

Engineering cerebral folding in brain organoids

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Valuable implement to the animal neurological models: Neurological diseases remain the largest cause of death and disability. The discovery of effective therapies is chiefly hindered by the lack of realistic neurological models. Unlike other tissues, it is infeasible or unethical to access primary human neural samples in bulk. But animal models often fail to replicate the complex human-specific neuronal factors present *in vivo*. Conventional *in vitro* cultures lack native three-dimensional (3D) morphologies, polarity, and receptor expression, as well as tissue-level interactions (Jensen et al., 2018).

Human brain organoids (HBOs) are viable solutions to model complex human brain tissues. Their use has become essential in studying the pathology of various diseases, including the newly discovered neural infection of the SARS-CoV-2 virus (Ramani et al., 2020). Organoids are stem cell-derived 3D cultures that often self-organize into tissue-like structures (Jensen et al., 2018). Unlike the conventional *in vitro* cultures, they mimic the complex morphology, architectures, and even functionality of the deriving human tissues. This mimicry permits their use in the etiology study in immediate relevance. Besides, organoids can arise from patient-derived cells, providing the hope of personalized medicine (Jensen et al., 2018). HBOs are organoids guided in a neuroepithelium path, recapitulating the human brain's key structures and cell lineages. For instance, the lumen (brain cavity) and the surrounding ventricular zone are present in HBOs, but missing in other *in vitro* models. Such vital brain features supply neuron stem cells that maintain proper cerebral development. Their disruption results in abnormal neurogenesis or catastrophic neurodegeneration.

Herein, we focus on the studies of gyrification, another key human brain structure that organoids may form. Gyrification is an essential and unique folding process of the human cortical brain. By increasing neuronal packing volume, gyrification maximizes the effective cortical surface area to support high-volume signaling and complex brain functionality (Tallinen et al., 2014). Its formation is not fully understood, but is considered the result of rapid growth and expansion of the cerebral cortex (an outer layer of the brain). As created from rapid growth and spatial confinement, the stresses lead to the buckling of the cortical layer into wavy structures, with outward ridges known as gyri and inward furrows called sulci (Figure 1A). As a unique feature for humans and some other primates, high-level gyrification

is suggested essential to complex behaviors (e.g., language, social communication) (Del Maschio et al., 2019). In contrast, the brains of small animals (e.g., commonly used rodents) exhibit little to no gyrification.

Gyrification-like folding in HBOs: Although gyrification-like folding sometimes appeared in the HBOs, it was seldomly characterized in these systems, substantially less than other structural features (e.g., lumens, ventricular zone). This is likely because the formation of these folding structures is highly inconsistent and mostly missing. More concerning, the formed folding is significantly weaker than the healthy human brain's. Recently, techniques were explored to develop gyrification with deep folding and high reproducibility. These techniques can be grouped into three categories, as elaborated in this section.

Induction through genetic manipulation: The phosphatase and tensin homolog (PTEN) gene was found to promote folding in HBOs. This gene mutation is pathologically linked to macrocephaly in humans (cortical overgrowth), by inducing rampant proliferation of neural progenitor cells and delaying terminal differentiation. Li and colleagues genetically inactivated PTEN in both human and mouse brain organoids through CRISPR-Cas9 (Li et al., 2017). After 4 weeks, the mutated human organoids exhibited clear signs of folding. After 6–8 weeks, PTEN-mutated HBOs had substantially increased in surface area, overall volume, and folding density (Figure 1B), while simultaneously decreasing sphericity. The same mutations also made the mouse organoids progressively larger, but did not significantly increase surface folding and remained smooth and spherical throughout their development.

This PTEN-mutated human organoid was then used as an infection model for Zika virus. Within only 10 days after the viral infection, the organoid model displayed severely hampered growth in both size and surface folding. After infection, PTEN-mutated HBOs shrank to 30%, and their folding area density decreased from ~1.7% to < 0.3%. Interestingly, the PTEN-mutated organoids were found significantly more susceptible to Zika viral infection. In particular, the regions associated with high-level folding exhibited substantially increased cell apoptosis. This is not surprising because the PTEN mutation enriched these folded regions with NP cells (Li et al., 2017), which are major targets of the Zika virus (Ramani et al., 2020). Thus, such genetic

manipulation successfully created high-level cortical folding and modeled the degenerated folding in diseased conditions. However, there is a major concern about the PTEN mutation, which is well known to lead to macrocephaly disorder and tumoral phenotype. By genetically inducing excessive neural proliferation, this model is questionable in representing a healthy brain vs. macrocephaly and tumoral conditions.

A similar study induced folding in HBOs through G protein-coupled receptors. Wang et al. (2020) discovered that the dopamine D1 receptor plays a vital role in the embryonic brain by influencing the differentiation and proliferation level of neural stem cells. By inhibiting the dopamine D1 receptors, they could increase the proliferation and hinder the differentiation of human neural stem cells, thus inducing excessive expansion and folding in cerebral organoids. This was accomplished in two routes, either by inhibiting the receptor directly with its inverse agonists or through CRISPR-Cas9 introducing a point mutation (A229T). The mutated organoids increased in volume from 0.5 to 0.7 mm³ and in surface area from 4 to 6 mm² compared to the control group (Figure 1C), while the sphericity was reduced to half the control value. The folding density increased from virtually zero to 4% of the total area in the mutated organoids. In contrast, the control group maintained a smooth surface with a folding density of essentially zero (Wang et al., 2020).

NR2F1 is another gene that potentially regulates brain folding. The *NR2F1* gene is implicated with Boonstra-Bosch-Schaff optic atrophy syndrome, a rare disorder related to the structurally malformed parietal and occipital cortex, causing vision impairment and intellectual disability in human patients. Bertacchi et al. (2020) explored the role of the *NR2F1* gene as an area-specific transcriptional regulator for brain morphology. In mouse animal models, they found eliminating NR2F1 expression up-regulated PAX6, a cortical area patterning gene that promoted neural proliferation and neurogenesis. The resulting mouse brains exhibited malformations similar to Boonstra-Bosch-Schaff optic atrophy syndrome patients. Similarly increased PAX6 expression was also observed in HBOs, where NR2F1 was genetically down-regulated. These studies demonstrated that NR2F1 controls factors which are typically associated with increased folding (i.e., cell proliferation, delayed differentiation) (Bertacchi et al., 2020). Although no increased folding was directly measured, they proposed that the NR2F1 gene orchestrates cortical size and folding, which are intriguing to HBO researchers.

All the above studies targeted the genes and transcription factors that regulate the levels of proliferation and differentiation in the brain. This is reasonable, as the HBOs aim to recreate a developmental process that

would take 4–5 times longer in the native human body. Thus, means to expedite this developmental process may sometimes be inevitable. This effect may be specific to the human organoids, as suggested in the PTEN study (Li et al., 2017). However, we should also be aware of its potential danger to introduce over-proliferation characteristics or even tumoral behaviors into the organoids.

Promotion through mechanical interaction: Another promising approach was explored that induces folding through the mechanical confinement during the embryoid body (EB) formation. EB is the precursor of HBOs and a special spheroid that forms three developmental germ layers (i.e., endoderm, ectoderm, mesoderm). Although spheroids were commonly generated using this method, no HBO formation has been explored via the microwell-cultured EBs until recently.

Our lab generated organoids through microwell-cultured EBs, devoid of using Matrigel. The resulting organoids demonstrated typical 3D organoid structures (e.g., lumen) in the conventional Matrigel-present methods. These 3D-printed microwells were tunable in shape and size, and subsequently, the physical confinement (Chen et al., 2020). The more confined microwells were found to generate larger organoids, suggesting promoted proliferation. Moreover, the folding level was also highly promoted by the confinement, measured by the wrinkling index (WI). WI is a 2D measurement of gyrification, defined as the ratio of the length of the organoid outline to the circumference of a circle with a similar area (Figure 1D). A higher WI indicates deeper folding. By day 20, the most optimized microwell achieved a wrinkling factor of more than 1.5. The device with these results was a high-resolution 3D printed device with a curved base. This value is comparable to that of a neonatal human brain, but achieved in a remarkably shorter period.

Karzbrun et al. (2018) cultured “organoids” in a microfluidic device, an even more confined space that squashed the EBs into a 150 μm tall laminated slice. These were not classical organoids, as the formation of 3D structures and culture lifespan were limited. Yet, the flattened layout provided a unique imaging advantage, so that individual cell movement was successfully traced and demonstrated inter-layer migration of cells during the wrinkling formation (Karzbrun et al., 2018). Also, the strong confinement in the z-dimension seemed to introduce deep folding, based on the WI measurement (Figure 1E). By day 20, their organoids achieved a WI > 2, which is even higher than the microwell-formed ones, although only 2D.

Rothenb ucher and colleagues created another brain organoid with a flattened morphology, calling them engineered flat brain organoids. They accomplished this by

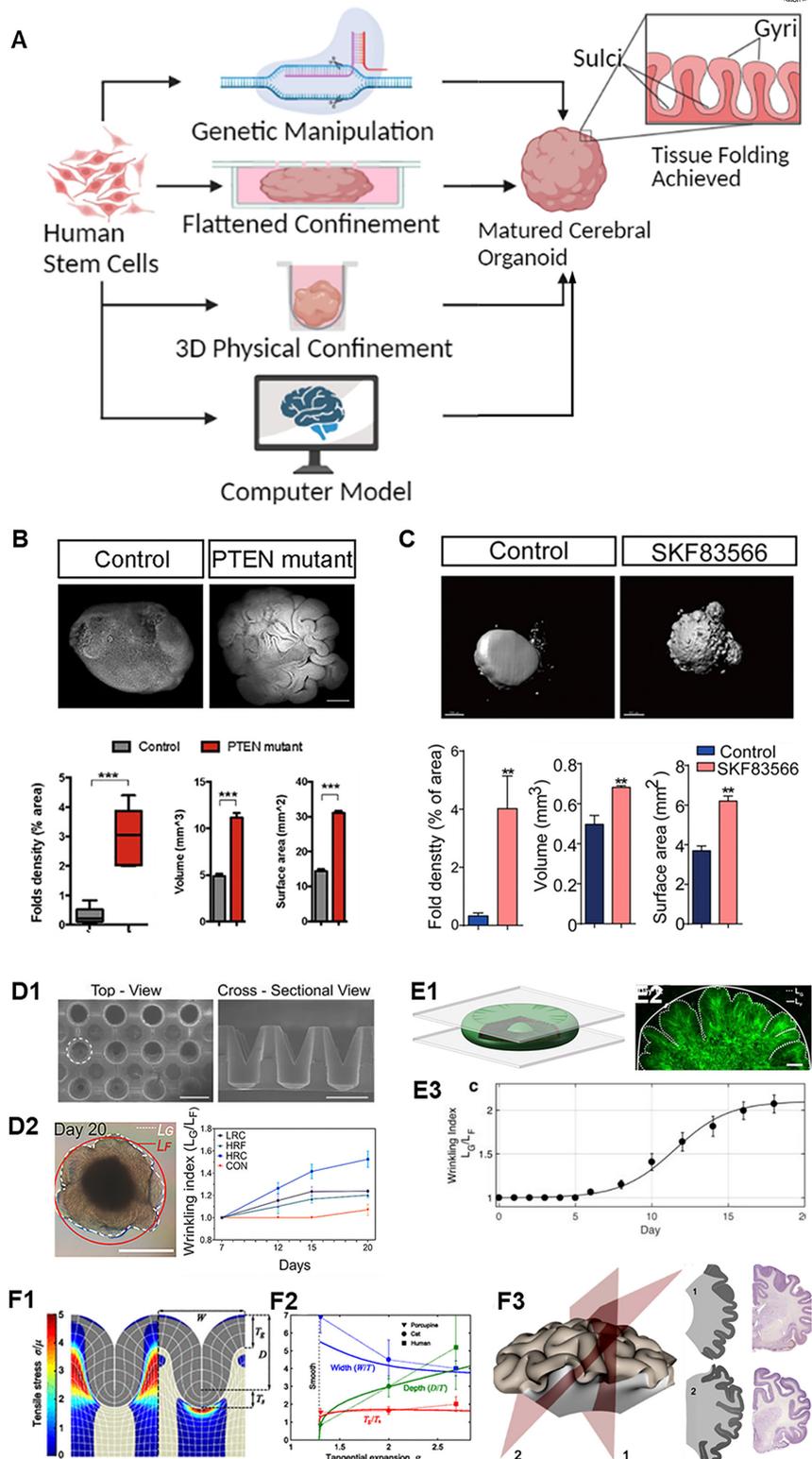


Figure 1 | Approaches that create and study the gyrification in brain organoids. (A) A desired human organoid needs to achieve gyrification, tissue folding that contains gyri and sulci (right, inset). The general strategy for realizing brain folding in cerebral organoids includes four approaches. First, genetic manipulation of (B) *PTEN* gene (Li et al., 2017) and (C) *DRD1* expression (Wang et al., 2020). Both mutated organoids showed significantly higher values than non-mutated control in folding density, organoid volume, and surface area (Li et al., 2017; Wang et al., 2020). Second, (D) 3D confinement was provided by (D1) 3D-printed microwell devices (Chen et al., 2020). (D2) The derived organoids with a high wrinkling index in 20 days, even without ECM embedding (Chen et al., 2020). Thirdly, (E) flattened confinement was provided by (E1) microfluidic device that squashed EBs in a 150 μm tall space. The derived organoids demonstrated (E2) 2D folding with (E3) a high wrinkling index (Karzbrun et al., 2018). Last, (F) mathematical modeling. (F1) A finite element model simulated the formation of sulci as a balancing response to the mechanical instability induced by tangential expansion. (F2) The sulci dimensions were simulated as a function of the tangential expansion and relative brain size. (F3) The slice images of simulated gyrification topography were highly consistent with the histology of raccoon brain (Tallinen et al., 2014). Image A was created using BioRender.com. Images B, C, D1, D2, E2, E3, F1, F2, and F3 were adapted from the corresponding references with permission from the publishers.

seeding EBs on a sheet of are honeycomb-shaped scaffold, which was 3D-printed out of polycaprolactone. The flattened morphology was created to better facilitate the diffusion of nutrients, and better tuning the tissue characteristics. Strong folding was observed after 20 days of culturing, although the gyrification level was not quantified. The researchers believed that the elongated cell migration path and a high number of starting cells gave rise to a high number of NPs, leading to gyrification (Rothenbücher et al., 2021). However, this folding occurred primarily in the ventricle zone, unlike other systems that generate folding in more matured cortical layers.

Theoretical studies: Cortical folding can be easily realized in mathematical models, which could provide a powerful complement to future *in vitro* HBO studies. Although impossible to function directly as the etiology models, the theoretical studies provided exciting insights into the underlying mechanism of folding. Engstrom and colleagues' mathematical model recreated out-of-phase oscillations (miss-alignment of thick portions between layers). This behavior exists in the cerebellum and HBOs, but contradicts the theory that the elastic instability/mismatch induces tissue folding (Engstrom et al., 2018). Per the simulated result, it requires the exchanges of neighbor cells in a fluidlike matter to attain the cerebellum's unique shapes. Their model can also be used to infer the tissue cell types and quantity from tissue morphology.

Tallinen et al. (2014) built a finite element model to simulate the folding process and deep sulci formation. Their model demonstrated that gyrification is a nonlinear consequence of mechanical instability (buckling) driven by the tangential expansion of the gray matter constrained by the white matter. Various folding characteristics were derived through this model, including gyrification extent, sulcus dimensions, and folding morphology (Figure 1F). These characteristics were determined by the tangential expansion rate and relative brain size, highly consistent with *in vivo* observations and their physical model (Tallinen et al., 2014).

Summary and future perspectives: Most of the above studies increased gyrification to or near the human brain level. But the based measurements vary from the volume, surface area, WI, sphericity, to folding density. Lack of standardized measurement or conversion makes it hard to compare these studies to each other and to the native brain that uses the 3D gyrification index (the area ratio of surface to convex hull). Furthermore, these studies neglected the subtle variation of folding levels within the brain, which ranges widely from one region to another (Del Maschio et al., 2019). It is critical for the models to fine-tune the folding level to the desired range accordingly. Levels of folding also vary with the organism's age,

an important consideration when setting up experiments (Del Maschio et al., 2019).

Further studies are still needed. Genetic methods of inducing folding often relied on over-proliferative neural growth. This raises concerns about yielding a tumoral genotype, which needs to be comprehensively assessed in follow-up studies. In contrast, the physical-constraint methods through micro-devices elegantly circumvented this problem by posing an alternative to genetic manipulation. Their moderate effects on promoting proliferation and folding were thought to attribute to the constraint-induced mechanical instability. Follow-up studies should define the underlying molecular mechanism of how HBOs translate mechanical instability into folding stimuli. Furthermore, response tests of these micro-engineered models to neurodegenerative conditions (ZIKA, traumatic brain injury, etc.) are desired. This could provide insight into whether the mechanically induced folding can be used to model diseases, compared to genetically mutated ones.

The mathematical models have demonstrated powerful prototypability by providing rapid results with high-volume iterations. They can also complement the biological models with more faithful features. For example, the PTEN-mutated organoids often lack deep sulcus, which is easy to create in the finite element model. But currently, further applications suffer from the lack of live model support in initiating parameters and verifying the results. Better integration of mathematical and live models in organoid folding would foster unprecedented new opportunities that further our understanding of how gyrification occurs.

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References

Bertacchi M, Romano AL, Loubat A, Tran Mau-Them F, Willems M, Faivre L, Khau van Kien P, Perrin L, Devillard F, Sorlin A, Kuentz P, Philippe C, Garde A, Neri F, Di Giaimo R, Oliviero S, Cappello S, D'Incerti L, Frassoni C, Studer M (2020) NR2F1 regulates regional progenitor dynamics in the mouse neocortex and cortical gyrification in BBSOAS patients. *EMBO J* 39:e104163.

Chen C, Rengarajan V, Kjar A, Huang Y (2020) A Matrigel-free method to generate matured human cerebral organoids using 3D-printed microwell arrays. *Bioact Mater* 6:1130-1139.

Del Maschio N, Fedeli D, Sulpizio S, Abutalebi J (2019) The relationship between bilingual experience and gyrification in adulthood: A cross-sectional surface-based morphometry study. *Brain Lang* 198:104680.

Engstrom TA, Zhang T, Lawton AK, Joyner AL, Schwarz JM (2018) Buckling without bending: a new paradigm in morphogenesis. *Phys Rev X* 8:041053.

Jensen G, Morrill C, Huang Y (2018) 3D tissue engineering, an emerging technique for pharmaceutical research. *Acta Pharm Sin B* 8:756-766.

Karzbrun E, Kshirsagar A, Cohen SR, Hanna JH, Reiner O (2018) Human brain organoids on a chip reveal the physics of folding. *Nat Phys* 14:515-522.

Li Y, Muffat J, Omer A, Bosch I, Lancaster MA, Sur M, Gehrke L, Knoblich JA, Jaenisch R (2017) Induction of expansion and folding in human cerebral organoids. *Cell Stem Cell* 20:385-396.

Ramani A, Müller L, Ostermann PN, Gabriel E, Abida-Islam P, Müller-Schiffmann A, Mariappan A, Goureau O, Gruell H, Walker A, Andrée M, Hauka S, Houwaart T, Dilthey A, Wohlgemuth K, Omran H, Klein F, Wiczorek D, Adams O, Timm J, et al. (2020) Sars-cov-2 targets neurons of 3d human brain organoids. *EMBO J* 39:e106230.

Rothenbücher TS, Gürbüz H, Pereira MP, Heiskanen A, Emneus J, Martinez-Serrano A (2021) Next generation human brain models: Engineered flat brain organoids featuring gyrification. *Biofabrication* 13:011001.

Tallinen T, Chung JY, Biggins JS, Mahadevan L (2014) Gyrification from constrained cortical expansion. *Proc Natl Acad Sci U S A* 111:12667-12672.

Wang Q, Dong X, Lu J, Hu T, Pei G (2020) Constitutive activity of a G protein-coupled receptor, DRD1, contributes to human cerebral organoid formation. *Stem Cells* 38:653-665.

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