REVIEW

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# The rise of three-dimensional human brain cultures

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**Pluripotent stem cells show a remarkable ability to self-organize and differentiate *in vitro* in three-dimensional aggregates, known as organoids or organ spheroids, and to recapitulate aspects of human brain development and function. Region-specific 3D brain cultures can be derived from any individual and assembled to model complex cell–cell interactions and to generate circuits in human brain assembloids. Here I discuss how this approach can be used to understand unique features of the human brain and to gain insights into neuropsychiatric disorders. In addition, I consider the challenges faced by researchers in further improving and developing methods to probe and manipulate patient-derived 3D brain cultures.**

Understanding the principles that underlie the assembly of cells into tissues and of tissues into organs is a fundamental goal in biology. Such understanding requires not just observation, but also the ability to construct and deconstruct complex, developing structures. This has been particularly challenging when studying the central nervous system (CNS) in humans, in part because of its complexity, but also because of poor accessibility to all stages of development and lack of functional tissue preparations. In other branches of medicine, such as haematology and oncology, easy access to tissue samples has led to a comprehensive understanding of organ development and substantial therapeutic advances. Therefore, there is a pressing need to develop functional, realistic and personalized models of the developing human brain so that we can better understand its unique biology and gain mechanistic insights into neuropsychiatric disorders.

Several recent conceptual and technological advances are now converging to make human brain tissue more accessible for study. First, the ability to culture pluripotent stem cells, including human embryonic stem (hES) cells, *in vitro*[1,2]. Second, the possibility to reprogram somatic cells into induced pluripotent stem (iPS) cells[3] and subsequently to promote their differentiation into neurons[4], or to shortcut this process and directly derive neurons[5,6]. Third, progress in building 3D brain cultures as well as advances in biomaterials, CRISPR–Cas9-based genome engineering[7] and highly-parallel single-cell transcriptomics[8]. Combined, these advances open opportunities for understanding the assembly of the human brain and how this may go awry in disease. This review discusses advances in building 3D human brain cultures, such as neural organoids or spheroids, and describes how these cultures may help researchers capture normal and abnormal organogenesis *in vitro*. While these approaches may bring access to previously inaccessible aspects of human biology, such as developmental processes in late human gestation, they are still models. As George Box pointed out, "all models are wrong but some are useful,"[9] and their value ultimately resides in their ability to provide testable predictions. Therefore, an important goal of this overview will also be to highlight the advantages and disadvantages of the various approaches for engineering *in vitro* models of the human nervous system.

## From pluripotent cells to brain cells in a dish

What principles guide organogenesis? Immanuel Kant astutely described life as a "self-organized, self-reproducing" process[10]. Self-organization implies the formation of ordered structures from relatively homogeneous elements in the absence of an external pattern. In embryology, this involves a dynamic process that starts with a relatively homogenous group of cells that are capable of differentiation and self-patterning and that respond to external forces. The combined action of internal (genetic, biochemical) and external (mechanical) inputs, as well as stochastic events, lead to symmetry breaking, cell rearrangements and non-uniform but controlled spatiotemporal growth. These processes result in emergent properties of the developing structure. For instance, self-assembly involves a rearrangement of elements. The concept of self-assembly originated in chemistry, as seen in Rayleigh–Bernard convection, but it has been extensively described in living organisms. Single dissociated cells obtained from amphibians will meaningfully self-sort when pH is restored[11] (Fig. 1a). Similarly, a single hydra can be dissociated into single cells, which then reassemble to recreate the entire organism[12]. Cell arrangement mediated by surface proteins is, however, not the only mechanism for self-assembly. For example, periodic waves of gene expression, which can be synchronized across groups of cells, have been shown to participate in the self-assembly of dissociated cells from the presomatic mesoderm[13].

### Neural differentiation of pluripotent cells

Human organogenesis follows many of the same developmental patterns seen in other species. The nervous system develops from a single tube that undergoes disproportionate enlargement of the anterior side (Fig. 1b). This process of generating biological tissue shape, also known as morphogenesis, involves local proliferation and patterning, complex cell–cell interactions, cell fate specification and long-distance migration. For instance, the formation of the cerebral cortex in humans involves massive proliferation of progenitors in various domains located close to the ventricle, followed by the orderly generation and arrangement of glutamatergic neurons, starting with lower layers and finishing with upper layer neurons positioned close to the pia[14]. Other cells, such as GABAergic neurons, migrate into the cerebral cortex after being specified in distant regions, and corticogenesis continues postnatally with the generation of glial cells. In other parts of the nervous system, the final arrangement can involve *en masse* physical movements of cells, such as in the case of the evagination and invagination that underlie optic cup formation.

Methods for inducing the differentiation of mouse and human pluripotent stem (mPS and hPS) cells *in vitro* can, surprisingly, recapitulate some of these elaborate processes even in 2D cultures[15]. Colonies of hPS cells can be micropatterned to recapitulate gastrulation-like events[16]. Contrary to expectations, this phenomenon occurs without cell motility

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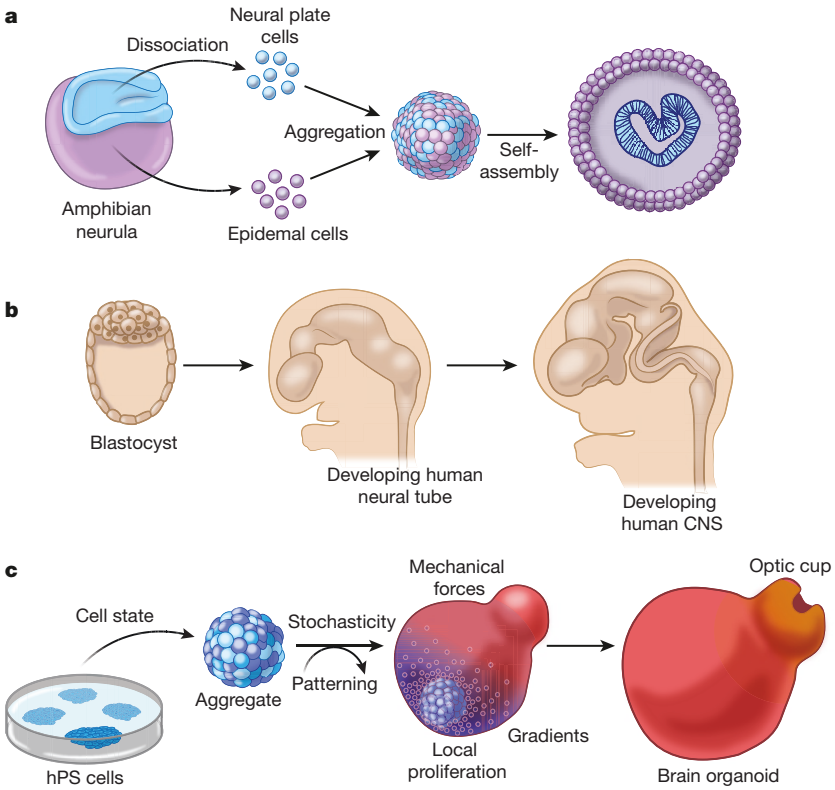


**Figure 1 | Self-organization and organogenesis. a**, Mixed single cells dissociated from the neural plate or the epidermis of an amphibian neurula can self-assemble and generate epidermis-like layers around a neural tube-like structure. **b**, Development of a complex CNS from a neural tube derived from hPS cells isolated from the inner mass of a blastocyst. **c**, Self-organization of brain organoids from hPS cells depends on cell state (for example, 'naive' versus 'primed'), size of initial aggregate, self-patterning or external patterning with growth factors and small molecules, local proliferation, mechanical forces and stochastic factors.

and may be the result of a Turing 'reaction–diffusion' mechanism based on signalling molecules[17] or an 'edge-sensing' mechanism in which cells respond differentially according to their position within the colony[18]. Early efforts to derive neural cells from pluripotent stem cells *in vitro* triggered differentiation by giving cells more degrees of freedom as small aggregates called embryoid bodies[4] and leveraged the fact that the predominant germ layer fate is ectodermal, even in *Xenopus*[19]. Subsequently, double inhibition of the SMAD pathway in hPS cells grown in high density 2D cultures was shown to be sufficient to generate a high proportion of neural precursors[20]. Neuroepithelial cells display a high degree of polarity and form neural rosettes around a pseudo-lumen[21]. This is a common pattern of neural cell organization, which has also been observed in CNS tumours and the ectodermal component of teratomas. The default fate of these neural precursors is forebrain, and minimal intervention is required to recapitulate the sequential generation of layer-specific cortical neurons in both rodent and human 2D cultures[22–25]. This anterior or rostral default state can be overturned, and cells can be converted to more caudal fates by patterning with small molecules and growth factors to generate midbrain, striatal or spinal cord neurons[26,27]. Moreover, stromal co-culture of mPS cells can induce the formation of 3D structures that include diverse cell types, such as eye-related structures[28].

However, there are limitations to stem cell differentiation in 2D cultures. Interactions with plastic surfaces prevail over interactions between cells or between cells and the extracellular matrix (ECM). Gradients of patterning molecules, gases and nutrients are dispersed and the interactions of growth factors with heparin sulfate or proteoglycans are altered. Apical–basal polarity is changed and migration is not constrained; at low density, neural progenitors move in a slow, amoeba-like way, but at high density they glide rapidly[29]. The stiffness of plastic dishes is not physiological and many cells isolated from organs or tumours become flat when cultured in 2D, altering their proliferation rate and differentiation status. Not surprisingly, drug screening trials in 2D cultures yield different results from those carried out in 3D cultures[30]. These issues have prompted the development of culture systems that recapitulate more complex cell–cell interactions and cell diversity, mature to later stages in development and that show higher levels of functionality (see Box). Because of the intention to more closely model the cytoarchitecture of organs, these 3D cultures are referred to as organoids or organ spheroids.

Starting with a small number of pluripotent cells, 3D cultures rely upon genetically encoded self-organization[31] to generate *in vitro*, and without an existing pattern, polarized floating structures that resemble *in vivo* tissue (Fig. 1c). As with all nonlinear systems, the state of initial elements is fundamental. For example, seeding density can influence fate choices. Early heterogeneity in cell states ('naive' or ground state versus 'primed'), stochastic processes and cell–cell interactions in aggregates contribute to symmetry breaking early on in 3D ensembles. Exogenous molecules or physical confinement of these aggregates, local differentiation and subsequent secretion of patterning molecules give rise to molecular gradients and reaction–diffusion phenomena. These processes in turn can cause localized proliferation and changes in mechanical forces, further leading to specialization and reassembly of cells.

The generation of an optic cup is probably the best way to illustrate the surprising capacity of pluripotent stem cells to self-organize in 3D cultures[32,33]. In this case, modulation of the Wnt pathway is used to develop retinal epithelium and vesicle-like structures. These structures show local mechanical autonomy, with proliferation and cytoskeletal changes in cells at key locations resulting in spontaneous curving and formation of the optic cup. Self-organizing phenomena and even multi-germ layer lineages have also been observed in cultures of non-brain tissues, such as kidney[34] and lung[35]. Moreover, gastrointestinal-related 3D cultures that include crypt-like structures can be derived from single stem cells isolated from primary tissue[36].

## Developments in 3D neural differentiation

What are the approaches for deriving 3D brain cultures, and what aspects can be recapitulated with this platform? Even the earliest reports of neural cultures describe efforts to maintain intact tissue architecture *in vitro*. As early as 1907, Harrison took frog neural tube and established hanging drop cultures by attaching tissue fragments to a glass coverslip in coagulated serum or lymph, so that "growing nerves could be brought under direct observation while alive"[37]. This 3D preparation could be maintained for weeks *in vitro* and was helpful in culturing the poliovirus in spinal cord cells from monkeys. Later, roller tubes and semipermeable membranes were used to grow slices of brain tissue in organotypic cultures[38]. This system maintains some of the 3D architecture and connectivity of the source tissue and can be subsequently grafted *in vivo* for vascularization (for example, into the lateral angle of the eye[39]). It can also be used to derive tissue explants, in which groups of cells or slices from different brain regions are kept in close juxtaposition to allow specific cell–cell interactions. But to grow neural stem cells that produce various CNS cell lineages, 3D aggregation into structures called neurospheres is essential[40]. The ability to isolate neural stem cells and to differentiate hPS cells in 2D prompted several approaches for deriving 3D brain tissue (Fig. 2).

One direction has been to build upon culture chamber systems, which have proved useful in identifying growth factors by cellular and subcellular isolation. This top-down approach, known as organ-on-a-chip, uses physical channels to position cell types, create gradients and control the flow of nutrients, and to provide spatial and temporal control of the cellular environment. For instance, microchip models of the blood–brain barrier include endothelial cells on one side of a membrane and neurons, pericytes and astrocytes on the other side, and can test the effects of cytokines[41]. This system uses reverse-engineering principles and provides rigorous control of variables, but depends on detailed knowledge of the organ and its physiology.

An alternative approach has been to rely on spontaneous morphogenesis in cell aggregates, such as organoids or organ spheroids. Adult stem cells or cells differentiated from pluripotent stem cells as well as tumour cells, can be used to derive organoids in suspension or embedded into extracellular matrices. This has been elegantly demonstrated by the Clevers laboratory for the gastrointestinal tract[36,42–44]. Organoid approaches allow more degrees of freedom in long-term cultures that give rise to cell diversity, complex cell–cell interactions and unique physical structures. When starting with hPS cells, there are two ways of differentiating organoids: undirected and directed (Fig. 2).

In directed differentiation approaches, aggregates of hPS cells are instructed to acquire an ectodermal fate and subsequently specified to become region-specific organoids or organ spheroids. The pioneering methods developed by the late Yoshiki Sasai involved 3D aggregation of mPS[45] or hPS[46] cells and culture in U- or V-bottomed wells followed by 2D plating of differentiated cells at a later stage. These experiments showed that the size of the initial clusters and the use of small molecules for survival were essential, and that lineage reporters and mechanical dissection can enrich the cultures for specific brain regions. An alternative approach used neural specification in 2D followed by 3D cultures of rosettes, yielding a combination of dorsal and ventral forebrain and maturation up to the first trimester stage of brain development[47]. Our group introduced a simple method for deriving dorsal forebrain in 3D that involves lifting intact colonies of human iPS cells, followed by neuralization and culture exclusively in suspension, without an extracellular matrix or culture in a bioreactor[48]. These spherical cultures grow up to 4 mm in diameter, contain equal proportions of deep and superficial layer cortical neurons as well as non-reactive astrocytes, and after approximately 9–10 months mature to resemble postnatal stages[49]. Similarly, the Song and Ming groups used patterning and miniaturized spinning bioreactors to obtain forebrain organoids and to derive midbrain or hypothalamus organoids[50].

In undirected organoid differentiation, such as the techniques developed by the Knoblich group, hPS cells are suspended and grown in an extracellular matrix, such as Matrigel, in spinning bioreactors[51,52]. Owing to the lack of inductive signals, these 3D cultures exhibit a variety of brain region identities and non-neural fates. Single-cell transcriptomic studies in undirected organoids[53,54] confirmed that dorsal and ventral forebrain cells are mixed with cells from other brain regions, such as retina, hindbrain and midbrain, and co-exist with choroid plexus and mesodermal cells. Recent work showed that individual organoids can acquire different fates and demonstrated the presence of various cell classes found in the mouse retina[54]. These differentiation techniques have a higher degree of stochasticity than directed differentiation, and early conditions could have large effects. Unconstrained organization leads to unique morphologies and levels of maturation, and the challenges in identifying and removing certain populations of cells can lead to non-physiological cell–cell interactions. Variability in undirected organoids may be related to inconsistency in neural induction[55], and the tendency has been to constrain cell fates using small molecules[56,57] or fibre microfilaments[55]. On the other hand, the high degree of diversity in these cultures may allow researchers to explore human CNS diversity and to map disease genes onto specific cell types.

Several directed approaches have been used to derive CNS regions in 3D cultures. Differentiation of hES cells in 40% oxygen and with up to 2% Matrigel dissolved in the medium[58] can yield forebrain cultures that show rolling and the formation of curvature with rostro-caudal polarization. This approach also generates ventral forebrain regions as well as abundant choroid plexus[59], but mostly dorsal forebrain when oxygen is removed in a subsequent modification[60]. By manipulating Wnt and bone morphogenetic protein (BMP) signalling, the fate of these cultures can be shifted more medially to derive hippocampus-like 3D cultures with both granule and pyramidal neurons[61]. Alternatively,

**BOX 1**

### 2D or 3D—that is the question

With many available options and the excitement surrounding 3D culture techniques, deciding what differentiation approach to use may not be easy. Both 2D and 3D neural differentiation methods have advantages and disadvantages for answering different questions.

Two-dimensional neural cultures can be used to study the neural stem cells and disease mechanisms underlying defects in neural progenitors. For instance, Iefremova *et al.*[60] first identified defects in organoids derived from individuals with Miller–Dieker syndrome, but to dissect the mechanism, they switched to a 2D culture system to find alterations in N-cadherin–β-catenin–Wnt signalling in radial glia. The scalability of 2D neural cultures make this system more useful for large-scale drug testing or for genome-wide CRISPR–Cas9 screens. Imaging assays and some morphological studies (for example, of dendrite complexity) are also easier to implement in 2D. Directed monolayer differentiation approaches can also provide the high-purity cultures necessary for therapeutic transplantation studies.

Three-dimensional neural cultures can be used over long periods—for almost two years[49]—and provide access to a large diversity of cell types and functional maturation states. The cytoarchitecture and cell–cell interactions are reminiscent of *in vivo* neural tissue. The cross-talk between specific cell types, such as astrocytes and neurons or oligodendrocytes, in the context of synaptogenesis or myelination, may be more informative in a 3D setting. Certain cellular phenotypes can best be studied in 3D. For instance, modular brain assembloids can be used to model inter-regional communication and dissect cell-autonomous versus non-autonomous effects[68]. Cortical interneurons display minimal migration in 2D systems, but accurately recapitulate saltatory movements in 3D cultures when compared to fetal tissue[68].

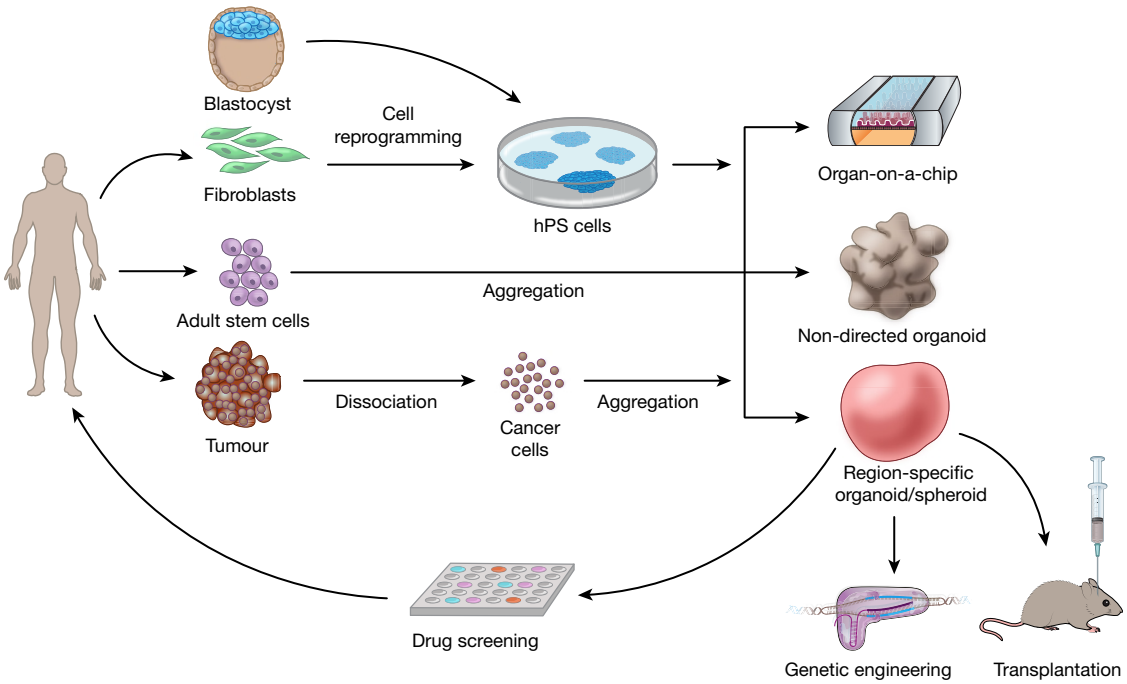


**Figure 2 | Different approaches for deriving human brain 3D cultures.** hPS cells derived from a blastocyst or by reprogramming of somatic cells, adult stem cells or cancer cells derived from primary tissue can be used to derive microfluidics-based organs-on-a-chip (top), undirected organoids (middle), and region-specific brain organoids or organ spheroids (bottom). These 3D cultures can be manipulated with CRISPR–Cas9 genome-editing technologies, transplanted into animals or used for drug screening.

growing hPS cells at high density in spinner flasks can yield large numbers of motor neurons[62].

Some of the region-specific organoid approaches result in unique features, such as the presence in of neuromelanin in midbrain organoids[63], an insoluble, dark polymer that becomes apparent postnatally in the substantia nigra[64,65]. Another unique aspect is the ability to generate distinct niches in the same preparation. Large aggregates of mES cells in the presence of sonic hedgehog (Shh) can generate oral ectoderm on the surface that can invaginate and contact a hypothalamic primordium present within, thus mimicking the development of the adenohypophysis[66]. In the presence of high fibroblast growth factor 2 (FGF2), insulin and FGF19, cerebellum-like organoids develop an elongated, polarized cerebellar plate that generates precursors for GABA ($\gamma$-aminobutyric acid)-releasing Purkinje cells and, at the edge, a rhombic-lip region containing granule progenitors[67]. However, in these cerebellum organoids as well as in forebrain organoids that include multiple domains (ventral, dorsal, medial), interactions between these regions are spatially unpredictable and asking specific development- or disease-related questions has been challenging. To address this issue, we introduced controlled assembly of 3D brain cultures[68] in what I will refer to as brain assembloids, to direct and probe more complex cell–cell interactions and to generate, as in electrical engineering, circuits from parts (Fig. 3).

## Developing human brain assembloids

It has been particularly challenging to study cell migration, inter-regional interactions and circuit assembly in the human CNS because it is not possible to obtain intact tissue at later stages of *in utero* development. For instance, the formation of cortical circuits involves not just connectivity between layers of glutamatergic excitatory neurons, but also the integration of around 20% GABAergic interneurons[69,70]. Interestingly, these interneurons are generated not in the dorsal forebrain, like glutamatergic cells, but in the ventral forebrain (subpallium)[69,71–73] and must migrate for months over long distances, beginning at mid-fetal human development[72]. Dysfunctional cross-talk between these two cortical cell types is thought to contribute to the pathophysiology of several neuropsychiatric disorders, including epilepsy and autism spectrum disorders (ASD)[74,75], but high-resolution probing and manipulation of cortical ensembles in humans has not been possible. To address this problem, we specified subdomains of the forebrain that functionally interact in development (Fig. 3a) and generated region-specific organoids resembling either the dorsal pallium or the subpallium, and subsequently fused them[68]. This modular system enabled us to monitor the saltatory migration of interneurons towards the cerebral cortex and to identify phenotypes in patient-derived cells. It also demonstrated that interneurons successfully integrate into a synaptically connected microcircuit. This approach has been subsequently used by other groups to model forebrain interactions[56,76].

Another way to build assembloids is by directly mixing cells of different lineages or by adding cells or biomaterials that have organizer-like capabilities. For instance, neural progenitors, endothelial cells, mesenchymal cells and microglia or macrophages have been mixed in a peptide-functionalized hydrogel and then used to test neurotoxicity[77]. Moreover, specific populations of spinal cord neurons derived from mES cells have been incorporated into aggregates to build rhythmically active circuits[78]. Wokman *et al.* built a gut–neural assembloid using neural crest cells and intestinal organoids[79], in which neural cells migrated into the mesenchyme of the intestinal-like tissue, self-organized and gave rise to rhythmic waves. When transplanted into rodents, these organoids showed electromechanical coupling and propagating contractions. CNS assembloids could also be built to study myelination by the addition or *in situ* generation of oligodendrocytes, especially as *in vitro* myelination methods are currently limited, or to model primary or metastatic brain cancer by the addition of tumour cells or assembly with cancer organoids.

Assembloids have the potential to capture more complex inter-regional brain interactions, building upon models in rodents that utilize spatially positioned brain explants. For instance, in rodent cortico-thalamic explants, only multipolar neurons of the thalamus project towards the cortex; these stop on layer 4 pyramidal neurons, even when placed close to the pia[80,81]. Vice versa, cortical neurons from deep layers project into the thalamus. The developmental stage for establishing these interactions matters, and the unique cytoarchitecture of the sensory cortex is present only when explants are not cut tangentially[82]. The generation of cortico-thalamic assembloids using fusion or spatio-temporally controlled patterning (Fig. 3b) would allow the study of early thalamic projections

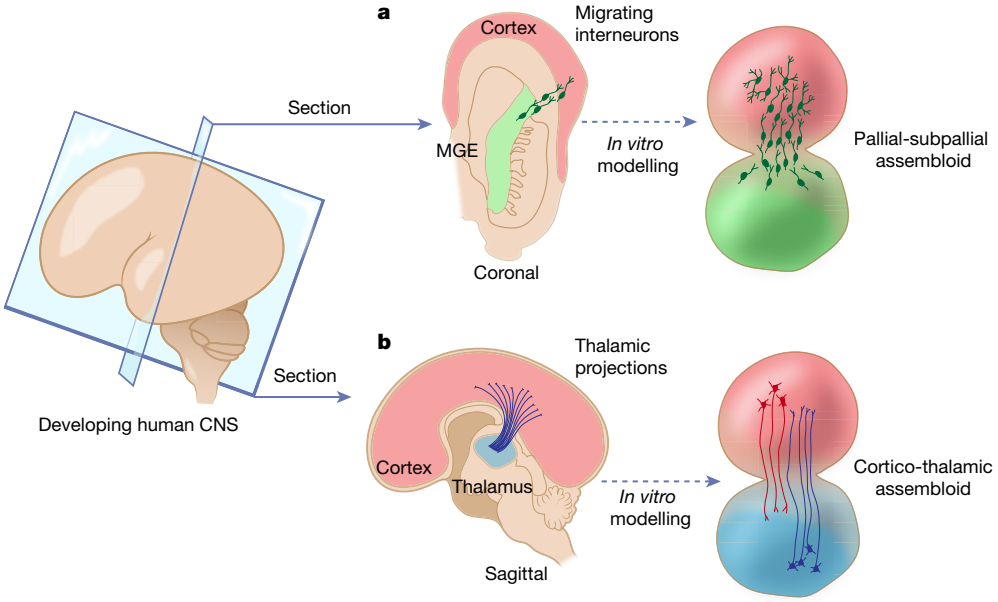


**Figure 3 | Human brain assembloids.** Cross-sections through the developing human brain at gestational week 17, showing the cerebral cortex (red), the medial ganglionic eminences (MGE, green) and the thalamus (blue). Interneurons from the MGE migrate tangentially to populate the dorsal pallium. Thalamic neurons project to the cortical subplate and then onto layer 4 cortical neurons, while deep layer cortical neurons project back to the thalamus. **a**, Pallial–subpallial assembloids show modelling of GABAergic interneuron migration and functional integration into cortical circuits. **b**, Cortico-thalamic assembloids illustrate projections from deep layer cortical neurons onto thalamic neurons and projections of thalamic neurons onto layer 4 cortical neurons.

into the human subplate and the role of thalamic activity in building cortical networks. Moreover, it could be used as a platform to investigate patient-derived cultures and verify, for instance, the role of dopamine receptors in mediating thalamocortical dysfunction in individuals with 22q11.2 deletion syndrome[83]. Similarly, a modular approach to specifying and assembling other circuits could bring insights into dysfunctions of cortico-striatal projections, cortico-spinal tracts, the meso-cortical pathway or cortico-hippocampal projections. Importantly, functional interactions between these cell types may lead to novel features for interrogation *in vitro*, such as the maturation of muscle fibres following innervation, the development of spines on medium spiny neurons or the modulation of synaptic plasticity by neurotransmitters.

## Applications of human 3D brain cultures

Three-dimensional neural cultures derived from human and other primate pluripotent stem cells are now being used to answer questions about brain development and evolutionary innovation, and to gain insights into human disease.

### Human brain development and evolution

The development of the CNS in humans takes a very long time: the generation of astrocytes continues into the first year of life, interneurons migrate for up to two years after birth[84], and myelination is completed only in the second or third decade of life[72]. Therefore, it is not surprising that knowledge regarding the biology of the stem cell niche, lineage specification and the mechanisms of maturation in human and non-human primate nervous systems is limited. One of the advantages of 3D cultures is that they allow long-term culture (months to years), which could open a window into at least the early stages of human CNS maturation. Forebrain organoids, for which comparison to available data sets from primary brain samples is possible, reach mid-fetal stages of cortical maturation after 3 months *in vitro*[48,50]. When we maintained such organoids for 20 months *in vitro*, they matured to postnatal stages, as shown by comparison at the single-cell level to primary cortical tissue[49]. More specifically, after about 9–10 months, glial cells switched from an early proliferative state to a mature astrocyte state with different morphologies and physiological effects on neurons[49]. This suggests that there may be an intrinsic molecular clock that keeps maturation on track, consistent with studies showing that when transplanted into rat or mouse cortex, human cells still take months to mature[22].

Compared to other primates, the human cerebral cortex displays a striking expansion, a larger diversity of cortical progenitors, more upper layer neurons and potential differences in cortical interneurons[72,85]. But why does the development of the human brain take so long, and how do these unique aspects arise? Initial studies with primate-derived 3D cultures suggest that differences in corticogenesis among species may result from cell-autonomous differences in the proliferation of neural progenitors[86]—more specifically, from differences in the cell cycle between humans and chimpanzees[87]. In parallel, studies with neural-crest-derived cells are starting to uncover the regulatory mechanisms that underlie facial development[88]. Comparison of corticogenesis across species in 3D brain cultures will require novel, inventive tools and analytical approaches to capture cell diversity and maturation while accounting for species-related differences in gestation. Assembloids may be particularly relevant in this regard for understanding differences in connectivity, such as the reorganization of corticofugal neurons in primates[89]. Sensory input is absent in these cultures and their cytoarchitecture is still primitive, but it is possible that an organoid system could allow us to study neurons present only in species with larger brains, such as von Economo neurons[90], or recently identified human-specific subtypes of parvalbumin neurons[91]. Genome engineering might even allow us to use organoids to study the effects of genetic variants found in Neanderthal or Denisovian genomes on corticogenesis.

### Disease modelling

Human 3D brain cultures already show great promise in modelling monogenic, polygenic and infectious human disorders. Organoids derived from patients with microcephaly who have mutations in the cell cycle-related gene *CDK5RAP2* display an abnormal plane of division in cells located in identifiable ventricular-like zones[51]. Organoids in which the tumour suppressor gene *PTEN* is deleted show increased proliferation and delayed neural differentiation, and this phenotype can be manipulated pharmacologically[57]. The 17p13.3 deletion leading to Miller–Dieker syndrome, a severe form of lissencephaly, has been challenging to study

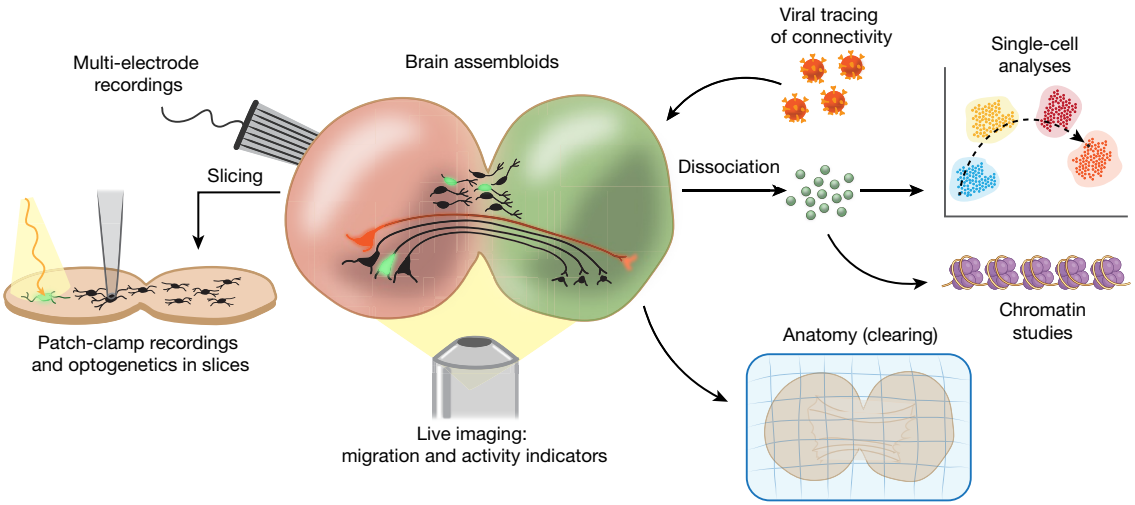


**Figure 4 | Methods for probing 3D brain assembloids.** Single-cell analyses (for example, transcriptomics, proteomics), chromatin studies (for example, chromatin immunoprecipitation and sequencing (ChIP–seq), assay for transposase-accessible chromatin using sequencing (ATAC–seq)), 3D reconstructions after application of tissue transparency methods, viral tracing to assess connectivity (retrograde labelling of neurons, red), live imaging of migration and neuronal activity (for example, genetically encoded calcium or voltage indicators), electrophysiology (patch-clamps, multi-electrode recordings) and optogenetic probing in slices or in intact 3D brain assembloids.

in animal models because mice are naturally lissencephalic. Two groups have developed forebrain organoid models of Miller–Dieker syndrome and found abnormalities in radial glia that can be rescued genetically and pharmacologically[59,60].

Using human iPS cells from multiple patients with ASD associated with macrocephaly, Mariani *et al.* derived organoids containing both dorsal and ventral domains and found changes in the proportion of GABAergic interneurons[92]. These cortical defects were related to overexpression of the forebrain transcription factor *FOXG1*. In forebrain assembloids derived from patients with Timothy syndrome, a monogenic disease associated with ASD and epilepsy, we found defects in the migration of cortical interneurons that could be restored pharmacologically by modulating the mutated L-type calcium channel[68]. Neurodegenerative disorders have been more challenging to model *in vitro* owing to their late onset, but an early study of 3D human neural cultures carrying Alzheimer's disease-related mutations showed that they recapitulated both β-amyloid and Tau pathology[93].

Gut organoids have already been successfully used to study host–microbe interactions, such as *Helicobacter pylori* infection in gastric organoids[94]. Similarly, human neurons have been essential for identifying the CNS cells that are affected by the microcephaly-related Zika virus infection and for identifying drugs that could reduce infection[50,95,96]. Cortical organoids derived from patients with a *TREX1*-dependent autoimmune disorder showed increased apoptosis and reduced size, and these abnormalities were mediated by astrocyte-dependent neurotoxicity[97].

More complex phenotypes in patient-derived cells can be assessed after transplantation into rodents. Hepatocytes, endothelial and stromal cells derived in 3D cultures and subsequently transplanted into mice show expansion of grafts by about 50-fold and secretion of human proteins[98]. Intact 3D brain cultures transplanted into the rodent CNS may integrate better than single-cell suspensions into neural circuits. More importantly, this integration could be shaped by vascularization, interactions with microglia or perhaps even sensory-like activity, and thus offer a unique system for asking questions about higher-level circuit function and dysfunction in disease.

## Hurdles and future steps

Brain 3D organoids and assembloids are promising new tools in our arsenal for asking biological and disease-related questions. But there are issues that will need to be addressed. First, brain 3D cultures only approximate the appearance and architecture of neural tissue. They are smaller (maximum dimensions of 4 mm) than the regions they model, and the internal cytoarchitecture is not always predictable. Radial glia are not attached to a superficial pial surface and although deep and superficial layers separate, it is difficult to derive pristine lamination *in vitro*. White matter regions, which are greatly expanded in primates and contain about two billion neurons in the human brain[85], are not visible in 3D cultures. Second, specific cell types are either absent, or present in ratios that differ from those found *in vivo*. Microglia, which are of non-ectodermal origin (born in the yolk sack), must migrate into the CNS before the blood–brain barrier closes. Oligodendrocytes are also less abundant in 3D cultures than *in vivo*[68], but they can be added to 3D ensembles. Similarly, organized meninges and capillaries are not present. Nonetheless, in the absence of circulation, exogenously added endothelial cells may secrete growth factors similar to those secreted in organotypic cultures[99]. Moreover, *in vitro* metabolic demands differ from those *in vivo*, and metabolic needs in the developing brain are species-specific[100]. The human brain develops in a low oxygen environment, but we do not know how changes in oxygen tension affect the development of millimetre-wide 3D cultures. Corticogenesis also involves apoptosis. This cell death can release neurotrophic factors[101], but it is unclear how cell debris is cleared. Third, human 3D brain cultures lack physiological sensory input and other aspects of developmental plasticity, such as critical periods. What happens, for instance, to corticospinal neurons in long-term cultures in the absence of spinal cord neurons? Future studies will need to address these questions.

### Quality control

Predictability is one of the main requirements for disease modelling and drug screening *in vitro* with any platform. Because of a lack of developmental axes and, for some approaches the stochastic differentiation individual 3D brain culture methods should be tested for reproducibility, accuracy, and scalability. It will be important to measure differentiation noise and, as with all dynamic systems, to identify the initial conditions that can drive large effects. Many methods are based on key steps involving Matrigel, which has unpredictable biochemical effects, or the use of up to 10% fetal bovine serum, which varies by supplier or lot and can activate glial cells. How do these conditions affect reproducibility and the reactive state of the cells? How do the cells in 3D brain cultures compare to *in vivo* cell types?[102] Direct comparisons with the developing brain at early stages are relatively straightforward, and most 3D brain cultures map to the first trimester forebrain. But at later stages, when

the proportions of cell types may diverge, these comparisons are challenging and require the comparison of single cells from culture with primary cells[49]. Finally, how scalable and easy to probe are 3D brain cultures? There are certain challenges in obtaining fast readouts in non-homogenous tissue and, for drug screening, penetrability must be considered. Nonetheless, there is evidence that large-scale production and long-term differentiation of 3D brain cultures is possible even in an academic setting[49,62].

One approach for addressing these challenges is to introduce quality control steps and to use directed differentiation approaches, which are more predictable. For instance, Arora *et al.*[103] used an automated micropipette system to identify intestinal pre-organoids from hindgut cultures. Size and morphology, expression of selected markers, live dyes for survival or brain-region-specific reporters can each be used in different combinations as read-outs. In disease models, a large fraction of the variance is driven by inter-individual differences[104–106] and therefore large sample sizes and the use of isogenic hPS cell lines is essential.

### Tools and biomaterials

Another strategy for increasing predictability, recapitulating key developmental features *in vitro* and obtaining reliable read-outs is to develop new biomaterial approaches and to apply novel tools for probing and manipulating brain organoids or assembloids (Fig. 4).

Light-sheet microscopy, which is fast and causes minimal photodamage, as well as other advanced microscopy techniques combined with genetically encoded calcium or voltage indicators, have the potential to capture the activity of whole 3D brain cultures over long periods of time. These imaging technologies can also be used to investigate tissue self-organization, activity waves during early development and the early emergence of disease phenotypes[107,108]. Highly parallel single-cell transcriptomics, and soon large-scale proteomics, will be able to provide insights into the diversity of cell types and cell states, lineage progression and defects in differentiation in patients and across species[49,53,54,87]. Tissue transparency methods, such as CLARITY[109], as well as anterograde and retrograde viral labelling techniques[110], can be used to map neuronal connectivity. Last, electrophysiological recordings with multi-electrode recordings or in slices combined with optogenetic techniques can capture network dynamics, including the potential emergence of neural oscillations. Acquiring multi-level read-outs on a large scale will require the use of advanced mathematical tools to comprehend self-organization principles and the cytodynamics underlying these complex processes, and to reliably identify disease-related phenotypes.

Novel biomaterials are required for both controlling neural patterning and for breaking symmetry in a predictable way, but also to advance the maturation and scalability of these cultures and the emergence of inaccessible biological processes. Stiffness influences morphogenesis and differentiation. Bio-scaffolds can also compartmentalize space, as shown for 3D cultures of salivary gland[111] or lacrimal gland[112]. The ECM has a unique composition in the developing human brain[113] and the perineuronal nets are thought to regulate neural plasticity[114]. Most of the biomaterials used to date for organoid cultures are insufficiently defined and have unpredictable effects on differentiation. In this regard, hydrogels, which are hydrophilic polymers that can be generated using a large variety of natural or synthetic materials (for example, poly-ethyleneglycol (PEG) or poly-vinyl-alcohol) hold great promise. Geometric confinement of hPS cells to PEG-patterned substrates, for instance, facilitates self-organization of cardiac lineages and results in beating cardiac microchambers[115]. Hydrogels are programmable, which is important for brain cultures where reproducing the non-uniform environment is probably more essential than scaling up. By manipulating their pore size and topology (that is, void space) and physical properties (for example, elasticity and topography), hydrogels can be assembled into higher-order architectures[116]. Mechanical forces and cell patterning can be modulated locally. 'Writing', soft lithography and other bioprinting strategies[117] can be used to achieve 3D micropatterning by embedding hydrogels with particles that release or sequester small molecules, growth factors, aptamers, nanoparticles or active peptide sequences. This compartmentalization could create transient organizers and morphogen gradients. Next-generation hydrogels and synthetic ECM will need to improve cell viability over larger scales (up to centimetres), incorporate dynamic features such as pH and oxygen sensing, and eliminate toxic agents.

We still know little about ECM in the developing human brain, but a reverse engineering approach could be used to de-cellularize brain tissue and use hydrogels to derive physiological scaffolds for 3D neural differentiation. Subsequently, ECM components that are necessary for deriving specific features in 3D brain cultures can be used to generate synthetic biomaterials and increase scalability. These experiments could also be informative for achieving predictable self-organization of specific brain regions.

### Novel features in human 3D brain cultures

The combination of biomaterials and state-of-the-art technologies for manipulating human 3D brain cultures has the potential to give rise to novel features *in vitro* and accelerate the study of human brain development and disease. A more permissive environment and extensive growth may lead to a deeper understanding of cortical folding and clarify how size is coupled with timing. For instance, will maturation depend on ensuring larger sizes of tissue *in vitro*, providing external stimulation, or achieving myelination? This is important because accelerating functional maturation up to later stages of postnatal development in brain assembloids could facilitate the study of neural circuits and help us understand how neural oscillations arise, and could ultimately inform models of neurodegeneration. As these and other features emerge and more elaborate transplantations in rodents and other species are being planned, discussions on the ethical aspects of this work should be pursued[118–120]. Engaging the public using accurate descriptions, for example by avoiding the use of terms such as 'mini-brains', will be essential.

## Outlook

This is an exciting new field and as with many technologies, it may follow a 'hype' cycle[121] in which we overestimate its effects in the short run and underestimate its effects in the long run. A better understanding of the complexity of this platform, and bringing interdisciplinary approaches will accelerate our progress up a 'slope of enlightenment' and into the 'plateau of productivity'.

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