

Human adherent cortical organoids in a multiwell format

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Abstract

Summary

In the growing diversity of human iPSC-derived models of brain development, we present here a novel method that exhibits 3D cortical layer formation in a highly reproducible topography of minimal dimensions. The resulting adherent cortical organoids develop by self-organization after seeding frontal cortex patterned iPSC-derived neural progenitor cells in 384-well plates during eight weeks of differentiation. The organoids have stereotypical dimensions of $3 \times 3 \times 0.2$ mm, contain multiple neuronal subtypes, astrocytes and oligodendrocyte lineage cells, and are amenable to extended culture for at least 10 months. Longitudinal imaging revealed morphologically mature dendritic spines, axonal myelination, and robust neuronal activity. Moreover, adherent cortical organoids compare favorably to existing brain organoid models on the basis of robust reproducibility in obtaining topographically-standardized singular radial cortical structures and circumvent the internal necrosis that is common in free-floating cortical organoids. The adherent human cortical organoid platform holds considerable potential for high-throughput drug discovery applications, neurotoxicological screening, and mechanistic pathophysiological studies of brain disorders.

eLife assessment

This paper describes an **important** advance in an in vitro neural culture system to generate mature, functional, diverse, and geometrically consistent cultures, in a 384-well format with defined dimensions and the absence of the necrotic core, which persists for up to 300 days. The well-based format and conserved geometry make it a promising tool for arrayed screening studies. Some of the evidence is **incomplete** and could benefit from a more direct head-to-head comparison with more standard culture methods and standardization of cell seeding density as well as further data on reproducibility in each well and for each cell line.

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Introduction

Using human embryonic or induced pluripotent stem cell (hiPSC) derived models to investigate the developing brain in health and disease has yielded considerable success (Marton and Paşca, 2020). The approaches have been varied, including single cell hiPSC-derived models grown in a monolayer (Sarkar et al., 2018 [↗](#); Zhang et al., 2013 [↗](#)), multiple neural cell types in 2D neural networks (Astick and Vanderhaeghen, 2018 [↗](#); Bardy et al., 2015 [↗](#); Gunhanlar et al., 2018 [↗](#); Shi et al., 2012 [↗](#)), 3D free-floating regionalized neural organoids (Paşca et al., 2015; Qian et al., 2016a; Xiang et al., 2019 [↗](#); Zhang et al., 2023 [↗](#)), and hiPSC-derived free-floating unguided neural organoids (Gomes et al., 2020 [↗](#); Lancaster et al., 2013; Paşca et al., 2022; Pellegrini et al., 2020 [↗](#); Renner et al., 2017 [↗](#); Sawada et al., 2020 [↗](#)). Among the consistent findings across models is that increasing cellular and topographical complexity has appeared to come at the cost of increased variability (Eichmüller and Knoblich, 2022 [↗](#); Kelava and Lancaster, 2016 [↗](#)). Therefore, a major current technical challenge is to identify hiPSC-derived models that recapitulate higher-order neural complexity with reduced heterogeneity.

Existing 3D models suffer from considerable variability due to the complex and heterogeneous nature of the free-floating structures (Cederquist et al., 2019 [↗](#); Lancaster et al., 2013, 2017 [↗](#); Paşca et al., 2015; Renner et al., 2017 [↗](#); Velasco et al., 2019 [↗](#); Yoon et al., 2019 [↗](#)). A further challenge, in particular with free-floating organoids, is the necrotic core that emerges when tissue volumes exceed the limits of oxygen and nutrient diffusion beyond a radius of ~300-400µm (Lancaster et al., 2017 [↗](#); Qian et al., 2016b [↗](#)). Although recent progress has been made with slicing organoids prior to the emergence of necrosis followed by organotypic air-liquid interface culture (Giandomenico et al., 2019 [↗](#); Qian et al., 2020 [↗](#)), even sliced organoids have to be repeatedly re-cut to prevent necrosis (Qian et al., 2020 [↗](#)), which is both laborious and risks introducing another potential source of variability. The generation of vascularized organoids would be the ultimate solution to reduce the necrotic core and the inherent stress that is observed in cortical organoids (Bhaduri et al., 2020 [↗](#); Fan et al., 2022 [↗](#)). It will be beneficial to have increased diffusion through microfluidics or vascularization (current efforts summarized in Matsui et al., 2021 [↗](#)) and further study the mutually beneficial interaction of neural cells and vascular cells (Crouch et al., 2022 [↗](#); Mansour et al., 2018 [↗](#); Wang et al., 2023 [↗](#)).

Here we propose a simplified approach to generating long-term hiPSC-derived adherent cortical organoids with reproducible dimensions and the potential for high-throughput screening in a 384-well format. The resulting adherent cortical organoids can be maintained in long-term culture and contain neurons with dendritic spines and robust activity, as well as several classes of glial cells including oligodendrocyte precursor cells, myelinating oligodendrocytes, and morphologically distinct sub-types of astrocytes.

Results

Self-organized topography of iPSC-derived adherent cortical organoids

Adherent cortical organoids reproducibly self-organized into layered radial structures in 384-wells plates within 8 weeks of seeding with hiPSC-derived forebrain-patterned neural progenitor cells (NPCs). Three different hiPSC source cell lines were used in this study, including commercially available NPCs, and NPCs generated using a modified version of our previously described protocol (Gunhanlar et al., 2018 [↗](#)) (Figure 1A [↗](#)). NPCs were capable of neural rosette formation and expressed SOX2, Nestin, and the frontal cortical NPC-marker FOXG1 (Figure 1B [↗](#), C, D, Figure

S1A). The initial 4 weeks after seeding the NPCs in Neural Differentiation Medium (ND) were characterized by proliferative expansion of NPCs and the emergence of early neural differentiation markers (**Figure 1E** [↗](#), **Figure S2**). Between 4– and 8-weeks post-seeding, neurons and glial cells emerged with a consistent spatial organization (**Figure 1E** [↗](#), **F**, **Figure S1B**, **Figure S2**), in which the central region was densely packed with cell bodies while the periphery contained circumferentially and radially organized processes originating from cells in the centre. Typically, cortical organoids defined as a single radial structure per well were observed in ~80% of the wells seeded with NPCs after 60 days of differentiation. The structural integrity of single structure organoids remained quite stable and slowly diminished over time to about 50% of intact single structure organoids after 1 year in culture (**Figure S1D**). Organoid structure formation was highly dependent on the proliferation rate of NPCs, that can differ substantially between differentiation batches and hiPSC clones. Plotting the interaction between proliferation and the amount of NPCs required to be seeded for the successful generation of adherent cortical organoids, showed a significant correlation ($r^2=0.67$) that can be used as a guideline for testing a range of NPC densities (**Figure S1C**). For each NPC line an optimal seeding density was estimated based on the proliferation rate of that NPC line. Multiple densities were seeded around the estimated optimal density and after 6 weeks it was visually determined which NPC density enabled adherent cortical organoid generation. Typically, too sparse seeding density generated neural networks lacking structure, while excessive densities resulted in overgrowth, leading to reduced structural organisation and compromised long-term survival.

Cell type distribution and layer formation

The spatial organisation that evolved over the first 8 weeks after seeding was paralleled by a shift in cell type distribution. Tau+/MAP2– axons exhibited long extensions in a circular pattern, while MAP2+ dendrites exhibited orthogonally-oriented radial outgrowth (**Figure 2A** [↗](#), **Figure S3**).

Overall, a reduction in progenitor markers (SOX2 day 14: 58.4%, day 56: 18.8% $P = <0.001$, PAX6 day 14: 34.5%, day 56: 8.0% $P = 0.20$) and a significant increase in neuronal cortical layer markers (CTIP2 day 14: 0.5%, day 56: 14.0% $P = <0.001$, CUX1 day 14: 1.3%, day 56: 24.4% $P = <0.001$) were observed (**Figure S2**). Cortical layer markers exhibited an inside-out pattern of development in which expression of the deep layer excitatory neuronal marker CTIP2 emerged before the upper layer marker CUX1 (**Figure S2**, **Figure 2C** [↗](#)). After 6–8 weeks following seeding, a self-organised rudimentary segregation of deep– and upper layer neurons emerged as shown by a clear macroscopic separation of deep– and upper layer neurons, although some neurons were spatially intermixed and some neurons were double-positive for CTIP2 and CUX1. Segregation was also observed between CUX1 and CUX2 positive cells as CUX2 is typically expressed over a wider range of upper cortical layers than CUX1 and also marks intermediate progenitors (Molyneaux et al., 2007 [↗](#)) (**Figure 2C** [↗](#), **Figure S4**). Analogous to the broad distribution of cortical cell subclasses, the majority of the neurons were glutamatergic, while GAD67+ interneurons were also present (**Figure 2D** [↗](#)), constituting ~10% of the NeuN-positive neuronal population consistently for all three source hiPSC lines (**Figure S5**).

Adherent cortical organoids contain multiple glial cell types

Within 8 weeks of seeding, a population of GFAP+/S100β+ astrocytes emerged. Many astrocytes had their soma located in the central region with process outgrowth radially (**Figure 2E** [↗](#), **Figure S6**), while other astrocytes exhibited subtype-specific morphologies including fibrous astrocytes (**Figure 2F** [↗](#)), protoplasmic-like astrocytes (**Figure 2G** [↗](#)) and interlaminar astrocytes (**Figure 2H** [↗](#)). GFAP/PAX6 double-positive radial glia were present at the outskirts of the densely populated centre of the organoid with processes growing radially outwards (**Figure 2I** [↗](#)). Similar to free-floating organoids, adherent cortical organoids survived for longer periods compared to monolayer neural cultures grown on larger surfaces. The longevity allows for the development of cell types not usually seen in a monolayer culture that can typically be cultured up to a maximum

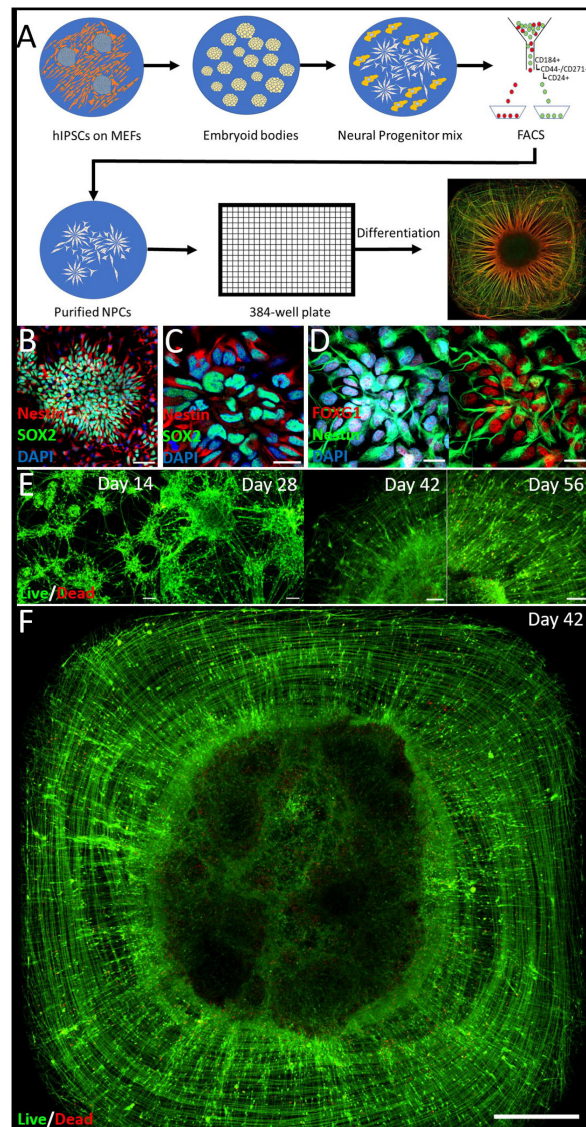


Figure 1

Adherent cortical organoid model.

A Schematic representation of the differentiation protocol. **B/C/D** Representative NPCs from three different iPSC lines with markers SOX2, Nestin and FOXG1 (scale bar B, 50 μm ; C/D, 20 μm). **E** Representative time course showing self-organization during differentiation, starting with radial organization between day 28 and day 42, seeding density 1500 NPCs per well (green is Live-stain from Viability/Cytotoxicity kit; scale bars left to right 100 μm , 150 μm , 100 μm , 100 μm). **F** Full well showing radial organization at day 42 in culture where only a few dead cells are visible in red in the dense centre of the structure, seeding density 1500 NPCs per well (scale bar, 500 μm).

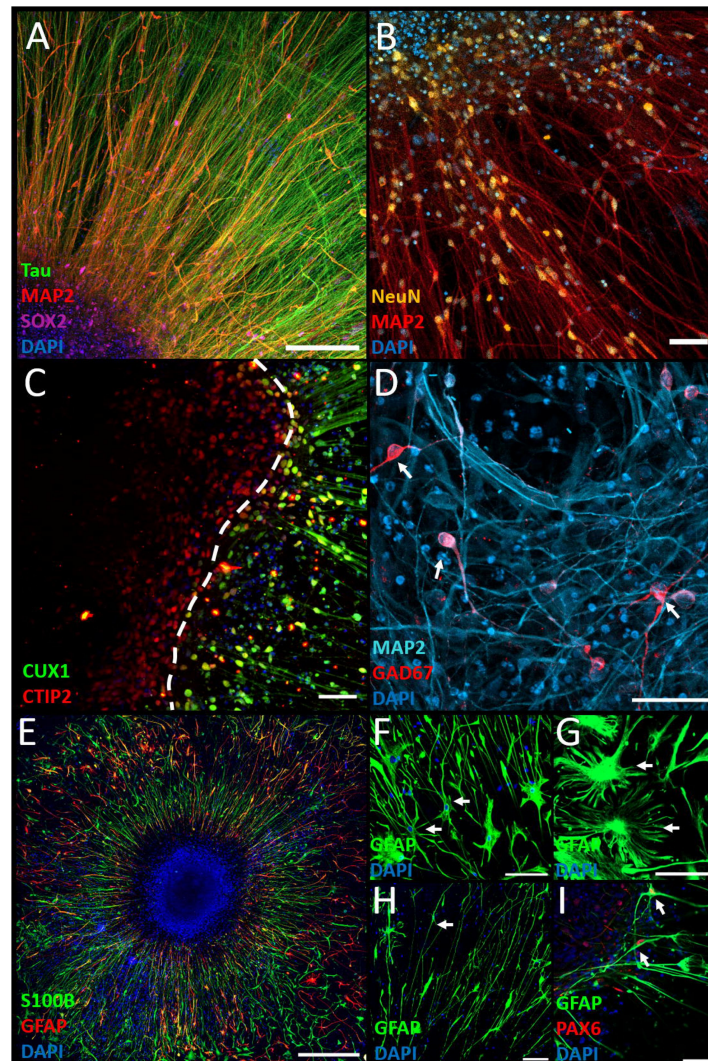


Figure 2

Adherent cortical organoids show an organized network of neuronal and astrocyte subtypes.

A MAP2+ somas and dendrites alongside Tau+/MAP2- axons show segregation of dendritic and axonal compartments, with SOX2+ progenitors concentrated in the center of the organoid (Day 75, 200 μm). **B** MAP2+ and NeuN+ cells indicate mature neurons (Day 72, 50 μm). **C** Deep-layer cortical marker CTIP2 and upper layer marker CUX1 show rudimentary segregation of cortical layers in expected inside-out pattern (Day 64, 50 μm). **D** A subset of the MAP2-positive neurons are GAD67+ (white arrows), indicating the presence of an interneuron population in the cortical organoids (Day 60, 50 μm). **E** Astrocyte markers GFAP and S100β show the general radial pattern of astrocyte outgrowth (Day 66, 500 μm). **F/G/H** GFAP staining reveals the morphologies of different astrocyte subtypes (white arrows), including fibrous astrocytes (**F**, Day 65, 100 μm), protoplasmic astrocytes (**G**, Day 65, 50 μm) and interlaminar astrocytes (**H**, Day 65, 100 μm). **I** Co-localization of astrocyte marker GFAP and PAX6 marks radial glia (white arrows) (Day 65, 50 μm).

of 2 to 3 months. By 6 weeks after seeding NPCs we observed the emergence of oligodendrocyte precursor cells (OPC), as shown by the expression of NG2 (**Figure 3A** [↗](#)). NG2+ cells remained present until at least 4 months (**Figure 3B** [↗](#)/**C**). Staining for Myelin Basic Protein (MBP) revealed the emergence of MBP+ oligodendrocytes around 4 months after NPC seeding when the organoids were continuously grown in the presence of T3 (2ng/ml) (**Figure 3D** [↗](#)). At 5 months, the oligodendrocytes showed increasingly mature morphologies (**Figure 3E-I** [↗](#)) and exhibited MBP co-localization along NF200+ axons (**Figure 3F** [↗](#)/**I**).

Adherent cortical organoids show synaptic connectivity and functional activity

Neurons within adherent cortical organoids exhibited clear evidence of synaptogenesis (**Figure 4A-D** [↗](#)). Sparse labelling of excitatory neurons with AAV9.CamKII.eGFP revealed the presence of Synapsin-positive (Syn+) mushroom-shaped dendritic spines (**Figure 4E** [↗](#)). To assess the functional activity of the cortical organoids, we used the genetically-encoded calcium indicator GCaMP6s under the control of the human Synapsin promoter (**Figure 4F** [↗](#)), allowing cell-type specific quantification of neuronal activity. Calcium imaging revealed robust synchronous network-level bursting (NB) (1.4 ± 0.07 NB/min) in which the vast majority of recorded neurons participated. In addition, substantial desynchronized activity was also observed (3.9 ± 0.5 events/min) during time periods outside of network-level bursting (**Figure 4G-J** [↗](#), **Figure 4** [↗](#) – **Video 1**).

Discussion

The study of early human brain development and related diseases has long been hampered by the inherent complexity of the human brain and the inaccessibility of living brain tissue at cellular resolution. Technological advances in induced pluripotent stem cell technology have now facilitated the opportunity to obtain living human neurons derived from specific individuals.

We describe here a platform to model early human frontal cortical development with high reproducibility and simplified organization. While 3D floating organoids, or sliced organoids, nicely recapitulate layered cortex formation, they are subject to variation in the relative contribution of cortical tissue within the organoid, forming multiple cortical patches along the edges and complicating structured analysis (Eichmüller and Knoblich, 2022 [↗](#); Giandomenico et al., 2019 [↗](#); Quadrato et al., 2017 [↗](#)). Moreover, 3D floating organoids suffer from necrosis in the core of the organoid due to lack of oxygen and nutrient diffusion. Recently, other protocols have been published starting from rosette formation with a focus on very early development and leading to single structure free floating cortical organoids (Pagliaro et al., 2023 [↗](#); Tidball et al., 2023 [↗](#)). Our platform predefines a rosette-forming iPSC-derived cortical NPC population that self-organizes into adherent singular radial structures in a standard 384-well format. Other examples have highlighted the benefits of a multi-well format or systematic individual structure formation (Knight et al., 2018 [↗](#); Medda et al., 2016 [↗](#)). Our platform now integrates these features to yield individual adherent layered cortical structures with robust functional synaptic connectivity and neuronal activity and including advanced glial cell types such as myelinating oligodendrocytes and subclasses of astrocytes. The small reproducible format of the organoids in a 384-well format has the distinct advantage of being able to image entire organoids without slicing or clearing and to perform spatiotemporal functional analysis by fluorescence-based calcium imaging. We confirmed the self-organizing potential and reproducibility of adherent cortical organoids across multiple hiPSC lines and using different sources of NPCs, controlling the seeding density for the proliferation rate of the specific NPC batch. Seeding NPCs with frontal cortical identity in the defined geometry of a 384-well plate enabled the development of long-term functional neural networks in a complex radial structure resembling early human cortical development. Future

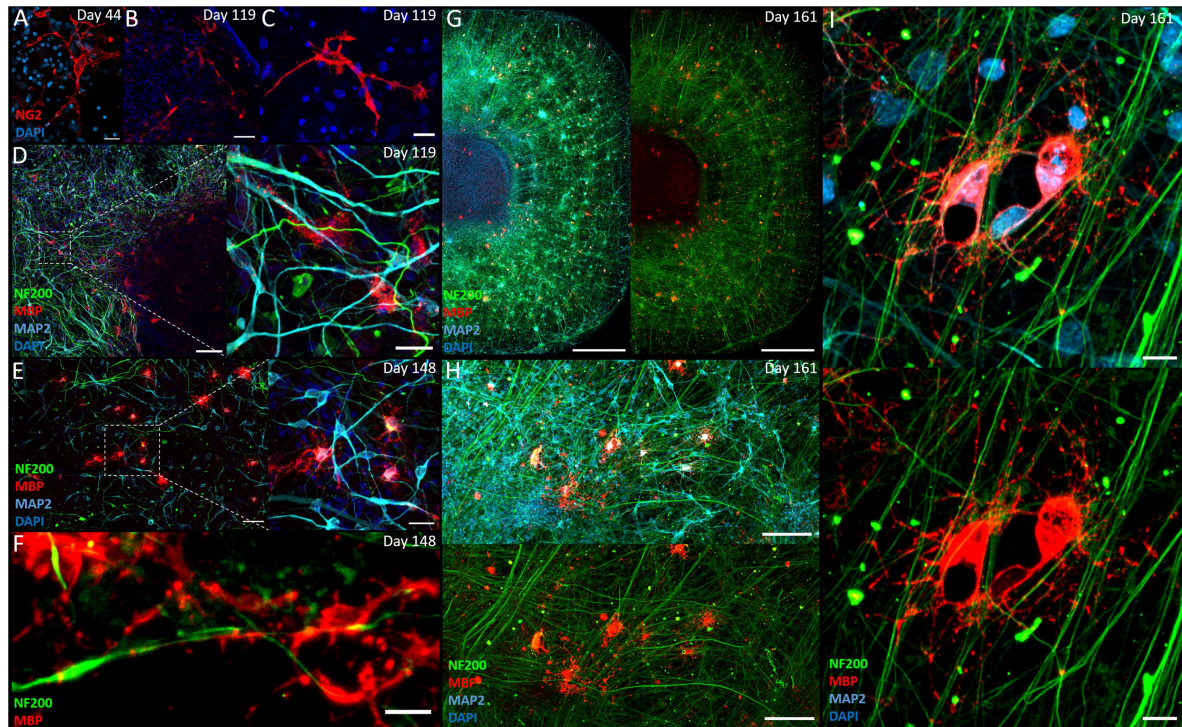


Figure 3

Adherent cortical organoids form oligodendrocyte lineage cells.

A Adherent cortical organoids show OPCs as early as 44 days, indicated by OPC marker NG2 (Day 44, 20 μ m). **B/C** The NG2+ OPCs are still present in the cortical organoids after 4 months (Day 119, B 50 μ m, C 20 μ m). **D** Young oligodendrocytes start to emerge after 4 months indicated by rudimentary MBP staining (Day 119 100 μ m and 20 μ m). **E/F** After 5 months, MBP-positive oligodendrocytes show more mature morphology and initial wrapping of NF200+ axons (Day 148; E, 50 μ m and 20 μ m, F 5 μ m). **G/H/I** Oligodendrocyte distribution at day 161 where the MBP+ oligodendrocytes sit between axons bundles and co-localize with NF200+ axons. (G/H/I Day 161, G 500 μ m, H 100 μ m, I 10 μ m)

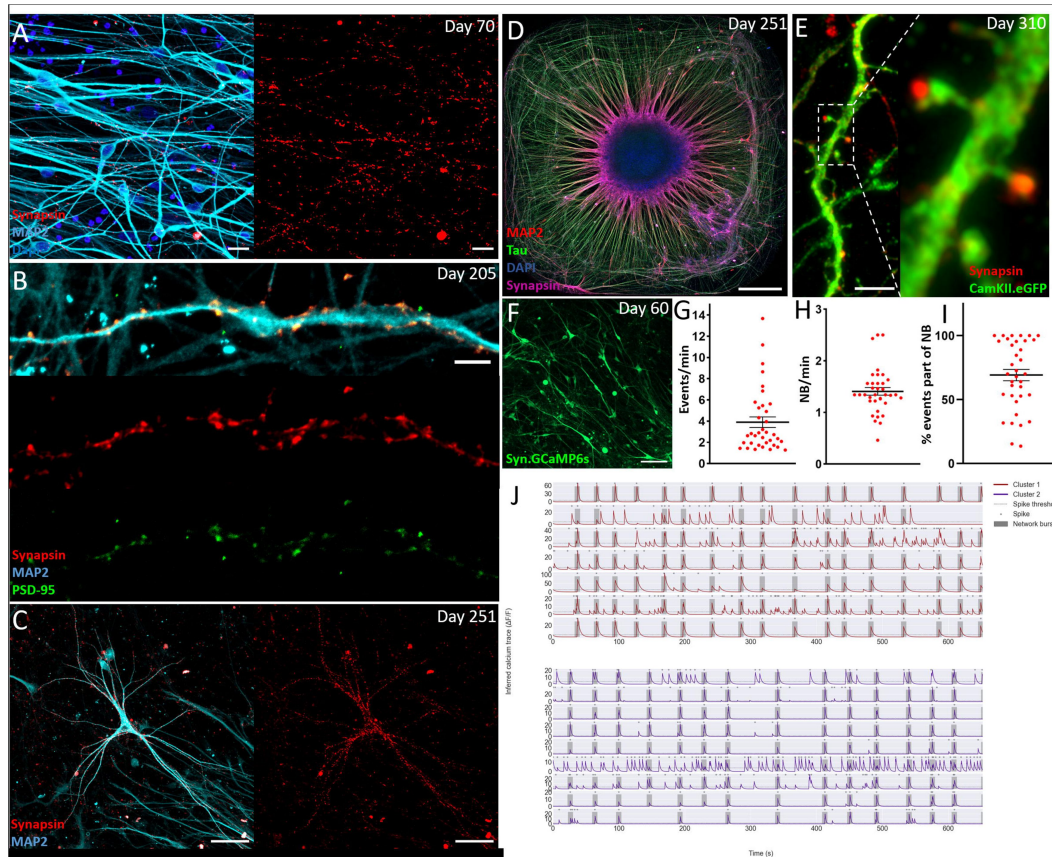


Figure 4

Adherent cortical organoids show synaptic connectivity and network bursts.

A Synapsin staining shows synapse formation along MAP2+ dendrites (Day 70, 20 μ m). **B** Co-localization of pre-synaptic marker Synapsin and post-synaptic marker PSD-95 (Day 205, 20 μ m). **C** MAP2+ dendrites and soma are decorated with Synapsin+ synapses (Day 251, 100 μ m). **D** Overview of entire well, showing alignment of Synapsin staining with MAP2 (Day 251, 500 μ m). **E** Sparse labeling of neurons with AAV9.CamKII.eGFP allows detailed imaging of glutamatergic dendritic spines with mature mushroom morphology, contacting pre-synaptic Synapsin puncta (Day 310, 5 μ m). **F** Snapshot of radially organized neurons transduced with AAV1.Syn.GCaMP6s.WPRE.SV40 (Day 60, 100 μ m). **G** Calcium events per minute from 36 cells from 7 recordings (each showing a cluster of neurons) from 2 different cortical organoids at day 61. **H** Network bursts per minute from 37 cells from 6 recordings from 2 different cortical organoids. **I** Percentage of events that are part of network bursts indicates that the majority of cells are involved in network burst activity. **J** Representative calcium traces from 2 different clusters in which cells are showing individual activity as well as network bursts where multiple cells are active simultaneously.

studies aimed at single cell gene expression analysis and advanced image-based analysis solutions in this platform will be interesting in the context of the increasing knowledge on single cell topographical, typological and temporal hierarchies in the developing human cortex (Bhaduri et al., 2021 [↗](#); Nowakowski et al., 2017 [↗](#), 2016 [↗](#); Uzquiano et al., 2022 [↗](#)).

These functional adherent cortical organoids in a multi-well format should be amenable to high-throughput screening applications, mechanistic pathophysiological studies of neurodevelopmental and neuropsychiatric disorders, and pharmacological and phenotypic screening of disease phenotypes during early cortical development. Moreover, toxicological studies for novel therapeutic compounds also show an increasing need for testing specific effects in human neuronal models, whereas studies using rodent models have often shown poor predictive power for drug safety and efficacy in human central nervous system disorders (van Esbroeck et al., 2017 [↗](#)).

Adherent cortical organoids also have some limitations, as the current iteration of adherent cortical organoids exhibits limited cortical layering and regional specification, that seem to be more advanced in floating whole brain organoids. However, these drawbacks are offset by significantly enhanced ease and higher-throughput possibilities of downstream analysis applications.

Taken together, we present a novel platform for cellular-level human brain modeling using adherent cortical organoids that exhibit high reproducibility and robust neuronal activity. The ability to reliably generate human cortical organoids in multi-well plates combined with neural network functionality offers a unique potential for brain disease modeling and therapeutic screening applications.

Materials and Methods

Generation of Neural Progenitor Cells

NPCs from 3 different source hiPSC lines were used. NPC-line 1: in house generated NPCs from human iPSC line WTC11 (Provided by Bruce R. Conklin, The Gladstone Institutes and UCSF, #GM25256, RRID:CVCL_Y803, Miyaoka et al., 2014). NPC-line 2: commercially available hNPCs from Axol Biosciences (ax0015). NPC-line 3: NPCs derived using the protocol of Shi et al., (Shi et al., 2012 [↗](#)) from hiPSC line IPSC0028 (Sigma-Aldrich, RRID:CVCL_EE38). Line 1 NPCs were generated as previously described from hiPSCs grown on a mouse embryonic fibroblast (MEF) feeder layer (Gunhanlar et al., 2018 [↗](#)). After passage 3, NPC cultures were purified using fluorescence-activated cell sorting (FACS). NPCs were detached from the culture plate using Accutase (Stem Cell Technologies) and CD184⁺/CD44⁺/CD271⁺/CD24⁺ cells (Yuan et al., 2011 [↗](#)) were collected on a FACS Aria III Cell Sorter (BD Bioscience) and expanded in NPC medium consisting of: DMEM/F12, 1% N2 supplement, 2% B27-RA supplement (Thermo Fisher Scientific), 1 µg/ml laminin (L2020, Sigma-Aldrich), 20 ng/ml basic fibroblast growth factor (Merck-Millipore, Darmstadt, Germany) and 1% penicillin/streptomycin (Thermo Fisher Scientific). NPCs were differentiated to adherent cortical organoids between passage 3 and 7 after sorting.

Neural differentiation

384-well plates (M1937-32EA, Life Technologies) were coated with 50 µg/ml laminin in dH₂O (Sigma, L2020) for 30 minutes at 37 °C. The NPCs in NPC medium were dissociated with Accutase (Stem Cell Technologies), live cells were counted with Trypan Blue (Stem Cell Technologies) in a Burkert counting chamber. The NPCs were seeded in the wells of a 384-well plate at defined densities for each cell line. Specifically, the optimal seeding density was determined by visual inspection of the organoids between 28 to 42 days after seeding a range of cell densities in the 384-well plate wells. NPCs were seeded and differentiated in Neural Differentiation Medium:

Neurobasal medium (Thermo Fisher Scientific), 1% N2 supplement (Thermo Fisher Scientific), 2% B27-RA supplement (Thermo Fisher Scientific), 1% minimum essential medium/non-essential amino acid (Stem Cell Technologies), 20 ng/ml brain-derived neurotrophic factor (ProSpec Bio), 20 ng/ml glial cell-derived neurotrophic factor (ProSpec Bio), 1 μ M dibutyl cyclic adenosine monophosphate (Sigma-Aldrich), 200 μ M ascorbic acid (Sigma-Aldrich), 2 μ g/ml laminin (Sigma-Aldrich) and 1% penicillin/streptomycin (Thermo Fisher Scientific). For oligodendrocyte maturation the cells were grown in the presence of 2 ng/ml T3 (Sigma-Aldrich). Cells were refreshed every 2-3 days.

Immunocytochemistry

For live-dead staining, living cultures were incubated with LIVE/DEAD™ Viability/Cytotoxicity Kit according to manufacturer's instructions (Thermo Fisher Scientific). For immunocytochemistry adherent cortical organoids were fixed for 20-30 minutes using 4% formaldehyde in phosphate-buffered saline (PBS), washed with PBS and blocked for 1 hour by pre-incubation in staining buffer containing 0.05 M Tris, 0.9% NaCl, 0.25% gelatin and 0.5% Triton-X-100 (pH 7.4). Primary antibodies were incubated for 48-72h at 4 °C in staining buffer, washed with PBS and incubated with the secondary antibodies in staining buffer for 2h at room temperature. The cultures were embedded in Mowiol 4-88 (Sigma-Aldrich), after which confocal imaging was performed with a Zeiss LSM700 and Zeiss LSM800 confocal microscope using ZEN software (Zeiss, Oberkochen, Germany). The following primary antibodies were used: SOX2 (Merck-Millipore AB5603, 1:200); Nestin (Merck-Millipore MAB5326, 1:200); MAP2 (Synaptic Systems 188004, 1:100); NeuN (Merck ABN78, 1:200); GFAP (Merck-Millipore AB5804, 1:300); FOXP1 (Abcam AB18259, 1:200); CUX1 (Abcam AB54583, 1:200); CTIP2 (Abcam AB18465, 1:100); Synapsin 1/2 (Synaptic Systems 106003, 1:200); and PSD95 (Thermo Fisher Scientific MA1-046, 1:100); Tau (Cell Signaling Technology 4019, 1:200); S100 β (Sigma-Aldrich S2532, 1:200); Pax6 (Santa Cruz sc-81649, 1:100); NG2 (Gift from W. Stallcup Lab, 1:100); NF200 (Sigma-Aldrich 083M4833 1:200); MBP (Abcam AB7349, 1:100); GFP (Abcam ab13970, 1:100); GAD67 (Merck-Millipore MAB5406, 1:100) DAPI (Thermo Fisher Scientific D1306). The following secondary antibodies were used 1:200: Alexa-488, Alexa-555, Alexa-647 (Jackson ImmunoResearch, West Grove, PA, USA).

Sparse labelling of excitatory neurons

pENN.AAV9.CamKII.4.eGFP.WPRE.rBG (Addgene viral prep # 105541-AAV9) was added to the cortical organoids at day 278 (1.68×10^8 GC/well). The transduced cortical organoids were fixed and stained at day 310.

Calcium imaging

For calcium imaging the genetically encoded calcium indicator AAV1.Syn.GCaMP6s.WPRE.SV40 (Penn Vector Core, 100843-AAV1) was added to the organoids at day 42 of differentiation (1.5×10^8 GC/well). Recordings were performed at day 60 on a Zeiss LSM800 confocal microscope using ZEN software (Zeiss, Oberkochen, Germany). The recordings were made with a 20x/0.8NA Ph2 Plan-Apochromat objective, with a field of view of $150 \times 100 \mu$ m and a pixel size of 0.3 μ m. The acquisition rates of the recordings were between 4-5 f.p.s. 24 hours before the recordings, the medium was switched to BrainPhys Neural Differentiation Medium. BrainPhys Neuronal Media (Stem Cell Technologies), 1% N2 supplement (Thermo Fisher Scientific), 2% B27-RA supplement (Thermo Fisher Scientific), 1% minimum essential medium/non-essential amino acid (Stem Cell Technologies), 1% penicillin/streptomycin (Thermo Fisher Scientific), 20 ng/ml brain-derived neurotrophic factor (ProSpec Bio), 20 ng/ml glial cell-derived neurotrophic factor (ProSpec Bio), 1 μ M dibutyl cyclic adenosine monophosphate (Sigma-Aldrich), 200 μ M ascorbic acid (Sigma-Aldrich) and 2 μ g/ml laminin (Sigma-Aldrich). The calcium imaging recordings were processed using CNMF-E (Pneumatikakis et al., 2016). Calcium traces were then analysed using a custom script for event and network burst detection using an algorithm written in Python (v3.8.2) (code accessibility can be requested via Github).

Cyquant Proliferation Assay

CyQUANT™ Direct Cell Proliferation Assay, C35011 (Thermo Fisher Scientific) was used according to manufacturer specifications. For each time point and each of the 3 NPC lines 10-12 wells of a 96-well plate were seeded with NPCs (2500 NPCs per well). In addition, NPC lines generated from 2 different clones from IPS line MH0159020 (Rutgers University Cell and DNA Repository) were added to increase the dynamic range of the proliferation curve. At 24h and 96h NPCs were frozen at -80°C . All NPC lines and timepoints were thawed, lysed and measured together. Doubling time was calculated between 24h and 96h. None of the wells was confluent at 96h.

Statistical analysis

All data represent mean $\pm$ SEM. When comparing developmental markers in Figure S2 we used one-way ANOVA followed by Tukey-Kramer's multiple correction test. $n=3-6$ images taken over two wells, for each time point.

Acknowledgements

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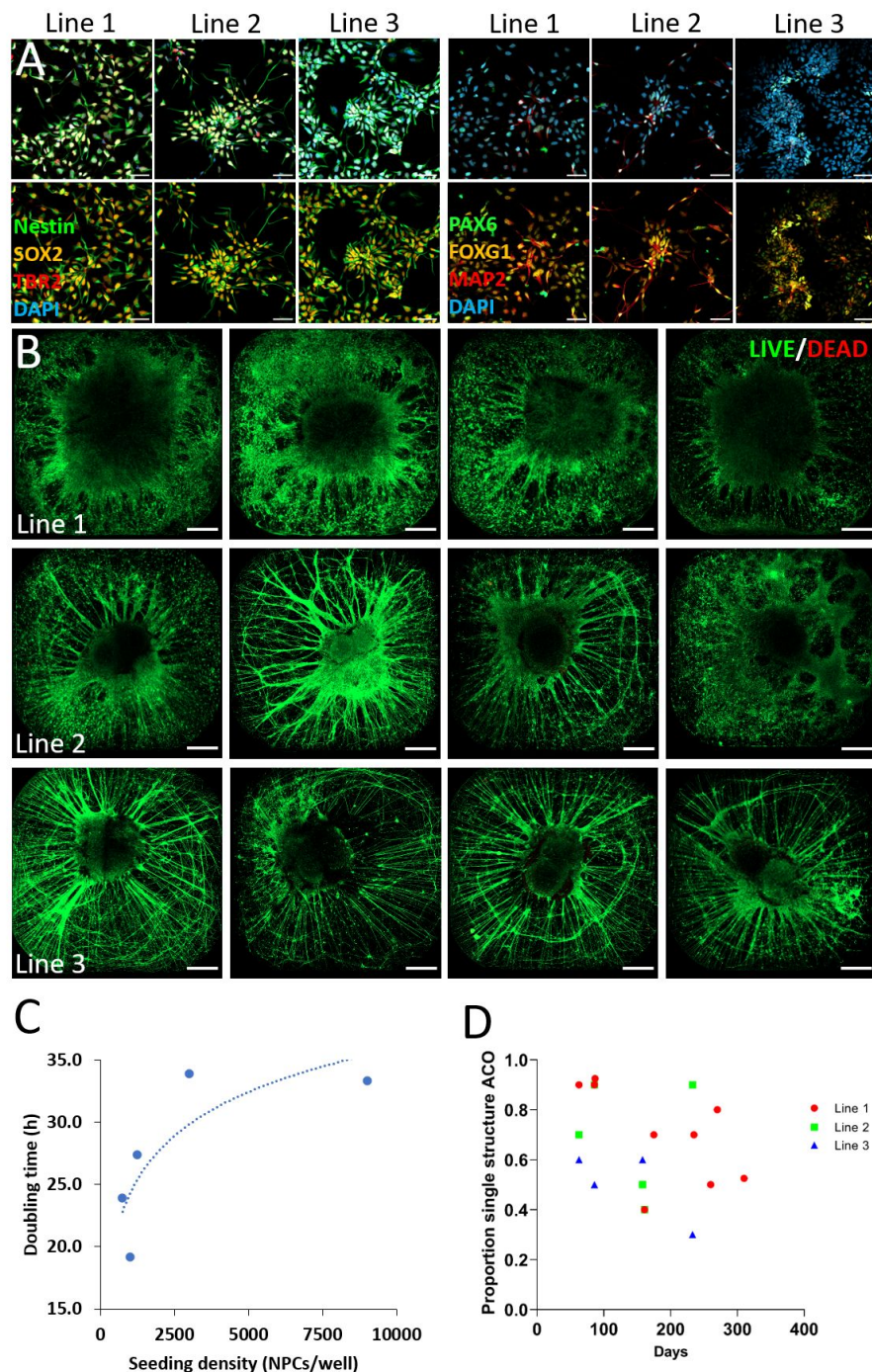
Author contributions

MvdK, SB and HS performed experiments, MU developed calcium imaging analysis algorithms, FdV and SAK supervised the work, MvdK, FdV and SAK wrote the paper.

Declaration of interests

The authors declare no competing interests.

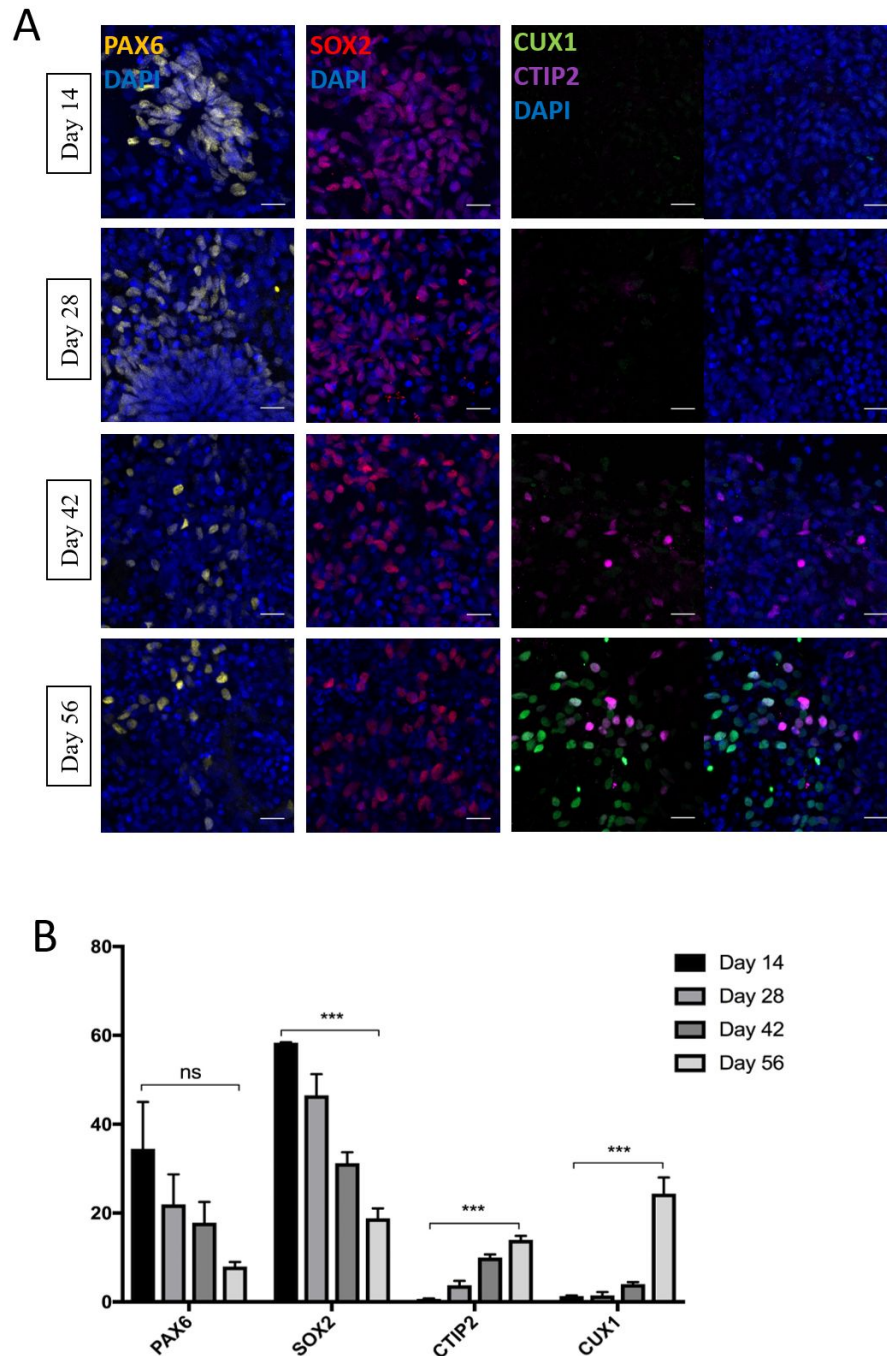
Supplementary figures



Supplementary Figure S1

Reproducibility of Neural Progenitor Cells and Adherent Cortical Organoids.

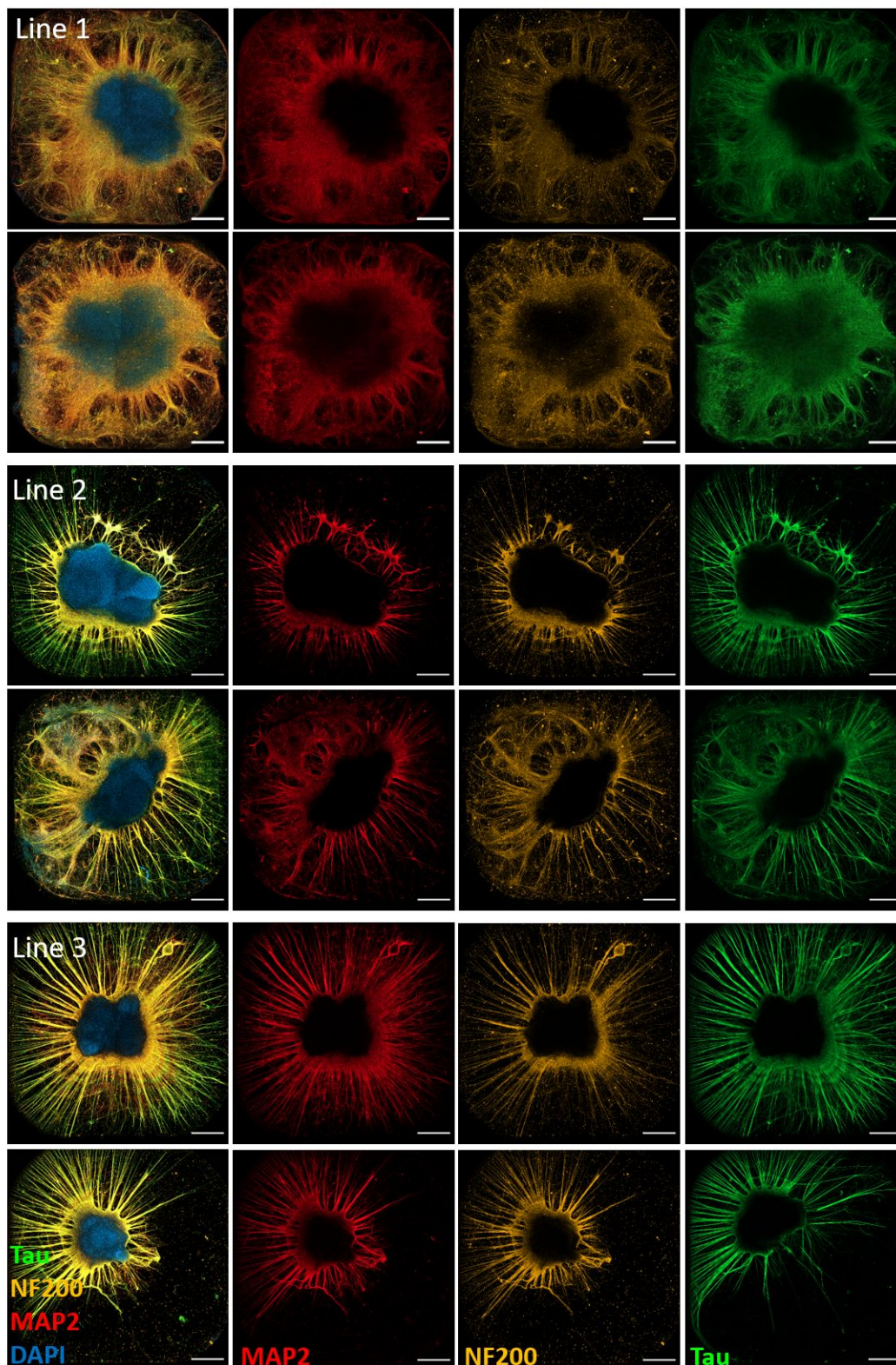
A NPCs are positive for SOX2, Nestin, FOXG1, PAX6 and TBR2 (scale bars 20 μ m) **B** LIVE/DEAD staining of adherent organoids (N=4) showing reproducibility of the structure formation. Seeding density line 1; 1250 NPCs, line 2; 750 NPCs, line 3; 1000 NPCs (scale bars 500 μ m). **C** Function of the necessary NPC seeding density to form adherent cortical organoids in relation to the doubling time as a measure of the proliferation rate of the NPCs. The doubling time of the NPCs explains more than half the variation ($r^2 = 0.67$) of the required seeding density showing that fewer cells need to be seeded for NPC lines with a higher proliferation rate. **D** Proportion of successful, single structure adherent cortical organoids. Each dot represents a different batch of adherent cortical organoids where the size of the batch ranges from 10-40 organoids.



Supplementary Figure S2

Neuronal maturation in cortical organoids is shown by a temporal shift in NPC and neuronal markers.

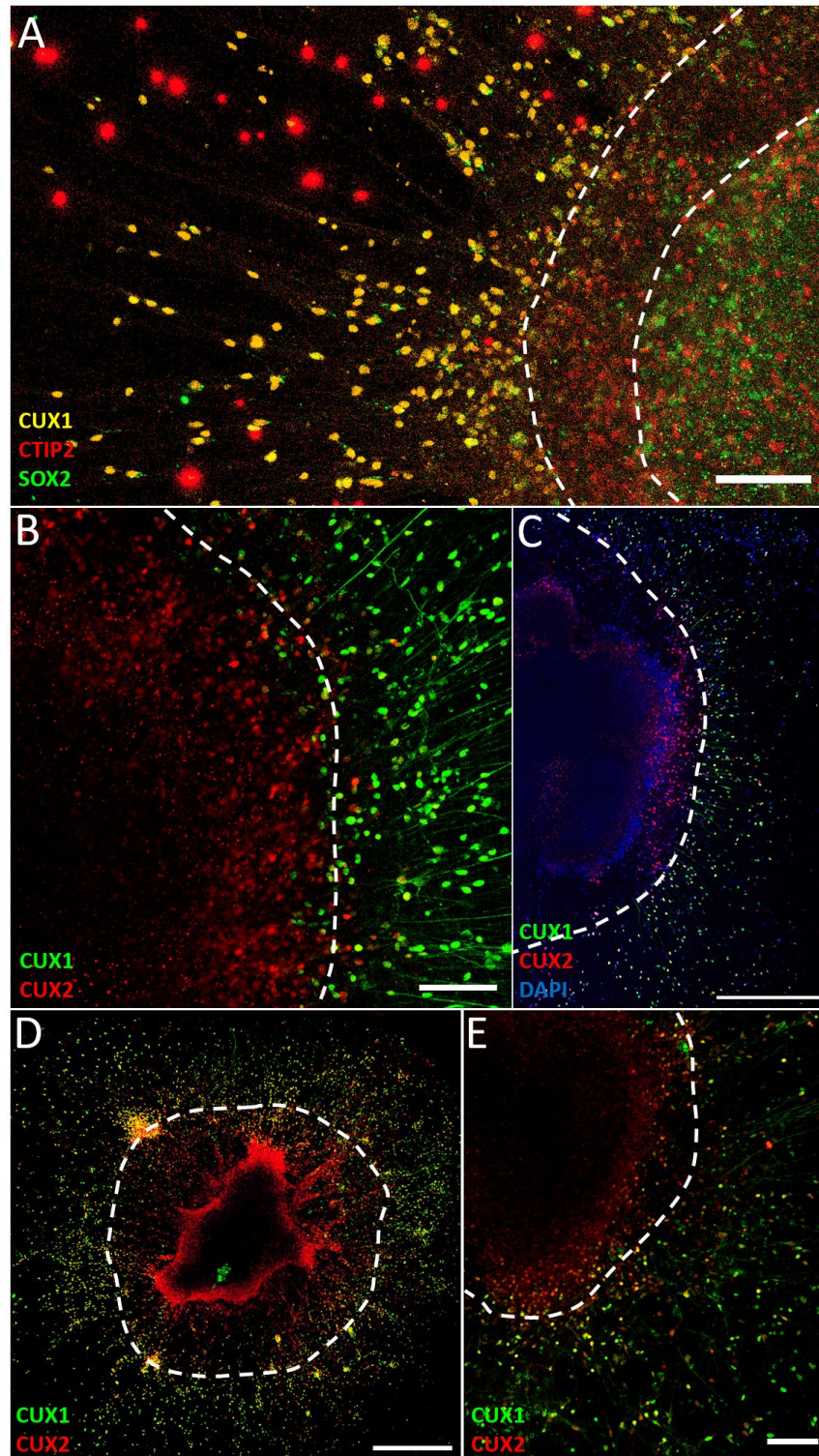
A Representative images exhibiting change in expression of NPC and neuronal markers over time. Number of cells positive for NPC markers SOX2 and PAX6 decreases over time. Deep-layer CTIP2+ neurons start appearing at day 28, while upper-layer CUX1+ positive neurons appear in higher numbers around day 56. (Scale bars 20 μ m). **B** The percentages of DAPI+ cells expressing each of the markers were quantified at four time points. Significant differences between week 2 and week 8 expression were seen for SOX2+ (down by 39.6 %), CTIP2+ (up by 13.5 %) and CUX1+ cells (up by 23.1 %). *** $p < 0.0001$, ANOVA one-way followed by Tukey-Kramer's multiple correction test. Error bars indicate SEM, $n=3-6$ images taken over two wells, for each time point.



Supplementary Figure S3

Distribution of axons and dendrites in adherent cortical organoids.

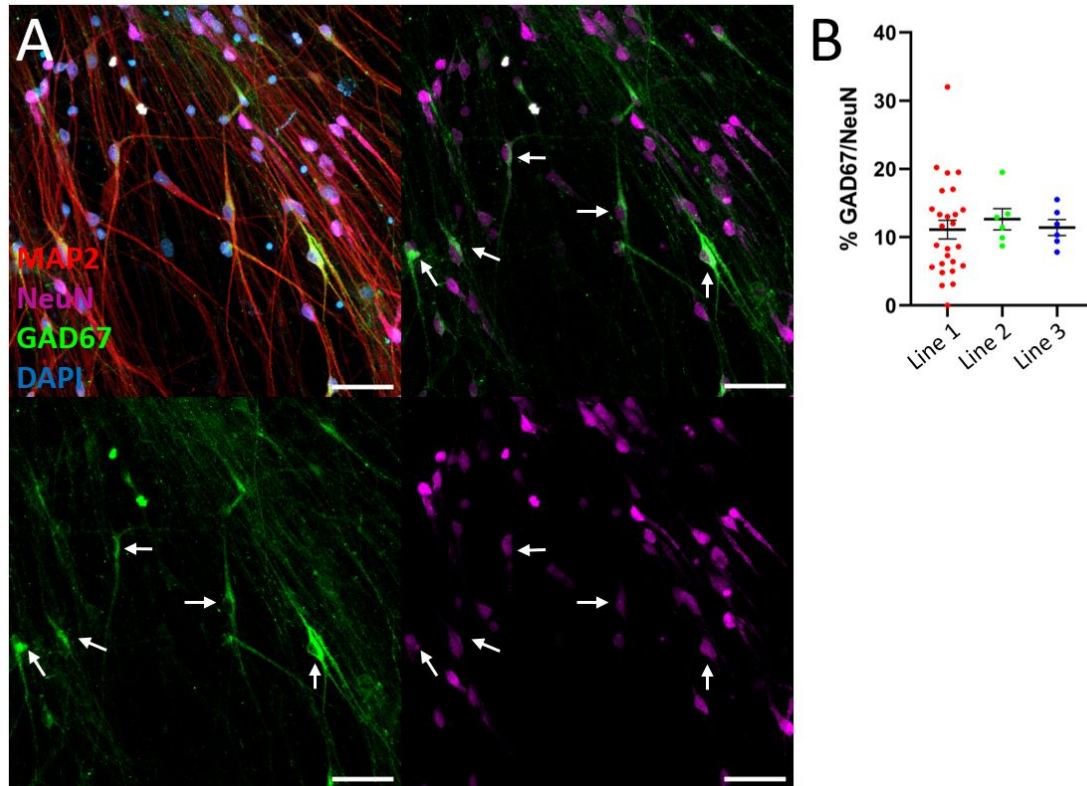
Radially organized MAP2+/Tau+ dendrites along with both radially and circumferentially organized NF200+/Tau+ axons in duplicate for each cell line (Day 63, scale bars 500 μ m)



Supplementary Figure S4

Cortical layering in the adherent cortical organoids.

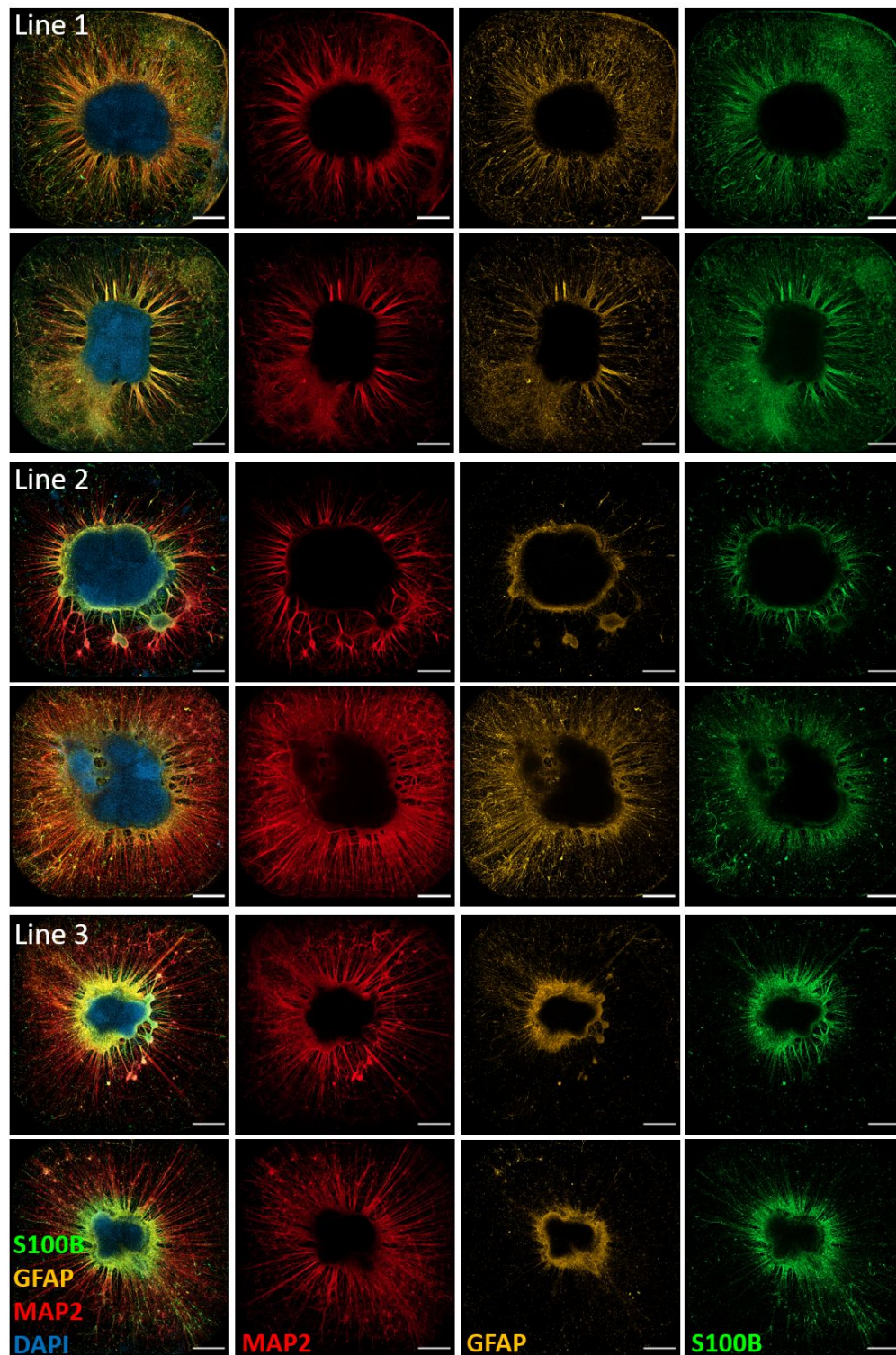
A Rudimentary separation of SOX2+ NPCs, CTIP2+ deep layer neurons and CUX1+ upper layer neurons (Day 67, scale bar 100 µm) **B-E** Separation of CUX1 and CUX2 expression (B day 70, scale bar 100 µm; C day 70, scale bar 500 µm; D day 67, scale bar 500 µm; E day 67, scale bar 100 µm)



Supplementary Figure S5

GAD67+ interneurons make up a small proportion of NeuN+ neurons in the adherent cortical organoids.

A Example image of GAD67+ interneuron proportion of NeuN+ neurons (Day 67, 50 μ m). **B** Quantification of GAD67+ proportion of NeuN+ neurons. Every point is the percentage of GAD67+ interneurons out of all NeuN+ neurons in a spatially randomized selected image with on average 130.6 ($\pm$ 16.1) NeuN+ nuclei per frame. (Line 1: N= 26 images of 6 different cortical organoids at day 65-67; Line 2/3: 6 images of 2 different organoids at day 65).



Supplementary Figure S6

Astrocyte distribution within Adherent Cortical Organoids.

The abundantly present GFAP+ and S100B+ astrocytes have a comparable distribution with the MAP2+ neurons in the adherent cortical organoids. The astrocytes show radial patterns with many somas located in the centre as well as a large presence outside the centre (Day 63, scale bars 500 μ m).

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Reviewer #1 (Public Review):

Summary:

Kroeg et al. describe a novel method for 2D culture human induced pluripotent stem cells (hiPSCs) to form cortical tissue in a multiwell format. The method claims to offer a significant advancement over existing developmental models. Their approach allows them to generate cultures with precise, reproducible dimensions and structure with a single rosette; consistent geometry; incorporating multiple neuronal and glial cell types (cellular diversity); avoiding the necrotic core (often seen in free-floating models due to limited nutrient and oxygen diffusion). The researchers demonstrate the method's capacity for long-term culture, exceeding ten months, and show the formation of mature dendritic spines and considerable neuronal activity. The method aims to tackle multiple key problems of in vitro neural cultures: reproducibility, diversity, topological consistency, and electrophysiological activity. The authors suggest their potential in high-throughput screening and neurotoxicological studies.

Strengths:

The main advances in the paper seem to be: The culture developed by the authors appears to have optimal conditions for neural differentiation, lineage diversification, and long-term culture beyond 300 days. These seem to me as a major strength of the paper and an important contribution to the field. The authors present solid evidence about the high cell type diversity present in their cultures. It is a major point and therefore it could be better compared to the state of the art. I commend the authors for using three different IPS lines, this is a very important part of their proof. The staining and imaging quality of the manuscript is of excellent quality.

Weaknesses:

(1) The title is misleading: The presented cultures appear not to be organoids, but 2D neural cultures, with an insufficiently described intermediate EB stage. For nomenclature, see: doi: 10.1038/s41586-022-05219-6. Should the tissue develop considerable 3D depth, it would suffer from the same limited nutrient supply as 3D models - as the authors point out in their introduction.

(2) The method therefore should be compared to state-of-the-art (well-based or not) 2D cultures, which seems to be somewhat overlooked in the paper, therefore making it hard to assess what the advance is that is presented by this work.

(3) Reproducibility is prominently claimed throughout the manuscript. However, it is challenging to assess this claim based on the data presented, which mostly contain single frames of unquantified, high-resolution images. There are almost no systematic quantifications presented. The ones present (Figure S1D, Figure 4) show very large variability. However, the authors show sets of images across wells (Figure S1B, Figure S3) which hint that in some important aspects, the culture seems reproducible and robust.

(4) What is in the middle? All images show markers in cells present around the center. The center however seems to be a dense lump of cells based on DAPI staining. What is the identity of these cells? Do these cells persist throughout the protocol? Do they divide? Until when? Addressing this prominent cell population is currently lacking.

(5) This manuscript proposes a new method of 2D neural culture. However, the description and representation of the method are currently insufficient.

(a) The results section would benefit from a clear and concise, but step-by-step overview of the protocol. The current description refers to an earlier paper and appears to skip over some key steps. This section would benefit from being completely rewritten. This is not a replacement for a clear methods section, but a section that allows readers to clearly interpret results presented later.

(b) Along the same lines, the graphical abstract should be much more detailed. It should contain the time frames and the media used at the different stages of the protocol, seeding numbers, etc.

<https://doi.org/10.7554/eLife.98340.1.sa2>

Reviewer #2 (Public Review):

Summary:

In this manuscript, van der Kroeg et al have developed a method for creating 3D cortical organoids using iPSC-derived neural progenitor cells in 384-well plates, thus scaling down the neural organoids to adherent culture and a smaller format that is amenable to high throughput cultivation. These adherent cortical organoids, measuring 3 x 3 x 0.2 mm, self-organize over eight weeks and include multiple neuronal subtypes, astrocytes, and oligodendrocyte lineage cells.

Strengths:

(1) The organoids can be cultured for up to 10 months, exhibiting mature dendritic spines, axonal myelination, and robust neuronal activity.

(2) Unlike free-floating organoids, these do not develop necrotic cores, making them ideal for high-throughput drug discovery, neurotoxicological screening, and brain disorder studies.

(3) The method addresses the technical challenge of achieving higher-order neural complexity with reduced heterogeneity and the issue of necrosis in larger organoids. The method presents a technical advance in organoid culture.

(4) The method has been demonstrated with multiple cell lines which is a strength.

(5) The manuscript provides high-quality immunostaining for multiple markers.

Weaknesses:

(1) Direct head-to-head comparison with standard organoid culture seems to be missing and may be valuable for benchmarking, ie what can be done with the new method that cannot be

done with standard culture and vice versa, ie what are the aspects in which new method could be inferior to the standard.

(2) It would be important to further benchmark the throughput, ie what is the success rate in filling and successfully growing the organoids in the entire 384 well plate?

(3) For each NPC line an optimal seeding density was estimated based on the proliferation rate of that NPC line and via visual observation after 6 weeks of culture. It would be important to delineate this protocol in more robust terms, in order to enable reproducibility with different cell lines and amongst the labs.

<https://doi.org/10.7554/eLife.98340.1.sa1>

Reviewer #3 (Public Review):

Summary:

Kroeg et al. have introduced a novel method to produce 3D cortical layer formation in hiPSC-derived models, revealing a remarkably consistent topography within compact dimensions. This technique involves seeding frontal cortex-patterned iPSC-derived neural progenitor cells in 384-well plates, triggering the spontaneous assembly of adherent cortical organoids consisting of various neuronal subtypes, astrocytes, and oligodendrocyte lineage cells.

Strengths:

Compared to existing brain organoid models, these adherent cortical organoids demonstrate enhanced reproducibility and cell viability during prolonged culture, thereby providing versatile opportunities for high-throughput drug discovery, neurotoxicological screening, and the investigation of brain disorder pathophysiology. This is an important and timely issue that needs to be addressed to improve the current brain organoid systems.

Weaknesses:

While the authors have provided significant data supporting this claim, several aspects necessitate further characterization and clarification. Mainly, highlighting the consistency of differentiation across different cell lines and standardizing functional outputs are crucial elements to emphasize the future broad potential of this new organoid system for large-scale pharmacological screening.

<https://doi.org/10.7554/eLife.98340.1.sa0>

Author response:

Public Reviews:

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Summary:

Kroeg et al. describe a novel method for 2D culture human induced pluripotent stem cells (hiPSCs) to form cortical tissue in a multiwell format. The method claims to offer a significant advancement over existing developmental models. Their approach allows them to generate cultures with precise, reproducible dimensions and structure with a single rosette; consistent geometry; incorporating multiple neuronal and glial cell types (cellular diversity); avoiding the necrotic core (often seen in free-floating models due to limited nutrient and oxygen diffusion). The researchers demonstrate the method's

capacity for long-term culture, exceeding ten months, and show the formation of mature dendritic spines and considerable neuronal activity. The method aims to tackle multiple key problems of in vitro neural cultures: reproducibility, diversity, topological consistency, and electrophysiological activity. The authors suggest their potential in high-throughput screening and neurotoxicological studies.

Strengths:

The main advances in the paper seem to be: The culture developed by the authors appears to have optimal conditions for neural differentiation, lineage diversification, and long-term culture beyond 300 days. These seem to me as a major strength of the paper and an important contribution to the field. The authors present solid evidence about the high cell type diversity present in their cultures. It is a major point and therefore it could be better compared to the state of the art. I commend the authors for using three different IPS lines, this is a very important part of their proof. The staining and imaging quality of the manuscript is of excellent quality.

We thank the reviewer for the positive comments on the potential of our novel platform to address key problems of in vitro neural culture, highlighting the longevity and reproducibility of the method across multiple cell lines.

Weaknesses:

(1) The title is misleading: The presented cultures appear not to be organoids, but 2D neural cultures, with an insufficiently described intermediate EB stage. For nomenclature, see: doi: 10.1038/s41586-022-05219-6. Should the tissue develop considerable 3D depth, it would suffer from the same limited nutrient supply as 3D models - as the authors point out in their introduction.

We appreciate the opportunity to clarify this point. We respectfully disagree that the cultures do not meet the consensus definition of an organoid. In fact, a direct quote from the seminal nomenclature paper referenced by the reviewer states: “We define organoids as in vitro-generated cellular systems that emerge by self-organization, include multiple cell types, and exhibit some cytoarchitectural and functional features reminiscent of an organ or organ region. Organoids can be generated as 3D cultures or by a combination of 3D and 2D approaches (also known as 2.5D) that can develop and mature over long periods of time (months to years).” (Pasca et al, 2022 doi10.1038/s41586-022-05219-6). Therefore, while many organoid types indeed have a more spherical or globular 3D shape, the term organoid also applies to semi-3D or non-globular adherent organoids, such as renal (Czerniecki et al 2018, doi.org/10.1016/j.stem.2018.04.022) and gastrointestinal organoids (Kakni et al 2022, doi.org/10.1016/j.tibtech.2022.01.006). Accordingly, the adherent cortical organoids described in the manuscript exhibit self-organization to single radial structures consisting of multiple cell layers in the z-axis, reaching ~200um thickness (therefore remaining within the limits for sufficient nutrient supply), with consistent cytoarchitectural topology and electrophysiological activity, and therefore meet the consensus definition of an organoid.

(2) The method therefore should be compared to state-of-the-art (well-based or not) 2D cultures, which seems to be somewhat overlooked in the paper, therefore making it hard to assess what the advance is that is presented by this work.

It was not our intention to benchmark this model quantitatively against other culture systems. Rather, we have attempted to characterize the opportunities and limitations of this approach, with a qualitative contrast to other culture methods. Compared to state-of-the-art 2D neural network cultures, adherent cortical organoids provide distinct advantages in:

(1) Higher order self-organized structure formation, including segregation of deeper and upper cortical layers.

(2) Longevity: adherent cortical organoids can be successfully kept in culture up to 1 year where 2D cultures typically deteriorate after 8-12 weeks.

(3) Maturity, including the formation of dendritic mushroom spines and robust electrophysiological activity.

(4) Cell type diversity including a more physiological ratio of inhibitory and excitatory neurons (10% GAD67+/NeuN+ neurons in adherent cortical organoids, vs 1% in 2D neural networks) and the emergence of oligodendrocyte lineage cells.

On the other hand, limitations of adherent cortical organoids compared to 2D neural network cultures are:

(1) Culture times for organoids are much longer than for 2D cultures and the method can therefore be more laborious and more expensive.

(2) Whole cell patch clamping is not easily feasible in the organoids because of the restricting dimensions of the 384well plates.

(3) Reproducibility is prominently claimed throughout the manuscript. However, it is challenging to assess this claim based on the data presented, which mostly contain single frames of unquantified, high-resolution images. There are almost no systematic quantifications presented. The ones present (Figure S1D, Figure 4) show very large variability. However, the authors show sets of images across wells (Figure S1B, Figure S3) which hint that in some important aspects, the culture seems reproducible and robust.

We made considerable efforts to establish quantitative metrics to assess reproducibility. We applied a quantitative scoring system of single radial structures at different time points for multiple batches of all three lines as indicated in Figure S1D. This figure represents a comprehensive dataset in which each dot represents the average of a different batch of organoids containing 10-40 organoids per batch. To emphasize this, we will adapt the graph to better reflect the breadth of the dataset. Additional quantifications are given in Figure S2 for progenitor and layer markers for Line 1 and in Figure S5 for interneurons across all three lines, showing relatively low variability. That being said, we acknowledge the reviewer's concerns and will modify the text to reduce the emphasis of this point, pending more extensive data addressing reproducibility across a wide range of parameters.

(4) What is in the middle? All images show markers in cells present around the center. The center however seems to be a dense lump of cells based on DAPI staining. What is the identity of these cells? Do these cells persist throughout the protocol? Do they divide? Until when? Addressing this prominent cell population is currently lacking.

A more comprehensive characterization of the cells in the center remains a significant challenge due to the high cell density hindering antibody penetration. However, dye-based staining methods such as DAPI and the LIVE/DEAD panel confirm a predominance of intact nuclei with very minimal cell death. The limited available data suggest that a substantial proportion of the cells in the center are proliferative neural progenitors, indicated by immunolabeling for SOX2 and Ki67. We will add additional figures to support these findings. Furthermore, we are currently optimizing the conditions to perform single cell / nuclear RNA sequencing to further characterize the cellular composition of the organoids.

(5) *This manuscript proposes a new method of 2D neural culture. However, the description and representation of the method are currently insufficient.*
 (a) *The results section would benefit from a clear and concise, but step-by-step overview of the protocol. The current description refers to an earlier paper and appears to skip over some key steps. This section would benefit from being completely rewritten. This is not a replacement for a clear methods section, but a section that allows readers to clearly interpret results presented later.*

We will revise the manuscript to include a more detailed step-by-step overview of the protocol.

(b) *Along the same lines, the graphical abstract should be much more detailed. It should contain the time frames and the media used at the different stages of the protocol, seeding numbers, etc.*

As suggested, we will also adapt the graphical abstract to include more detail.

Reviewer #2 (Public Review):

Summary:

In this manuscript, van der Kroeg et al have developed a method for creating 3D cortical organoids using iPSC-derived neural progenitor cells in 384-well plates, thus scaling down the neural organoids to adherent culture and a smaller format that is amenable to high throughput cultivation. These adherent cortical organoids, measuring 3 x 3 x 0.2 mm, self-organize over eight weeks and include multiple neuronal subtypes, astrocytes, and oligodendrocyte lineage cells.

Strengths:

- (1) The organoids can be cultured for up to 10 months, exhibiting mature dendritic spines, axonal myelination, and robust neuronal activity.*
- (2) Unlike free-floating organoids, these do not develop necrotic cores, making them ideal for high-throughput drug discovery, neurotoxicological screening, and brain disorder studies.*
- (3) The method addresses the technical challenge of achieving higher-order neural complexity with reduced heterogeneity and the issue of necrosis in larger organoids. The method presents a technical advance in organoid culture.*
- (4) The method has been demonstrated with multiple cell lines which is a strength.*
- (5) The manuscript provides high-quality immunostaining for multiple markers.*

We appreciate the reviewer's acknowledgement of the strengths of this novel platform as a technical advance in organoid culture that reduces heterogeneity and shows potential for higher throughput experiments.

Weaknesses:

- (1) Direct head-to-head comparison with standard organoid culture seems to be missing and may be valuable for benchmarking, ie what can be done with the new method that cannot be done with standard culture and vice versa, ie what are the aspects in which new method could be inferior to the standard.*

In our opinion, it would be extremely difficult to directly compare methods because of substantial differences. Most notably, whole brain organoids grow to large and irregular globular shapes, while adherent cortical organoids have a highly standardized shape confined by the limits of a 384-well. Moreover, it was not our intention to benchmark this model quantitatively against other culture systems. Rather, we have attempted to characterize the opportunities and limitations of this approach, with a qualitative contrast to other culture methods.

(2) It would be important to further benchmark the throughput, ie what is the success rate in filling and successfully growing the organoids in the entire 384 well plate?

Figure S1D shows the success rate of organoid formation and stability of the organoid structures over time. In addition, we will add the number of wells that were filled per plate.

(3) For each NPC line an optimal seeding density was estimated based on the proliferation rate of that NPC line and via visual observation after 6 weeks of culture. It would be important to delineate this protocol in more robust terms, in order to enable reproducibility with different cell lines and amongst the labs.

Figure S1C provides the relationship between proliferation rate and seeding density, allowing estimation of seeding densities based on the proliferation rate of the NPCs. However, we appreciate the reviewers feedback and will modify the methods to provide more detail.

Reviewer #3 (Public Review):

Summary:

Kroeg et al. have introduced a novel method to produce 3D cortical layer formation in hiPSC-derived models, revealing a remarkably consistent topography within compact dimensions. This technique involves seeding frontal cortex-patterned iPSC-derived neural progenitor cells in 384-well plates, triggering the spontaneous assembly of adherent cortical organoids consisting of various neuronal subtypes, astrocytes, and oligodendrocyte lineage cells.

Strengths:

Compared to existing brain organoid models, these adherent cortical organoids demonstrate enhanced reproducibility and cell viability during prolonged culture, thereby providing versatile opportunities for high-throughput drug discovery, neurotoxicological screening, and the investigation of brain disorder pathophysiology. This is an important and timely issue that needs to be addressed to improve the current brain organoid systems.

We thank the reviewer for highlighting the strengths of our novel platform. We appreciate that all three reviewers agree that the adherent cortical organoids presented in this manuscript reliably demonstrate increased reproducibility and longevity. They also commend its potential for higher throughput drug discovery and neurotoxicological/phenotype screening purposes.

Weaknesses:

While the authors have provided significant data supporting this claim, several aspects necessitate further characterization and clarification. Mainly, highlighting the consistency of differentiation across different cell lines and standardizing functional

outputs are crucial elements to emphasize the future broad potential of this new organoid system for large-scale pharmacological screening.

We appreciate the feedback and will add more detail on consistency and standardization of functional outputs.

<https://doi.org/10.7554/eLife.98340.1.sa4>