using electrodes on the scalp of an individual

Isogenic cell lines
cells that have the same genetic background, except for an introduced or deleted target gene mutation. Usually, they are genetically engineered to model a specific condition, and the non-mutated cell line is used as a corresponding control

Microcephaly
a medical condition where the head circumference is smaller than a typically developing child

Optical activation
cellular response induced by activation of membrane light-sensitive channels

Optogenetics

technology used to control cellular activity by expressing genetically encoded light-sensitive channels

Patch-clamp recording
a technique frequently used in neurosciences to measure ionic currents and voltage passing across the membrane (action potentials) in individual neurons

Pluripotent stem cells
cells that are able to differentiate into all three germ layers of an animal embryo

Retrotransposition
insertion mechanism of genetic material that can self-amplify and integrate into the genome

Transcription activator-like effector nucleases (TALEN)
composed of a nuclease fused with a DNA-binding domain that can be modified to target and cleave specific DNA sequences

Virus-mediated circuit tracing
a modified rabies virus that allows the labeling of neurons connected through synapsis to reconstruct brain circuitry

Xeno-contamination
uncontrolled presence of material from a different species.

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Highlights

- Brain organoids are three-dimensional self-assembled structures that recapitulate crucial molecular and cellular steps of the brain development.
- Protocols exist to create brain organoids in a dish from human pluripotent stem cells derived from patients and healthy individuals.
- The incorporation of single-cell sequencing, genome editing, and optogenetics could improve the application of brain organoids.
- The human brain organoids are being used to understand monogenetic and syndromic neurodevelopmental disorders, as well as environmental causes of neurological conditions, such as Zika pathophysiology.

Outstanding questions box

- How can lessons learned from human brain organoids in a dish be translated to patients?
- Is the activity level of human brain organoids comparable to the natural neurodevelopment of the human brain?
- When does human complex neuronal network begin and can it be reproduced in the lab?

Box 1

Clinician's Corner

- The inaccessibility of the human embryonic brain is a major obstacle towards the understanding of the origins of many neuro-psychiatric disorders that starts *in utero*.
- The reconstruction of critical steps of human neurodevelopment will open experimental opportunities to test hypotheses about the contribution of embryonic/intrauterine environment and genetic variants to the human brain.
- Human brain organoids *in vitro* might one day be used clinically as diagnostic tools and platforms to personalize treatments.
- Pharma companies might incorporate human brain organoid models in their drug discovery pipeline to evaluate drugs in an environment similar to the one found in patients and to reduce animal experimentation.

Box 2

Mitigating ethical concerns

Aside from the technical bottlenecks, there is at least one crucial ethical question that arises from the idea of human brain organoids growing in labs across the world: could brain organoids ever develop signs of neural activity similar to real human brains? It seems a naive question but recognizing when cognition arises in the human brain is not trivial. For instance, by comparing metrics of baby electroencephalography with a neural activity profile obtained from a brain organoid, we might be able to correlate human-specific developmental steps in the formation of higher-order functions [71]. When compared with an aborted human material, it is already being reported that some components of this developmental process can be found in brain organoids, such as transcriptomic [9] and epigenetic profiles [10], cellular diversity and synaptic connections [26]. Taking these studies into consideration, it seems that the organoids possess the necessary parts to allow the formation of a network circuitry, however, how far they can functionally mature remains to be explored. For a long time, researchers have been studying pieces of human brain, isolated during surgeries for epilepsy or tumors. Isolated human tissue can retain certain neural oscillatory activity *in vitro* [76]. However, different from brain organoids, these tissues will only be alive in culture for a few weeks [77]. Contrary, brain organoids can be viable for months if not years, building up their connections over time and even process external information, such as light signals coming from connected retina cells [26]. Therefore, brain organoids could be connected to other sensory organs and show neural plasticity. Such discussion is perhaps, at present, a philosophical exercise. However, this possibility would open a new set of ethical concerns that would dictate what type of experiments could then be done using these more complex brain organoids. If brain organoid technology evolved to a level where its activity is similar to a newborn baby, a moral dilemma over their use could be considered [78]. Fortunately, scientists and ethicists are not ignoring these concerns but starting to discuss potential guidelines to support science advancement.

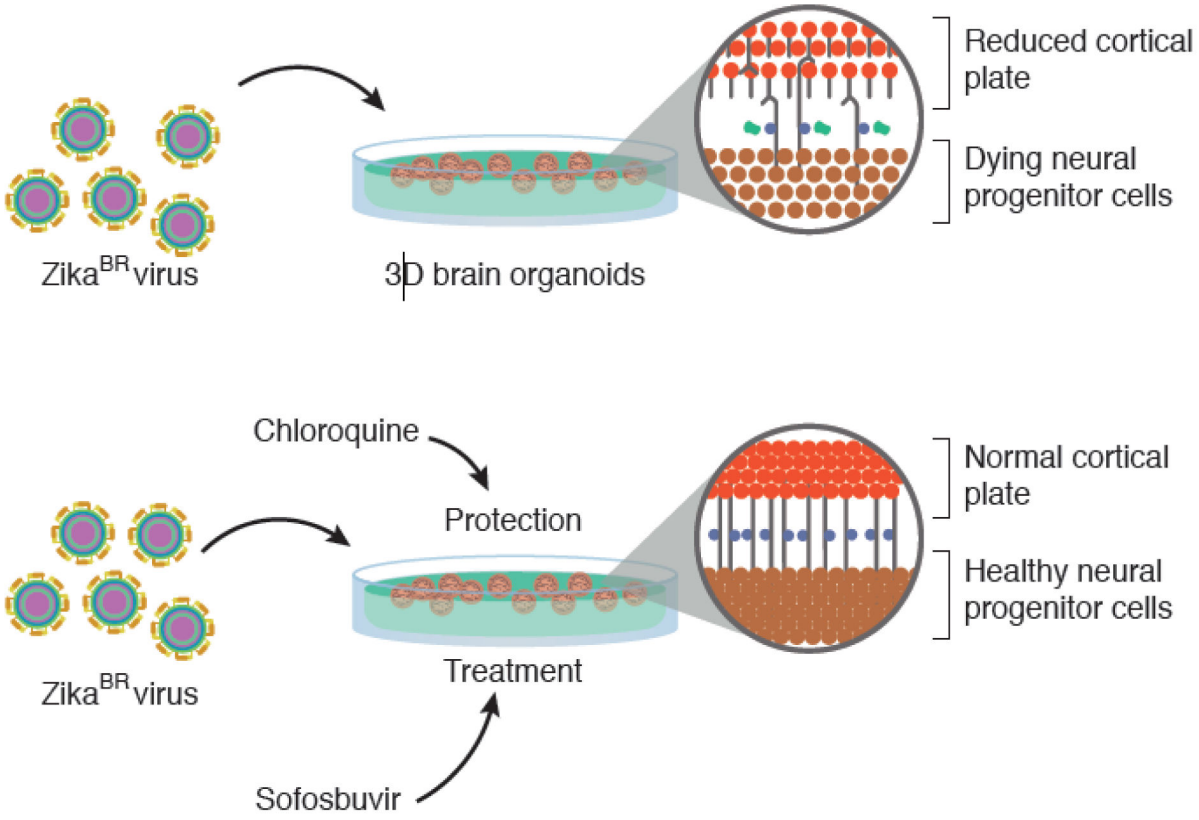

Figure 1 |. From cause to cure.

Together with animal models, human brain organoids were used to confirm the detrimental causal link between the Zika virus on prenatal brain development during the Brazilian outbreak. (Top) The circulating Zika virus in the northeast of Brazil was isolated from a case and used to infect human brain organoids in the lab. These human brain organoids were derived from iPSCs reprogrammed from adult skin fibroblasts from neurotypical individuals. Upon exposure, the Zika virus was able to infect neural progenitor cells (NPC) from ventricles inside the organoids and induce cell death. As a consequence, the migration and differentiation of the NPCs were significantly reduced leading to a reduction of the cortical thickness. Inspection of the cortical layers revealed that the Zika virus was killing early progenitor cells, responsible for cortical layer V/VI. (Bottom) The cell death induced by the Zika virus in these human brain organoids was so severe that it was also used to test drugs to prevent infection or inhibit viral replication. Successful drugs, such as Chloroquine and Sofosbuvir, lead to protection and treatment of Zika-infected human organoids respectively. Studies using human brain organoids and new neurotrophic viruses can rapidly point towards mechanisms, prevention, and potential treatments for susceptible populations.

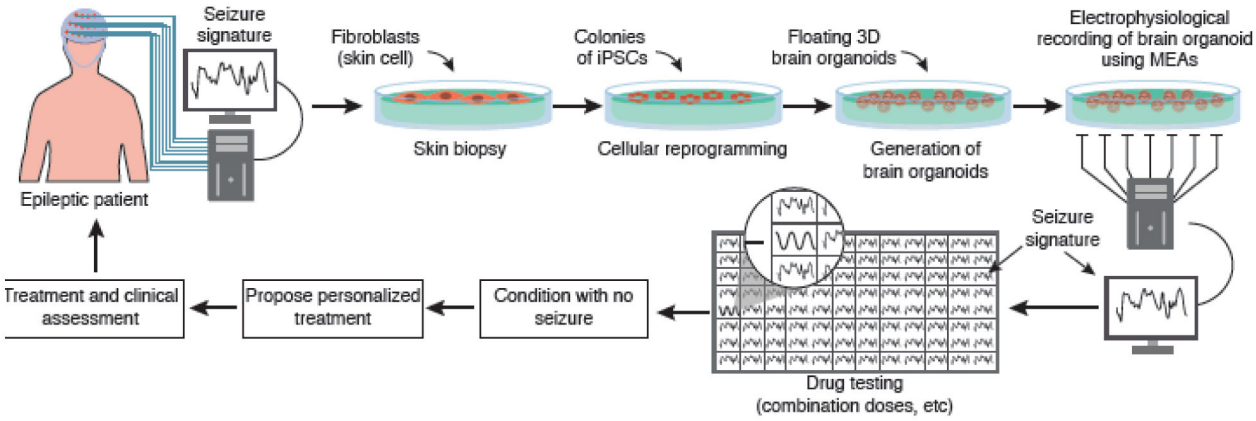

Figure 2 |. A proposed in vitro model to study the neuro-signature patterns of the neuronal activity of patients with epilepsy.

Human iPSC-derived brain organoids might one day be used for electrophysiological assays to investigate epilepsy and specific neuronal phenotypes related to a seizure signature in a dish. Patient-derived brain organoids could be used for drug screening, allowing optimization of the right medicine and dose, personalizing the treatment.