

ORIGINAL ARTICLE

Multiscale Imaging-Based Characterization of Human Cortical Organoids Generated from Induced Pluripotent Stem Cells

Deepak Agrawal¹, Aparna Sharma², Sujata Mohanty³, Prabhakar Singh⁴

HOW TO CITE THIS ARTICLE:

Deepak Agrawal, Aparna Sharma, Sujata Mohanty et. al, Multiscale Imaging-Based Characterization of Human Cortical Organoids Generated from Induced Pluripotent Stem Cells. Int. J Neuro Neurosurgery. 2026; 18(2): 57-67.

ABSTRACT

Human induced pluripotent stem cell (iPSC)-derived cortical organoids have emerged as transformative in vitro models for studying human neurodevelopment due to their remarkable ability to recapitulate early stages of corticogenesis in a three-dimensional environment. Unlike two-dimensional cultures, cortical organoids exhibit self-organizing properties that enable the formation of neuroepithelial regions, neuronal populations, and glial networks resembling the developing human cerebral cortex. In this study, we established a standardized and reproducible protocol for generating human cortical organoids from iPSCs and performed multiscale structural characterization using confocal microscopy, scanning electron microscopy (SEM), and transmission electron microscopy (TEM). Human iPSCs were cultured under feeder-free conditions and differentiated through embryoid body formation followed by neural induction and cortical differentiation. Mature organoids were analyzed to evaluate cellular organization, tissue morphology, and ultrastructural maturation. Confocal microscopy demonstrated well-organized cortical-like architecture characterized by uniform nuclear distribution, extensive GFAP-positive astroglial networks, and distinct neuronal cell bodies identified by NeuroTrace staining. SEM analysis revealed spherical organoids with dense and highly organized surface topology, neuroepithelial folding, and neurite-like projections suggestive of progressive neuronal connectivity and tissue remodeling. TEM confirmed neuronal maturation through the identification of well-defined neuronal cell bodies, intercellular junctions, and myelin sheath-like structure surrounding neuronal processes. These findings demonstrate that the generated cortical organoids successfully reproduce essential structural features of early human brain development.

AUTHOR'S AFFILIATION:

¹Professor, Department of Neurosurgery, JPN Apex Trauma Center, All India Institute of Medical Sciences, New Delhi, India.

²Department of Neurosurgery, JPN Apex Trauma Center, All India Institute of Medical Sciences, New Delhi, India.

³Stem Cell Facility, All India Institute of Medical Sciences, New Delhi, India.

⁴Department of Anatomy, All India Institute of Medical Sciences, New Delhi, India.

CORRESPONDING AUTHOR:

Deepak Agrawal, Professor, Department of Neurosurgery, JPN Apex Trauma Center, All India Institute of Medical Sciences, New Delhi, India.

E-mail: drdeepak@gmail.com

➤ Received: 16-06-2026 ➤ Accepted: 02-08-2026



Creative commons non-commercial CC BY-NC: This article is distributed under the terms of the creative commons attribution non-commercial 4.0 License (<http://www.creativecommons.org/licenses/by-nc/4.0/>) which permits non-commercial use, reproduction and distribution of the work without further permission provided the original work is attributed as specified on the Red Flower Publication and Open Access pages (<https://rfppl.co.in>)

The integration of multimodal imaging techniques provides robust validation of organoid architecture and maturation, establishing this platform as a reliable and biologically relevant model for studying neurodevelopment and neurological disease mechanisms. This work further contributes toward the standardization of cortical organoid generation and characterization protocols for translational neuroscience research.

KEYWORDS:

• Human induced pluripotent stem cells (hiPSCs) • Cortical organoids • Brain organoids • Neurodevelopment • Structural characterization

INTRODUCTION

The human cerebral cortex is one of the most structurally and functionally complex tissues in the body, responsible for higher cognitive functions including memory, perception, language, and decision-making.⁽¹⁵⁾ Cortical development involves a tightly regulated sequence of events including neural induction, neuroepithelial expansion, radial glial proliferation, neuronal migration, and layer-specific differentiation.⁽¹⁰⁾ Understanding these developmental processes is essential for elucidating the pathogenesis of neurodevelopmental and neurodegenerative disorders.⁽¹⁾ However, direct investigation of human brain development remains limited due to restricted accessibility to living neural tissue and the inherent differences between animal models and the human brain.⁽¹⁸⁾

Traditional two-dimensional (2D) neural cultures have provided important insights into cellular behavior, but they fail to replicate the three-dimensional cytoarchitecture, spatial organization, and cellular heterogeneity of the developing brain. As a result, these systems often lack the physiological relevance required to accurately model human neurodevelopmental processes. Recent advances in stem cell biology have led to the emergence of induced pluripotent stem cell (iPSC)-derived brain organoids, which represent a major breakthrough in developmental neuroscience.⁽⁶⁾ Brain organoids are self-organizing three-dimensional cellular structures generated from pluripotent stem cells that recapitulate several hallmarks of early brain development, including ventricular zone-like structures, neuroepithelial organization, neuronal differentiation, and cortical layer formation.⁽⁹⁾ Lancaster and colleagues first demonstrated that cerebral

organoids could mimic early human brain development and model microcephaly, establishing organoid technology as a powerful tool for neuroscience research.⁽⁸⁾ Since then, organoid systems have rapidly evolved and have been widely used for studying neurodevelopmental disorders, neurodegeneration, viral neuropathogenesis, and therapeutic responses.

Among various brain organoid models, cortical organoids are particularly valuable because they closely resemble the developing human cerebral cortex. These organoids exhibit region-specific differentiation, radial glial organization, and neuronal maturation patterns similar to those observed during human corticogenesis.⁽⁷⁾ Furthermore, cortical organoids have demonstrated the ability to generate diverse neuronal subtypes and complex neural networks, making them increasingly relevant for modeling higher-order brain functions and disease states.⁽¹³⁾

Despite these advancements, reproducibility and structural consistency remain major challenges in organoid research. Variability in organoid size, cellular composition, and maturation can significantly affect experimental outcomes and limit translational applicability.⁽⁵⁾ Additionally, comprehensive structural validation using multimodal imaging approaches is essential to confirm successful cortical differentiation and maturation. Previous studies have emphasized the importance of combining histological and ultrastructural techniques to accurately characterize organoid organization and developmental progression.⁽¹²⁾

Confocal laser scanning microscopy enables detailed visualization of cellular organization and spatial distribution of neuronal and glial populations within organoids. Scanning

electron microscopy (SEM) provides high-resolution analysis of three-dimensional surface morphology and tissue architecture, while transmission electron microscopy (TEM) offers ultrastructural insights into organelle organization, intercellular junctions, and neuronal maturation. The integration of these imaging modalities allows for comprehensive evaluation of organoid development across multiple spatial scales.

In the present study, we established a standardized and reproducible protocol for generating human *iPSC*-derived cortical organoids and performed extensive structural characterization using confocal microscopy, SEM, and TEM. Our objective was to validate the formation of cortical-like architecture and evaluate neuronal maturation through multiscale imaging analysis. By combining cellular, morphological, and ultrastructural assessments, this study aims to provide a robust framework for cortical organoid generation and characterization, thereby contributing to the advancement of organoid-based models in neuroscience research and translational medicine.

MATERIALS AND METHODS

Human *iPSC* Culture and Maintenance

Human *iPSCs* were cultured under feeder-free conditions to ensure reproducibility and minimize variability associated with feeder layers. Cells were maintained on Geltrex-coated culture plates in StemFlex™ medium, which supports pluripotency and robust proliferation. Cultures were incubated at 37°C in a humidified atmosphere containing 5% CO₂. Medium was replenished daily to maintain optimal nutrient levels and remove metabolic waste.

Cells were passaged every 3–4 days using a non-enzymatic dissociation method (0.5 mM EDTA) to preserve cell integrity and minimize stress-induced differentiation. Only *iPSCs* cultures with consistent morphology and confirmed genomic stability were used for downstream applications, ensuring reliability of organoid formation.

Generation of Cortical Organoids

Cortical organoids were generated using a stepwise differentiation protocol designed to mimic early human neurodevelopment.

Briefly, *iPSCs* were dissociated into single-cell suspensions using Accutase and seeded into low-attachment 96-well plates to promote the formation of embryoid bodies (EBs). The use of low-attachment conditions facilitated spontaneous aggregation and self-organization of cells.

Neural induction was initiated using dual-SMAD inhibition, a well-established method to suppress mesodermal and endodermal lineage differentiation while promoting neuroectodermal fate. Within 24–48 hours, compact EBs formed and began to exhibit early neural characteristics.

Subsequently, EBs were transitioned into cortical differentiation medium (CDM), supplemented with B27, N2, GlutaMAX, and ascorbic acid, to support neuronal lineage commitment and maturation. The developing organoids were then transferred to non-adherent culture dishes and maintained under continuous orbital shaking. This dynamic culture condition enhanced oxygen and nutrient diffusion, reducing the formation of necrotic cores and promoting uniform growth.

Media were refreshed regularly, and organoids were cultured over extended periods until they developed into mature, spherical structures with defined cortical-like regions. This protocol ensured reproducibility and consistent structural development across batches.

Sample Preparation for Imaging

Organoids designated for imaging were fixed using 4% paraformaldehyde to preserve cellular architecture. For confocal microscopy, samples were cryoprotected in sucrose, embedded in optimal cutting temperature (OCT) compound, and sectioned at 20–30 μm thickness using a cryostat. Sections were mounted on glass slides for staining and imaging.

Confocal Laser Scanning Microscopy

For immunofluorescence analysis, organoid sections were permeabilized using PBS containing Triton X-100 and incubated with primary antibodies against glial fibrillary acidic protein (GFAP) to identify astroglial cells. Following overnight incubation at 4°C, sections were treated with Alexa Fluor-conjugated secondary antibodies. Nuclear staining was performed using DAPI, while neuronal cell

bodies were visualized using NeuroTrace fluorescent Nissl stain. After washing, sections were mounted using antifade mounting medium and imaged using a confocal laser scanning microscope (Leica SP8). Z-stack imaging was performed to capture three-dimensional cellular organization within the organoids.

Scanning Electron Microscopy (SEM)

For SEM analysis, organoids were fixed in 2.5% glutaraldehyde, followed by washing in 0.1M phosphate buffer (pH 7.4). Samples were dehydrated through a graded ethanol series and subjected to critical point drying. Dried samples were mounted onto aluminum stubs and sputter-coated with a thin layer of gold-palladium to enhance conductivity. Imaging was performed at various magnifications to assess surface topology and structural organization, under an EVO18 scanning electron microscope (Zeiss).

Transmission Electron Microscopy (TEM)

For ultrastructural analysis, organoids were fixed with 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M phosphate buffer, followed by post-fixation in 1% osmium tetroxide for 1 hour. Samples were dehydrated in acetone, embedded in Araldite CY 212 epoxy resin, polymerized at 60° C and sectioned into

ultrathin slices (60–70 nm). Sections were stained with uranyl acetate and lead citrate to enhance contrast and examined using a transmission electron microscope (Talos F200S, Thermo Fisher Scientific, WA, USA) at 200 kV at a primary magnification of 2500X to 5500X. This allowed detailed visualization of intracellular structures, including organelles and membrane specializations.

RESULTS

Generation and Morphological Development of Human Cortical Organoids

Human *iPSC*-derived cortical organoids were successfully generated using a stepwise differentiation and self-organization protocol. During the early stages of culture, single-cell *iPSC* aggregates rapidly formed compact embryoid bodies (EBs) under low-attachment conditions. Following neural induction and transition into cortical differentiation medium, the developing organoids progressively increased in size and complexity. Over time, the organoids acquired a distinct spherical morphology with smooth external boundaries and dense cellular organization, indicating successful tissue compaction and structural maturation (Figure 1).

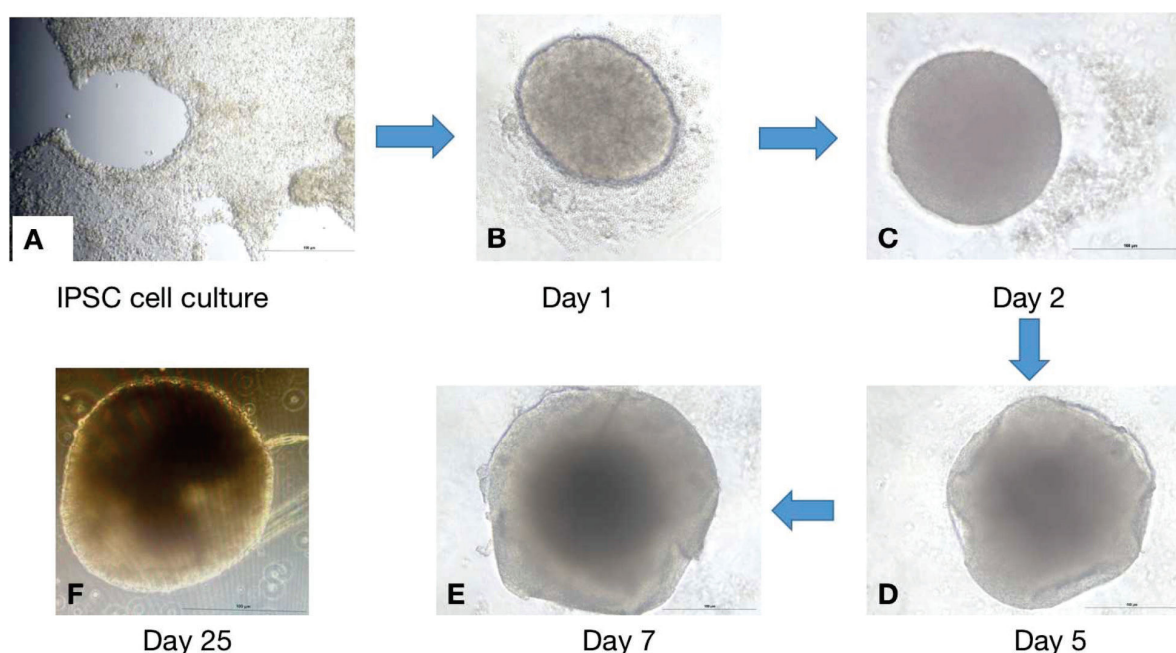


Figure 1: Generation and morphological progression of human *iPSC*-derived cortical organoids

Representative bright-field images illustrating sequential stages of cortical organoid formation from human induced pluripotent stem cells (*iPSCs*). Undifferentiated *iPSC* colonies were dissociated to generate embryoid bodies (Day 1), which progressively compacted into uniform spheroidal aggregates by Day 2. Continued neural induction resulted in enlargement and the appearance of a smooth neuroepithelial surface by Day 5, followed by increased tissue organization and structural maturation at Day 7. By Day 25, mature cortical organoids exhibited a well-defined spherical morphology with increased tissue density, indicating successful cortical differentiation and organoid maturation. Scale bars = 100 μ m.

The mature cortical organoids exhibited translucent peripheral regions suggestive of neuroepithelial organization and active cellular differentiation. The organoids maintained structural integrity throughout prolonged culture conditions, demonstrating the reproducibility and stability of the differentiation protocol. Morphologically, the organoids closely resembled early developing neural tissue, displaying characteristics associated with primitive cortical organization and tissue self-patterning.

Confocal Microscopy Reveals Organized Cortical-like Cellular Architecture

Confocal microscopy analysis demonstrated a highly organized and spatially coordinated cellular architecture within the human *iPSC*-derived cortical organoids (Figure 2). Immunofluorescence imaging using GFAP staining revealed extensive astroglial differentiation distributed throughout the organoid structure. GFAP-positive cells formed interconnected astroglial networks

that appeared continuous across multiple regions of the organoid, suggesting the establishment of a supportive glial microenvironment. The widespread distribution of astrocytic components indicates successful lineage specification and maturation of glial populations alongside neuronal differentiation.

DAPI nuclear staining revealed dense and uniformly distributed nuclei arranged in organized spatial patterns resembling early cortical layering. The nuclei appeared intact and evenly dispersed without evidence of fragmentation, condensation, or disorganization, indicating preserved cellular viability and tissue integrity. Distinct regions of increased nuclear density were observed, suggestive of localized proliferative zones analogous to ventricular-like neuroepithelial regions seen during early corticogenesis. The radial arrangement of nuclei further reflected coordinated tissue organization and cellular patterning within the organoid.

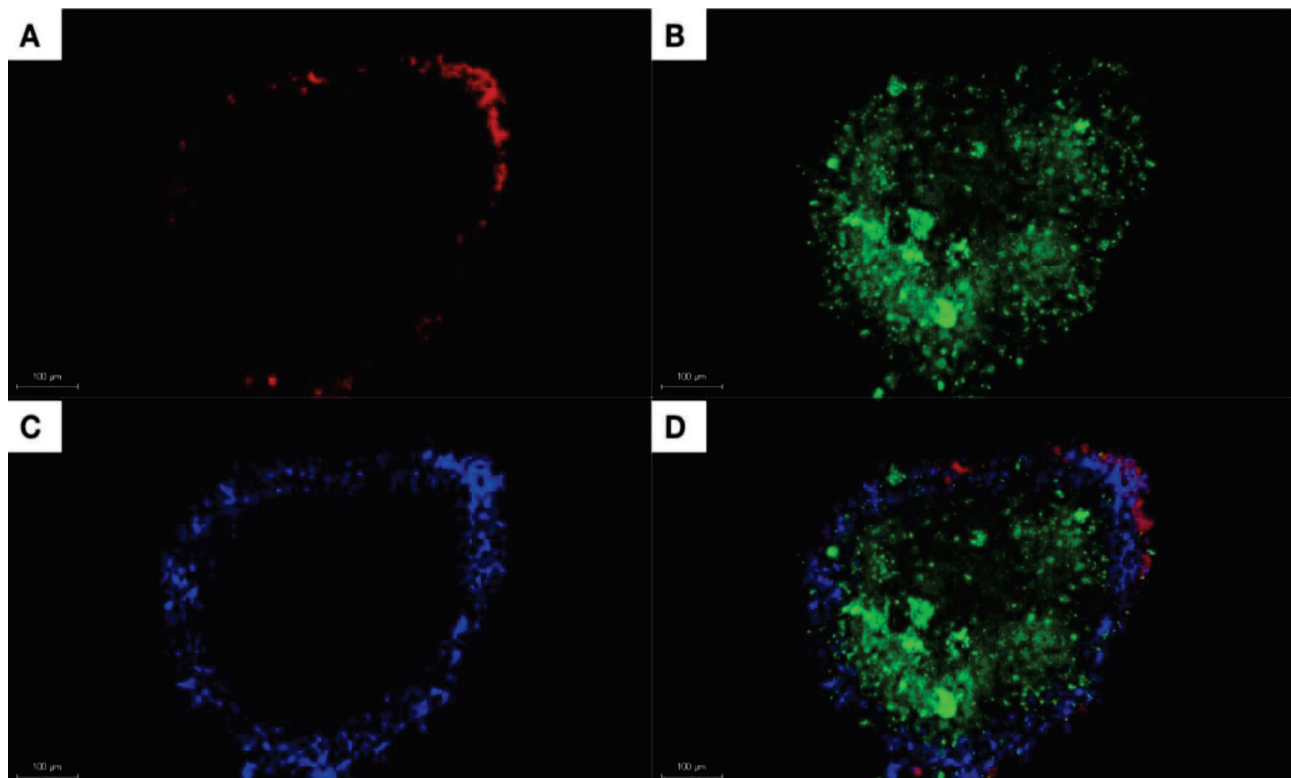


Figure 2: Confocal fluorescence characterization of human *iPSC*-derived cortical organoids. Representative confocal fluorescence images of mature human *iPSC*-derived cortical organoids demonstrating neuronal and glial organization.

(A) **NeuroTrace** fluorescent Nissl staining (red) highlighting neuronal cell bodies distributed throughout the organoid, (B) GFAP immunofluorescence (green) showing astrocytic networks within the cortical organoid, (C) DAPI nuclear staining (blue) demonstrating the overall cellular distribution and tissue architecture, (D) Merged image illustrating the spatial organization of **NeuroTrace**-positive neurons, GFAP-positive astrocytes, and DAPI-stained nuclei, confirming the establishment of a multicellular cortical-like structure. Scale bars = 100 µm.

NeuroTrace fluorescent Nissl staining demonstrated clearly identifiable neuronal cell bodies distributed throughout the organoid sections. The neuronal populations appeared integrated within the surrounding tissue architecture, supporting the formation of complex neuronal assemblies. Neuronal cells exhibited organized spatial orientation rather than random distribution, indicating progressive neural network formation.

Merged fluorescence images demonstrated coordinated co-localization of neuronal and astroglial populations within the same structural framework. This integrated cellular organization strongly supports the emergence of cortical-like tissue architecture and indicates successful recapitulation of critical aspects of early human cortical development. Overall, confocal microscopy confirmed the presence of structurally organized neural tissue with distinct neuronal and glial populations, validating effective cortical differentiation within the organoids.

SEM Demonstrates Complex Three-Dimensional Surface Topology and Neuroepithelial Folding

SEM provided detailed insights into the external morphology and three-dimensional microarchitecture of the cortical organoids (Fig. 3). At lower magnifications, the organoids displayed a well-defined spherical morphology with compact and highly organized external surfaces. The surface architecture appeared dense and structurally cohesive, indicating effective cellular compaction and tissue stability during maturation. Higher magnification imaging revealed intricate neuroepithelial folding patterns distributed across the organoid surface. These folds formed complex three-dimensional structures resembling early cortical morphogenetic organization. The neuroepithelial regions appeared as tightly packed cellular clusters arranged in layered and folded conformations, suggesting active tissue remodeling and self-organization processes. Such folding patterns are characteristic of early neurodevelopmental events and indicate progressive structural maturation within the organoids.

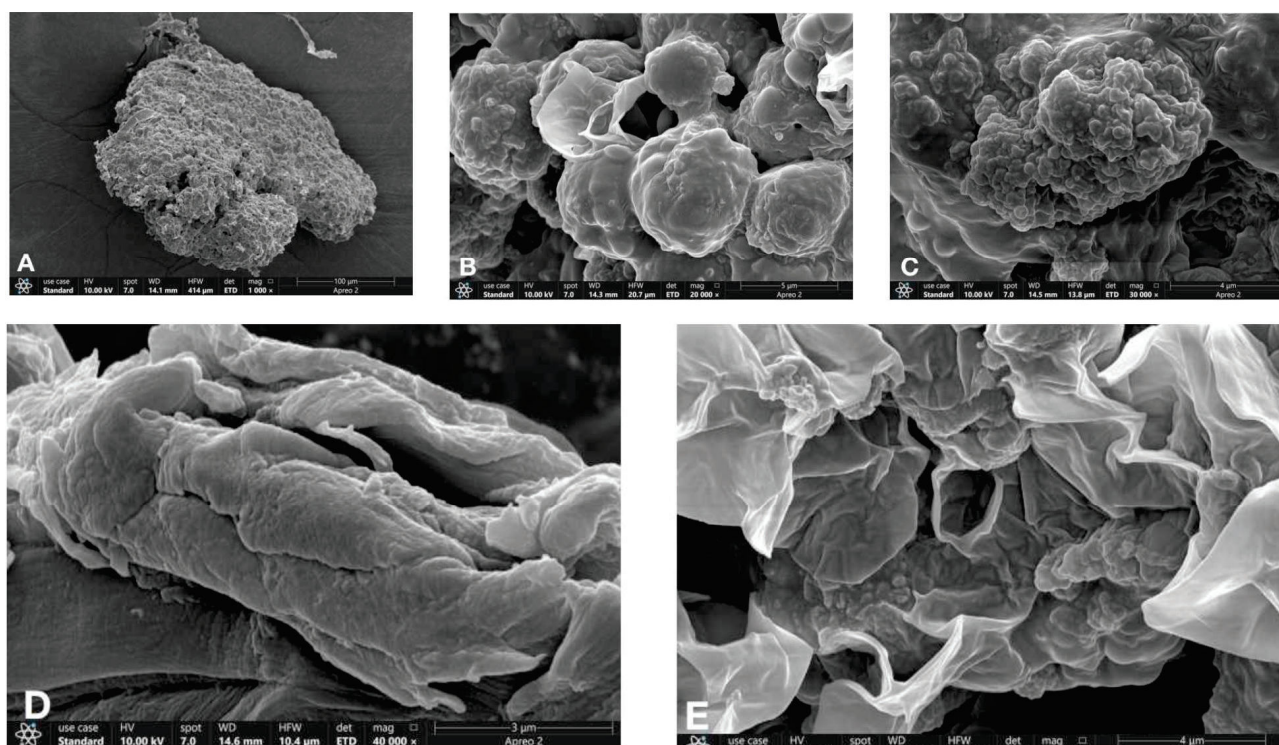


Figure 3: Scanning electron microscopy (SEM) characterization of human iPSC-derived cortical organoids.

Representative SEM images illustrating the surface morphology and ultrastructural organization of mature human iPSC-derived cortical organoids at increasing magnifications, (A) Low-magnification view showing the overall three-dimensional spheroidal architecture of the cortical organoid, (B) Higher-magnification image demonstrating densely packed cellular aggregates and a compact tissue surface, (C) Well-defined neuroepithelial folding and multilayered cellular organization, resembling early cortical tissue morphogenesis, (D) High-magnification view showing elongated neurite-like projections and closely apposed cellular processes suggestive of developing neuronal connectivity, (E) Ultrastructural image revealing prominent neuroepithelial folds, interconnected cellular networks, and extensive cell-cell interactions, consistent with progressive cortical organoid maturation. Scale bars: (A) 100 μ m; (B) 5 μ m; (C) 4 μ m; (D) 3 μ m; (E) 4 μ m.

The organoid surfaces also exhibited numerous neurite-like projections extending between adjacent cellular regions. These projections formed interconnected mesh-like networks across the surface, suggesting the establishment of cellular connectivity and communication pathways. The presence of these elongated projections indicates advancing neuronal differentiation and structural organization. In addition, the surface topology displayed varying elevations and interconnected ridges, contributing to the overall complexity of the tissue architecture. The dense cellular interactions observed across the organoid surface suggest coordinated growth dynamics and active extracellular structural organization. Collectively, SEM analysis demonstrated that the cortical organoids developed advanced three-dimensional organization with neuroepithelial folding and neuronal connectivity patterns resembling early cortical tissue formation.

TEM Confirms Ultrastructural Maturation and Neuronal Differentiation

TEM enabled high-resolution visualization of intracellular and ultrastructural features within the cortical organoids, providing definitive evidence of neuronal maturation (Figure 4). Distinct neuronal cell bodies with well-preserved cellular architecture were clearly observed throughout the tissue. The cells exhibited defined nuclear and cytoplasmic boundaries, indicating intact membrane integrity and cellular preservation during tissue processing.

The nuclei appeared structurally organized with uniform chromatin distribution, while the surrounding cytoplasm demonstrated organized intracellular composition. Neuronal processes extending from the cell bodies were visible within several regions of the organoid, suggesting active neuronal differentiation and structural connectivity.

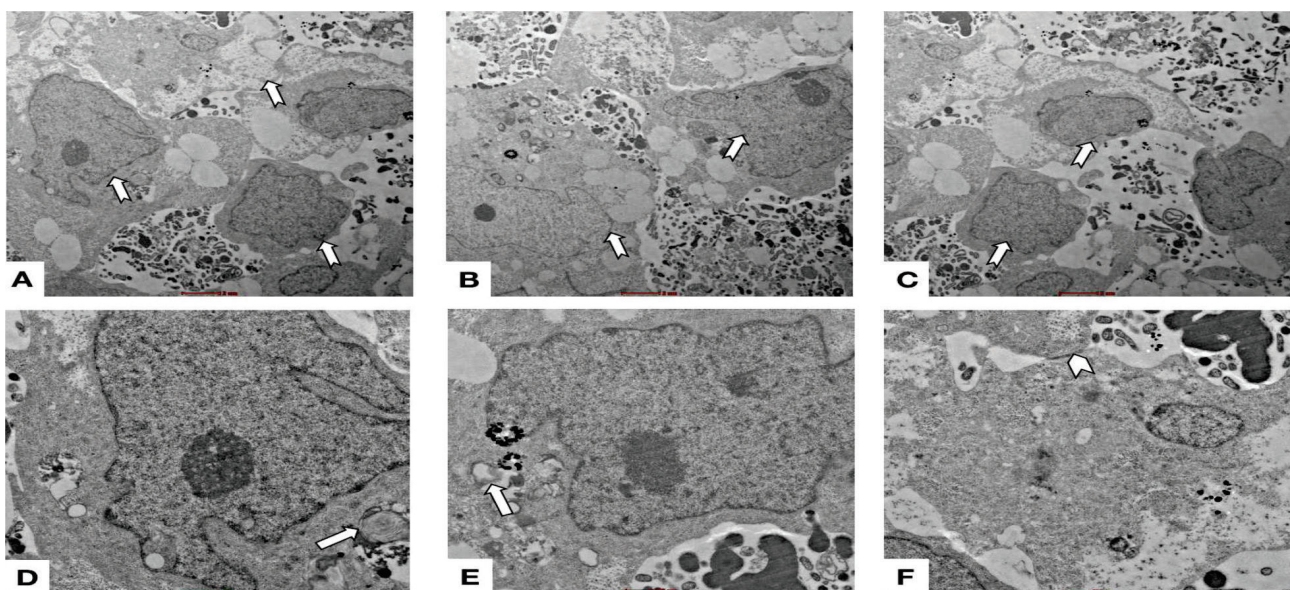


Figure 4: Transmission electron microscopy (TEM) characterization of human iPSC-derived cortical organoids

Representative TEM images demonstrating the ultrastructural features of mature human iPSC-derived cortical organoids, (A-C) Low-magnification images showing neuronal cell bodies with prominent nuclei and cytoplasm, extensive cell-cell contacts, and organized cellular architecture (arrows), (D) Higher-magnification image illustrating a mature neuronal cell body with a well-defined nucleus, cytoplasmic organelles, and adjacent neuronal processes (arrow), (E) TEM image showing closely apposed neuronal cells with distinct plasma membranes and intercellular junctions (arrow), indicative of tissue organization, (F) Ultrastructural view demonstrating a myelin sheath-like structure surrounding a neuronal process (arrow), together with surrounding neuropil, consistent with advanced structural maturation of the cortical organoid.

A notable finding was the presence of prominent myelin sheath-like structures surrounding neuronal projections. These structures suggest the initiation of axonal organization and early insulating mechanisms associated with neuronal

maturation. Although complete myelination is generally associated with later developmental stages, the identification of these membrane-associated structures indicates significant progression toward mature neural architecture.

TEM imaging also revealed organized intercellular interactions and clearly identifiable membrane boundaries between adjacent cells. The presence of intercellular contacts and closely associated cellular arrangements indicates coordinated tissue assembly and structural cohesion within the organoid. These features support the development of a complex and interconnected neural microenvironment.

The ultrastructural organization observed in the cortical organoids collectively confirms successful neuronal differentiation, tissue maturation, and establishment of cortical-like cellular architecture. The preservation of intracellular detail and membrane organization further validates the robustness of the organoid generation and fixation protocols used in this study.

Integrated Structural Validation Across Imaging Modalities

Collectively, the findings obtained through confocal microscopy, SEM, and TEM demonstrate a high degree of structural and cellular organization within the human *iPSC*-derived cortical organoids. Confocal imaging validated the presence of organized neuronal and astroglial populations, SEM revealed advanced three-dimensional tissue topology and neuroepithelial folding, and TEM confirmed ultrastructural maturation and neuronal differentiation.

The concordance of findings across these complementary imaging modalities strongly supports the successful generation of cortical organoids that recapitulate essential features of early human brain development. These results establish the organoids as structurally robust and biologically relevant *in vitro* models suitable for neurodevelopmental studies, disease modeling, and translational neuroscience research.

DISCUSSION

The present study demonstrates the successful generation and multiscale structural characterization of human *iPSC*-derived cortical organoids, highlighting their capacity to recapitulate key morphological and cellular features of early human brain development. By integrating confocal microscopy, SEM and TEM, this work provides a comprehensive and hierarchical evaluation of organoid

organization, spanning from tissue-level architecture to subcellular ultrastructure. Such a multimodal approach is essential for validating organoid systems, as it enables cross-confirmation of structural features across different spatial resolutions and is widely adopted in organoid research.⁽⁸⁾

At the cellular level, confocal microscopy revealed a highly organized arrangement of neuronal and astroglial populations within the organoids. The presence of GFAP-positive astroglial networks distributed throughout the structure indicates successful glial differentiation, which is a critical hallmark of advancing neural maturation. Astrocytes play essential roles in synaptic modulation, metabolic support, and maintenance of extracellular homeostasis, and their early emergence within organoids suggests the establishment of a supportive neural microenvironment.⁽³⁾ Furthermore, the organized nuclear distribution observed with DAPI staining reflects coordinated cellular proliferation and spatial patterning. The apparent layering and density gradients resemble early cortical plate formation and ventricular zone-like organization, suggesting that the developmental trajectory of these organoids closely parallels *in vivo* corticogenesis.⁽⁷⁾

The identification of neuronal populations through NeuroTrace staining further supports the successful differentiation of *iPSCs* into functional neural lineages. Importantly, the spatial integration of neurons and astrocytes observed in merged images indicates the formation of a coordinated cellular network rather than isolated populations. This level of organization is critical for modeling neurodevelopmental processes, as neuron-glia interactions are fundamental for synapse formation and neural circuit establishment.⁽¹¹⁾

At the tissue level, SEM analysis provided important insights into the three-dimensional organization and surface morphology of the organoids. The observed spherical morphology with a dense and organized surface network reflects uniform growth and structural integrity, key indicators of reproducibility in organoid cultures. The presence of neuroepithelial-like folding is particularly significant, as it mirrors early morphogenetic events during cortical development, where tissue folding contributes to expansion of surface area and

cellular compartmentalization.⁽¹⁴⁾ These folds likely represent zones of active proliferation and differentiation, analogous to neurogenic niches in the developing brain.

The detection of neurite-like projections extending across the organoid surface further indicates progressive neuronal maturation and connectivity. These projections suggest the initiation of intercellular communication and network formation, which are essential for functional neural activity.⁽²⁾ Similar structural features have been reported in advanced organoid systems demonstrating neuronal diversity and network dynamics.

At the ultrastructural level, TEM provided definitive evidence of neuronal differentiation and maturation. The presence of well-defined neuronal cell bodies with clear nuclear and cytoplasmic boundaries confirms cellular integrity and differentiation. The observation of myelin sheath-like structures surrounding neuronal processes is particularly noteworthy, as it suggests early stages of axonal organization. While complete myelination occurs later in development, early myelin-like features have been described in organoid models undergoing maturation.⁽⁶⁾ Additionally, the identification of intact membrane boundaries and intercellular junctions highlights the establishment of cohesive tissue architecture and cellular communication pathways. These ultrastructural features are consistent with previous reports describing synaptic organization and neuronal maturation in cerebral organoids.⁽¹⁶⁾ When considered collectively, the findings from confocal microscopy, SEM, and TEM demonstrate a high degree of concordance, reinforcing the validity of the organoid system. The ability to observe consistent structural features across multiple imaging modalities strengthens confidence in the reproducibility and biological relevance of the model. This integrative validation approach addresses a major limitation in organoid research—variability in structure and maturation—and contributes toward standardization of protocols.⁽¹⁷⁾

Despite these promising results, certain limitations should be acknowledged. One of the primary challenges in organoid systems is the lack of vascularization, which can lead to diffusion limitations and restrict long-term growth and maturation. This limitation has been widely discussed in the literature and

remains a major area of ongoing research.⁽¹⁹⁾ Additionally, while structural maturation is clearly demonstrated, functional validation through electrophysiological studies would further strengthen the findings.

Future directions may involve incorporation of vascular-like systems, co-culture with microglia, and bioengineering approaches to improve physiological relevance. Advanced techniques such as single-cell RNA sequencing and electrophysiological recordings could further elucidate the functional maturity of these organoids. Such approaches have already begun to reveal complex neuronal activity and circuit formation in organoid systems.⁽⁴⁾

In conclusion, this study provides robust evidence that human *iPSC*-derived cortical organoids can faithfully recapitulate key structural features of early brain development. The integration of multiscale imaging techniques not only validates the organoid model but also underscores its potential as a powerful platform for advancing our understanding of human neurodevelopment, disease modeling, and regenerative neuroscience.

CONCLUSION

This study establishes an effective method for generating and characterizing cortical organoids from human *iPSCs*. Through light microscopy, DAPI-based confocal imaging, SEM, and TEM, we demonstrated successful structural maturation and organization resembling early human cortical development. Light microscopy revealed neuroepithelial organization; confocal microscopy confirmed nuclear integrity and spatial layering; SEM visualized surface networks and cellular interconnectivity and TEM confirmed subcellular maturation, including mitochondria and synaptic vesicles. These findings validate the organoids as a reliable, reproducible *in vitro* model for studying cortical development, cell architecture, and neurobiological mechanisms relevant to human brain physiology and pathology.

Conflict of Interest: The authors declare no conflicts of interest.

Funding Information

This work was supported by the Indian Council of Medical Research (ICMR), Government of India.

REFERENCES

1. Alex AM, Buss C, Davis EP, Campos GL, Donald KA, Fair DA, Gaab N, Gao W, Gilmore JH, Girault JB, Grewen K, Groenewold NA, Hankin BL, Ipser J, Kapoor S, Kim P, Lin W, Luo S, Norton ES, O'Connor TG, Piven J, Qiu A, Rasmussen JM, Skeide MA, Stein DJ, Styner MA, Thompson PM, Wakschlag L, Knickmeyer R; ENIGMA ORIGINS Group (2023) Genetic influences on the developing young brain and risk for neuropsychiatric disorders. *Biol Psychiatry* 93(10):905–920. <https://doi.org/10.1016/j.biopsych.2023.01.013>
2. Cullen DK, Gordián-Vélez WJ, Struzyna LA, Jgamadze D, Lim J, Wofford KL, Browne KD, Chen HI (2019) Bundled three-dimensional human axon tracts derived from brain organoids. *iScience* 21:57–67. <https://doi.org/10.1016/j.isci.2019.10.004>
3. Di Lullo E, Kriegstein AR (2017) The use of brain organoids to investigate neural development and disease. *Nat Rev Neurosci* 18(10):573–584. <https://doi.org/10.1038/nrn.2017.107>
4. Di Stefano J, Di Marco F, Cicalini I, FitzGerald U, Pieragostino D, Verhoye M, Ponsaerts P, Van Breedam E (2025) Generation, interrogation, and future applications of microglia-containing brain organoids. *Neural Regen Res* 20(12):3448–3460.
5. Fan X, Hou K, Liu G, Shi R, Wang W, Liang G (2025) Strategies to overcome the limitations of current organoid technology—engineered organoids. *J Tissue Eng* 16:20417314251319475. <https://doi.org/10.1177/20417314251319475>
6. Giandomenico SL, Mierau SB, Gibbons GM, Wenger LMD, Masullo L, Sit T, Sutcliffe M, Boulanger J, Tripodi M, Derivery E, Paulsen O, Lakatos A, Lancaster MA (2019) Cerebral organoids at the air-liquid interface generate diverse nerve tracts with functional output. *Nat Neurosci* 22(4):669–679. <https://doi.org/10.1038/s41593-019-0350-2>
7. Kadoshima T, Sakaguchi H, Nakano T, Soen M, Ando S, Eiraku M, Sasai Y (2013) Self-organization of axial polarity, inside-out layer pattern, and species-specific progenitor dynamics in human ES cell-derived neocortex. *Proc Natl Acad Sci USA* 110(50):20284–20289. <https://doi.org/10.1073/pnas.1315710110>
8. Lancaster MA, Renner M, Martin CA, Wenzel D, Bicknell LS, Hurles ME, Homfray T, Penninger JM, Jackson AP, Knoblich JA (2013) Cerebral organoids model human brain development and microcephaly. *Nature* 501(7467):373–379. <https://doi.org/10.1038/nature12517>
9. Lee CT, Bendriem RM, Wu WW, Shen RF (2017) 3D brain organoids derived from pluripotent stem cells: promising experimental models for brain development and neurodegenerative disorders. *J Biomed Sci* 24:59. <https://doi.org/10.1186/s12929-017-0362-8>
10. Li ZX, Tu SX, Li YW, Hu ZB, Liu WT, Tao YC, Zhao L, Song NN, Chen JY, Zhang Q, Qi CC, Zhu HW, Ding YQ, Hu L (2026) Med13 is involved in the radial migration and contralateral projection of cortical neurons via PlxnA4. *Commun Biol* 9:394. <https://doi.org/10.1038/s42003-026-09704-w>
11. Pathak D, Sriram K (2023) Neuron-astrocyte omnidirectional signaling in neurological health and disease. *Front Mol Neurosci* 16:1169320. <https://doi.org/10.3389/fnmol.2023.1169320>
12. Qian X, Nguyen HN, Song MM, Hadiono C, Ogden SC, Hammack C, Yao B, Hamersky GR, Jacob F, Zhong C, Yoon KJ, Jeang W, Lin L, Li Y, Thakor J, Berg DA, Zhang C, Kang E, Chickering M, Nauen D, Ho CY, Wen Z, Christian KM, Shi PY, Maher BJ, Wu H, Jin P, Tang H, Song H, Ming GL (2016) Brain-region-specific organoids using mini-bioreactors for modeling ZIKV exposure. *Cell* 165(5):1238–1254. <https://doi.org/10.1016/j.cell.2016.04.032>
13. Quadrato G, Nguyen T, Macosko EZ, Sherwood JL, Yang SM, Berger DR, Maria N, Scholvin J, Goldman M, Kinney JP, Boyden ES, Lichtman JW, Williams ZM, McCarroll SA, Arlotta P (2017) Cell diversity and network dynamics in photosensitive human brain organoids. *Nature* 545(7652):48–53. <https://doi.org/10.1038/nature22047>
14. Rajagopalan V, Scott J, Habas PA, Kim K, Rousseau F, Glenn OA, Barkovich AJ, Studholme C (2011) Spatiotemporal morphometry of adjacent tissue layers with application to the study of sulcal formation. *Med Image Comput Comput Assist Interv* 14(Pt 2):476–483. https://doi.org/10.1007/978-3-642-23629-7_58
15. Sultana of, Bandaru M, Islam MA, Reddy PH (2024) Unraveling the complexity of human brain: structure, function in healthy and disease states. *Ageing Res Rev* 100:102414. <https://doi.org/10.1016/j.arr.2024.102414>
16. Truong HD, Ge Z, Chng E, et al (2026) Strategies to control cellular spatial organization in microphysiological systems. *Microsyst*

- Nanoeng 12:85. <https://doi.org/10.1038/s41378-025-01141-9>
17. Wang J, Qiao G, Tan H, Li MJ, Wang K, Xu J, Janowski M, Fu TM, Walczak P, Liang Y (2025) Intravital multimodal imaging of human cortical organoids for chronic stroke treatment in mice. *bioRxiv*. <https://doi.org/10.1101/2025.07.05.663297>
 18. Xiang Y, Park IH (2024) Brain organoids: from unguided to regionalized to nucleus-specific. *Life Med* 3(2):lnae014. <https://doi.org/10.1093/lifemedi/lnae014>
 19. Zhao X, Xu Z, Xiao L, Shi T, Xiao H, Wang Y, Li Y, Xue F, Zeng W (2021) Review on the vascularization of organoids and organoids-on-a-chip. *Front Bioeng Biotechnol* 9:637048. <https://doi.org/10.3389/fbioe.2021.637048>