organoids

MDPI

Article

Defined, Simplified, Scalable, and Clinically Compatible Hydrogel-Based Production of Human Brain Organoids

Eva Tomaskovic-Crook [1,2,3,4,*], Sarah Liza Higginbottom [1,2], Binbin Zhang [1], Justin Bourke [1,5], Gordon George Wallace [1] and Jeremy Micah Crook [1,2,3,4,*]

1 ARC Centre of Excellence for Electromaterials Science, Intelligent Polymer Research Institute, AIIM Facility, Innovation Campus, University of Wollongong, Squires Way, Fairy Meadow, NSW 2519, Australia
2 Arto Hardy Family Biomedical Innovation Hub, Chris O'Brien Lifehouse, Camperdown, NSW 2050, Australia
3 School of Medical Sciences, Faculty of Medicine and Health, The University of Sydney, Camperdown, NSW 2006, Australia
4 Illawarra Health and Medical Research Institute, University of Wollongong, Wollongong, NSW 2522, Australia
5 Ion Channels and Human Diseases Laboratory, Florey Institute for Neuroscience and Mental Health, Parkville, VIC 3052, Australia
* Correspondence: evatc@uow.edu.au (E.T.-C.); jcrook@uow.edu.au (J.M.C.)

Abstract: Human brain organoids present a new paradigm for modeling human brain organogenesis, providing unprecedented insight to the molecular and cellular processes of brain development and maturation. Other potential applications include in vitro models of disease and tissue trauma, as well as three-dimensional (3D) clinically relevant tissues for pharmaceuticals development and cell or tissue replacement. A key requirement for this emerging technology in both research and medicine is the simple, scalable, and reproducible generation of organoids using reliable, economical, and high-throughput culture platforms. Here we describe such a platform using a defined, clinically compliant, and readily available hydrogel generated from gelatin methacrylate (GelMA). We demonstrate the efficient production of organoids on GelMA from human induced pluripotent stem cells (iPSCs), with scalable production attained using 3D printed GelMA-based multiwell arrays. The differentiation of iPSCs was systematic, rapid, and direct to enable iPSCs to form organoids in their original position following seeding on GelMA, thereby avoiding further cell and organoid disruption. Early neural precursors formed by day 5, neural rosettes and early-stage neurons by day 14, and organoids with cellular and regional heterogeneity, including mature and electrophysiologically active neurons, by day 28. The optimised method provides a simplified and well-defined platform for both research and translation of iPSCs and derivative brain organoids, enabling reliable 3D in vitro modelling and experimentation, as well as the provision of clinically relevant cells and tissues for future therapeutics.

Keywords: brain organoid; hydrogel; gelatin methacrylate; GelMA; induced pluripotent stem cells; clinically compatible; scalable; 3D printing

Citation: Tomaskovic-Crook, E.; Higginbottom, S.L.; Zhang, B.; Bourke, J.; Wallace, G.G.; Crook, J.M. Defined, Simplified, Scalable, and Clinically Compatible Hydrogel-Based Production of Human Brain Organoids. *Organoids* **2023**, *2*, 20–36. https://doi.org/10.3390/organoids2010002

Academic Editors: Süleyman Ergün, Stefan Liebau and Bahram Parvin

Received: 19 September 2022
Revised: 28 November 2022
Accepted: 5 January 2023
Published: 11 January 2023

1. Introduction

Human brain organoids are artificial, self-assembling, micro-tissue constructs reminiscent of the immature brain. They are produced from human pluripotent stem cells that self-pattern into complex neural tissues with defined compartments [1,2]. As such, they offer significant potential for studying the molecular and cellular processes underlying fundamental aspects of brain development and maturation, including normal function and dysfunction. Other applications could include in vitro models for pharmaceutical screens and cell or tissue replacement. Importantly, depending on the biological processes being studied, as human tissue systems, brain organoids may complement or replace traditional animal models, being especially important for researching processes that are specific to the human brain.

There is a pressing need to develop organoid culture platforms that are uncomplicated, defined, reliable, scalable, economical, and standardized, ideally with qualified reagents, materials, and procedures that are amendable to current good laboratory and manufacturing practices (cGLPs and cGMPs respectively). Such systems will complement pre-existing methods and facilitate quality assured research and regulatory approval towards clinical compliance.

Chemically defined, tunable, degradable, and biocompatible hydrogels are increasingly being used for cell support and delivery in basic research and biomedical applications, such as drug discovery, toxicology screens, and regenerative medicine [3]. Whether integrating them into existing cell culture platforms, or using them to develop new and more translational methods, they serve to mimic the physical and biochemical characteristics of native extracellular matrices (ECM) and provide opportunities to better analyze the biology and control the behaviour of cells in vitro and in vivo. Hydrogel-based growth substrates have been incorporated to conventional 2D planar culture systems and featured with 3D bioprinting, for more defined and clinically-compliant support, and differentiation of human pluripotent stem cells (PSCs) [4,5], although typically human PSCs (including both embryonic stem cells or iPSCs) are cultured and differentiated using fibroblast feeder cells [6], and/or feeder-free with undefined tumour-derived Matrigel™ basement membrane preparation [7]. However, although enabling robust cell culture with chemically defined medium such as mTeSR™1 [8], and while Matrigel™ remains one of the most widely used substrates for human PSC support and differentiation, feeder-based systems are unnecessarily complex and Matrigel™ comprises undefined components, being derived from the Engelbreth-Holm-Swarm (EHS) mouse sarcoma. As such, both are problematic for regulatory approval, with the latter manifestly unsuitable for clinical use [9].

To overcome the shortcomings of the aforementioned approaches, biocompatible hydrogels can be used for developing simpler, cost-effective, defined, and clinically compliant platforms for PSC culture and differentiation, with the latter predictably extending to organoids. Here, we report using GelMA hydrogel to culture and differentiate human iPSCs to neuronal lineage and brain organoids (Figure 1). GelMA is an inexpensive and easy to handle derivative of collagen that comprises both natural cell-binding motifs, including RGD (arginine-glycine-aspartate; principal integrin-binding domain present within ECM) and matrix metalloproteinase (MMP)-sensitive degradation sites (for proteolytic breakdown and cell migration), and various amino acid side chain functionalities for covalent modification [10]. Additionally, GelMA remains stable at 37 °C, which is vital for tissue engineering. Organoids reproducibly displayed dorsal forebrain identity and characteristic cortical tissue architecture, with heterogeneous masses of densely packed cell soma, prolific neurite formation, functional neuronal subtypes, and cells that arranged to form tuboid structures. Finally, scalable production of organoids was attained with 3D printed GelMA-based multiwell arrays. Our GelMA-based culture system represents a robust and useful approach for generating brain organoids with high fidelity for research and translational application.

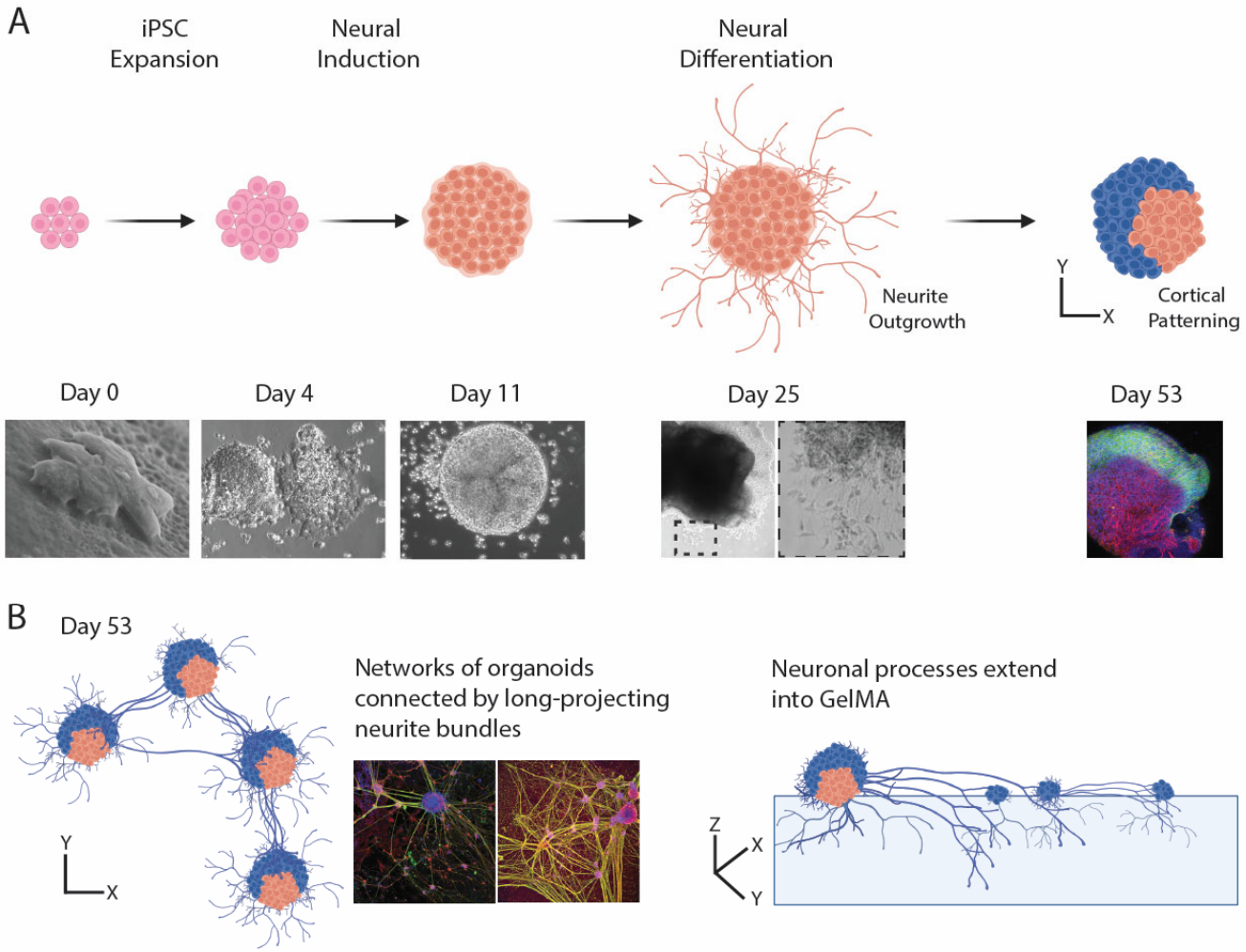

Figure 1. GelMa-based iPSC differentiation to human brain organoids. (**A**): Timeline illustrating the major stages for producing brain organoids from iPSCs on GelMA, including iPSC expansion, neural induction, and neural differentiation. (**B**): Networks of organoids can be propagated on GelMA hydrogel, connected via long-projecting neurite bundles, with neurite processes extending into the GelMA.

2. Materials and Methods

2.1. GelMA Synthesis

GelMA was synthesised as described previously [10]. Alternatively, ready-made GelMA can be purchased from Sigma-Aldrich (900496). Briefly, 10 g of Gelatin (from porcine skin, gel strength ~300 g bloom, type A; Sigma-Aldrich, St. Louis, MO, USA) was dissolved in 100 mL autoclaved Dulbecco's Phosphate Buffered Saline (DPBS, pH 7.4; Sigma-Aldrich, D8537) at 50 °C and stirred until fully dissolved (~3 h). A total of 8 mL methacrylic anhydride (Sigma-Aldrich, 64100 or 276685) was added gradually while the pH was maintained at 4.4 by dosing with alkaline solution using a pH-controlled dosing pump. The reaction was allowed to proceed for 3 h at 50 °C, before dilution with 300 mL DPBS. The solution was dialysed in distilled water (dH_2O; molecular weight cut-off: 12–14 kDa) at 40 °C for 7 days. After purification, the GelMA solution (a clear, colourless viscous liquid) was subjected to freeze drying, resulting in a bright white product with 65–70% yield. The degree of functionalization (73%) was calculated by Proton Nuclear Magnetic Resonance Spectroscopy in deuterium oxide. The freeze-dried product was stored at 4 °C in a dark and inert environment, until further use.

2.2. Preparation of GelMA Hydrogel Discs

GelMA hydrogel discs for conventional iPSC and organoid plate-based culture were made from pre-polymerised GelMA solution (5–10%; *w*/*v*), freshly prepared from 0.5–1 g freeze dried GelMA by dissolving in 9.5 mL DPBS at 37 °C, followed by the addition of 50 mg of the photoinitiator 2-hydroxy-1-(4-(hydroxyethoxy)phenyl)-2-methyl-1-propanone (Irgacure 2959, CIBA Chemicals, Basel, Switzerland, 29891301PS04) dissolved in 0.5 mL ethanol (ChemSupply, Gillman, Australia, EA043; 0.5% *w*/*v* final concentration). Discs of approximately 15 mm diameter and 1 mm thickness were produced from 0.2 mL pre-polymerised solution per well of a 24 well cell culture microplate. Crosslinking was achieved with exposure to UV light through an optical fibre connected to a Dymax Blue-Wave 75 UV spot lamp (Dymax Corporation, Torrington, CT, USA). The UV intensity was set at 100 mW/cm2 (at 365 nm) for 60 s. The hydrogel discs were subsequently soaked in 1 mL DPBS solution for 2 h at 4 °C, rinsed twice, soaked overnight in DPBS at 4 °C, and then stored light-safe in 1 mL DMEM/F12 (Life Technologies (Gibco), Carlsbad, CA, USA; 11330-057) at 4 °C, until further use [9].

2.3. Printing GelMA Hydrogel Multiwell Arrays

GelMA hydrogel multiwell arrays were made entirely from pre-polymerised GelMA solution (10%; *w*/*v*), freshly prepared from 1 g freeze dried GelMA by dissolving in 9.5 mL DPBS at 37 °C, followed by the addition of 50 mg of the photoinitiator 2-hydroxy-1-(4-(hydroxyethoxy)phenyl)-2-methyl-1-propanone (Irgacure 2959, CIBA Chemicals) dissolved in 0.5 mL ethanol (0.5% *w*/*v* final concentration). Printing was performed with a 3D-Bioplotter® System (EnvisionTEC, Gladbeck, Germany) fitted with a 30CC Optimum syringe barrel and piston (Nordson EFD, Westlake, OH, USA) and 200 µm printing nozzle (Nordson EFD). Pre-polymerised GelMA was loaded into the barrel and centrifuged at 300× *g* at 15 °C for 1 min to remove air bubbles, followed by printing onto autoclaved glass slides. After printing, crosslinking was achieved with exposure to UV light (300 s at 10 mW/cm^2), again to generate a soft gel with shear modulus of <500 Pa at 37 °C, followed by washing and storage until further use, as described above. Arrays comprised of 9 × 9 (81) wells, with each array constructed from 4 layers of GelMA (90° offset between layers), each layer comprising 10 parallel continuous GelMA hydrogel strands, 200 µm in diameter, with 500 µm spacing between strands. Each well was 500 µm in diameter, 800 µm deep, and ~160 µL volume.

2.4. Rheometry

The shear modulus was measured at 37 °C using a Physica MCR 301 Rheometer (Anton Paar GmbH, Graz, Austria) with a 15 mm parallel plate and a temperature control stage.

2.5. Human iPSC Culture

ATCC-BXS0116 (passages 7–17) and JMC1i-SS9 (passages 35–39) human iPSCs were cultured on GelMA hydrogel discs using commercially available cGMP mTeSR™1 cell culture medium (STEMCELL Technologies, Vancouver, BC, Canada; 05850; prepared as per the manufacturer's instructions). BXS0116 (ATCC ACS-1030) human iPSCs were derived from healthy donor bone marrow CD34+ cells and JMC1i-SS9 human iPSCs were derived in-house from healthy donor dermal fibroblasts. The cell lines were derived by Sendai viral expression of reprogramming factors Oct-4, Klf4, c-Myc, and Sox-2 (OKSM) and the Human STEMCCA Constitutive Polycistronic (OKSM) Lentivirus Reprogramming Kit (Millipore, Burlington, MA, USA; SCR544), respectively. Both cell lines were tested as mycoplasma negative using an e-Myco mycoplasma PCR detection kit (iNtRON Biotechnology, Gyeonggi-do, Republic of Korea; 25237) and approved for use by the University of Wollongong Human Research Ethics Committee (HE14/049). For each cell line, we employ a quality controlled, two-tiered banking process based on a Master Cell Bank, which is used to generate a Working Cell Bank for ensuing research. Prior to iPSC seeding,

GelMA discs were incubated in 5% CO_2 at 37 °C in 1 mL prewarmed culture media. Areas of iPSC differentiation were removed from cultures immediately prior to passaging for subculture. Passaging was performed by dissociating colonies with EDTA (Sigma-Aldrich, E8008) at 37 °C for 2 min, rinsing twice with 1 mL prewarmed DMEM/F-12, and seeding dissociated cell aggregates by employing a 1:6 to 1:12 split ratio. Cultures were maintained in a humidified 37 °C, 5% CO_2 incubator and monitored daily to ensure suitable timing of passaging (~60–70% confluency), and medium was changed every second day.

2.6. Human iPSC Differentiation and Generation of Brain Organoids

Dissociated iPSCs were seeded (2×10^5 cells) onto GelMA hydrogel and cultured for 4–6 days in mTeSR™1 cell culture medium (carefully replenished on the second day) prior to initiating neural induction. For GelMA multiwell array-based culture, iPSCs were seeded at the bottom of individual hydrogel wells and maintained as described above. On the fourth day, spent media was removed and neural induction was initiated using 1 mL per well of STEMdiff™ Neural Induction Medium (STEMCELL Technologies, 05835). Neural induction was performed over 7 days, again within a humidified 37 °C, 5% CO_2 incubator, with half media changes made every 2–3 days. On the seventh day, cells were transitioned in neural differentiation media, comprising Neurobasal® Medium (Life Technologies (Gibco), 21103-049; 50% final volume), DMEM/F-12 medium (50% final volume), Neurocult™ SM1 neuronal supplement (STEMCELL Technologies, 05711; 1% final concentration), N2 supplement-A (STEMCELL Technologies, 07152; 0.5% final concentration), brain-derived neurotrophic factor (BDNF; Peprotech (Lonza), Cranbury, NJ, USA, AF-450-02; 50 ng/mL final concentration), and L-Glutamine (Life Technologies (Gibco), 25030-081; 1% final volume). Culture media was always prewarmed and 1 mL added carefully to each well, with half media changes performed every 3–4 days, being careful to monitor both the acidity of media and morphology of cultures. Organoids were formed by day 28 and could be further matured and maintained (>3 years) in neural differentiation media with ongoing media changes every 3 to 4 days.

2.7. Scanning Electron Microscopy (SEM)

SEM of GelMA hydrogel surface porosity or iPSCs on hydrogel involved submersing samples in iPSC culture media for 24 h, freeze drying overnight using a Christ Alpha 2-4 LD Freeze Dryer (Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany), and coating with gold (20 nm) using an Edwards sputter coater. Samples were kept desiccated until analysed with SEM performed using a JSM-7500FA LV Scanning Electron Microscope (Joel, Tokyo, Japan).

2.8. Immunocytochemistry

Samples were fixed with 3.7% paraformaldehyde (PFA) in phosphate buffered saline (PBS, pH 7.4, Sigma-Aldrich) for 30 min at room temperature (RT). Samples were then blocked and permeabilized with 5% (v/v) goat serum (GS; (Sigma-Aldrich, G9023) in PBS containing 0.3% (v/v) Triton X-100 (Sigma-Aldrich, T9284) for 2 h at RT. Samples were subsequently incubated with primary antibodies against OCT4 (mouse, 1:200; STEMCELL Technologies, 60093), SSEA4 (mouse, 1:200; STEMCELL Technologies, 60062), TRA-1-60 (mouse, 1:200; Millipore, Temecula, CT, USA, MAB4360), TRA-1-81 (mouse, 1:200; Millipore, MAB4381), PAX6 (rabbit, 1:100; Sigma-Aldrich, HPA030775), nestin (NES) (mouse, 1:200; Millipore, MAB5326), synaptophysin (SYP) (rabbit, 1:500; Millipore, 04-1019; or rabbit, 1:250 Abcam, Eugene, OR, USA, ab32127), TUBB3 as recognised by the TuJ1 antibody (Chicken, 1:1000; Millipore, AB9354), v-Glut (1:200, Life Technologies (Invitrogen), Carlsbad, CA, USA, 482400), GAD67 (mouse, 1:200, Millipore, AB5406), doublecortin (DCX) (1:200, guinea pig, Millipore, AB2253), TBR1 (Rabbit, 1:300, Abcam, ab31940), GABA (rabbit, 1:200; Sigma-Aldrich, A2052), FOXG1 (Rabbit, 1:300, abcam, ab18259), and reelin (RELN) (Mouse, 1:300, Millipore, MAB5364) at 4 °C overnight. On the second day, samples were rinsed with 0.1% Triton X-100 in PBS three times and samples were incubated with species-specific Alexa

Fluor tagged secondary antibody (1:1000; Life Technologies (Invitrogen)) for 2 h at RT. Nuclei were labelled with Hoechst 33342 (5 µg/mL; Life Technologies (Invitrogen), H3570) or 4′,6-diamidino-2-phenylindole (DAPI, 1 µg/mL; Life Technologies (Invitrogen), D1306) at RT for 15 min. Samples were immersed in PBS on glass coverslip bottom culture plates and imaged with a Leica TCS SP5 II confocal microscope (Leica Microsystems, Wetzlar, Germany. Images were collected and analysed using Leica Application Suite AF (LAS AF) software and Image J (version 1.52h; http://imagej.nih.gov/ij, accessed on 31 March 2020).

2.9. Flow Cytometry

Single cell suspensions of iPSCs were prepared from colonies grown on GelMA by incubating in Gentle Cell Dissociation Reagent (STEMCELL Technologies) for 8 min at RT, collected in cold PBS, and passed through a 37 µm sieve to generate a single cell suspension. Cells were centrifuged at 300× *g* for 5 min prior to resuspension and fixing in 3.7% PFA for 10 min on ice. Fixation was followed by centrifugation at 500× *g* for 5 min and resuspension in PBS for storage at 4 °C until use. Cells were washed twice by centrifugation at 500× *g* for 3 min and resuspension in 0.1% Triton X-100 in PBS. Following further centrifugation at 500× *g* for 3 min, simultaneous blocking and permeabilization occurred by resuspension in 5% GS/PBS containing 0.3% Triton X-100 for a 30 min incubation at RT. After centrifugation at 500× *g* for 3 min, cells were resuspended in OCT4 (1:200, STEMCELL Technologies), IgG2b.K (1:200, Life Technologies (Invitrogen), TRA-1-60 (1:100, Millipore), TRA-1-81 (1:100, Millipore), or IgM (1:100, Life Technologies (Invitrogen)) unconjugated primary antibodies diluted in 5% GS/PBS. At the completion of a 30 min incubation at RT, cells were washed twice by centrifugation at 500× *g* for 3 min and resuspended in 2% GS/PBS containing 0.1% Triton X-100. Following washes, cells were incubated for 30 min at RT in Alexa Fluor 488 nm tagged secondary antibody (goat anti-mouse, 1:500, Life Technologies (Invitrogen)). Cells were again washed by centrifugation at 500× *g* for 3 min and resuspended in 2% GS/PBS containing 0.1% Triton X-100 prior to a final resuspension in 2% GS/PBS. Samples were analysed by an Accuri C6 flow cytometer (BD Life Sciences, San Jose, CA, USA).

2.10. Live Cell Labelling

Live cell labelling of organoids was performed using the membrane permeable fluorescent probes NeuroFluor™ CDr3 (STEMCELL Technologies, 01800) and NeuroFluor™ NeuO (STEMCELL Technologies, 01801) to selectively label iPSC-derived NPCs and iPSC-derived neurons, respectively. Dual labelling was performed according to the manufacturer's instructions. Briefly, following removal of culture medium, organoids were incubated with 1 mL of labelling medium at 37 °C in a 5% CO_2 incubator for 1 h. Labelling medium was then aspirated followed by addition of 1 mL fresh culture medium and further incubated at 37 °C in a 5% CO_2 incubator for 2 h. Medium was removed and 1 mL of fresh medium was added. Visualization of labelling was performed using a Leica TCS SP5 II confocal microscope. Images were collected and analysed using Leica Application Suite AF (LAS AF) software.

2.11. Calcium Imaging

Organoids were incubated with 2 µM Fluo-4, AM (Life Technologies (Invitrogen), F14201) in fresh culture medium for 30 min at 37 °C, followed by washing in HEPES buffered Hank's balanced salt solution (HEPES-HBSS; 137 mM NaCl, 5.4 mM KCl, 1.5 mM CaCl2, 0.44 mM KH_2PO_4, 0.5 mM $MgCl_2$, 0.4 mM $MgSO_4$, 0.3 mM Na_2HPO_4, 4 mM $NaHCO_3$, 5.6 mM D-Glucose, and 10 mM HEPES, pH 7.4; all chemicals from Sigma-Aldrich). The samples were mounted onto coverslips and observed on a Leica TCS SP5 II confocal microscope. The data were collected and quantified using LAS AF software (Leica). To induce intracellular calcium flux, GABA(A) receptor antagonist Bicuculline (50 µM; Sigma-Aldrich, 14340) was added with HEPES-HBSS for 3 min followed by further imaging.

2.12. Multielectrode Array Electrophysiological Recordings

After 55 days in culture (4 days iPSC expansion + 7 days neural induction + 44 days neural differentiation), samples were transferred from culture wells to 60 channel microelectrode arrays (MEAs) with 30 μm titanium nitride electrodes spaced 200 μm apart (60MEA200/30iR-ITO, Multi Channel Systems MCS GmbH, Reutlingen, Germany). Culture media was replaced with HEPES-HBSS based neuronal recording solution as previously described [11]. Glutamate (L-glutamic acid, Sigma-Aldrich, 49449) was added to a final concentration of 200 μM during recordings. Extracellular electrophysiological signals were recorded at 25 kHz (MEA2100 hardware, Multi Channel Systems), stored, and filtered offline with a 50 Hz high-pass 2nd order Butterworth filter (MC_Rack Ver. 4.6.2, Multi Channel Systems).

3. Results

3.1. Synthesis of GelMA Hydrogels

GelMA hydrogels were either cast to form discrete discs that were maintained in conventional commercially sourced polystyrene plates or printed to form multiwell arrays. Pre-polymerised GelMA solution (5–10%; *w*/*v*) was freshly prepared from freeze-dried GelMA [10]. GelMA hydrogel discs were 15 mm in diameter and 1 mm thick, while multi-well arrays comprised of 9 × 9 (81) wells, with each array constructed from 4 layers (90° offset between layers), with each layer comprising 10 parallel continuous strands, 200 μm in diameter, with 500 μm spacing between strands. Each well was 500 μm in diameter, 800 μm deep, and ~160 μL volume. After casting discs, or immediately following printing arrays, crosslinking was achieved with exposure to UV light (discs: 60 s at 100 mW/cm^2; arrays: 300 s at 10 mW/cm^2) to generate a soft gel with shear modulus of <500 Pa at 37 °C. The resulting hydrogels had highly porous surfaces in the absence and presence of cells (Figure 2A,B; respectively). Scanning electron microscopy (SEM) indicated the gel pores were networked with smaller pores connecting larger pores, suggestive of inherent permeability and penetrability.

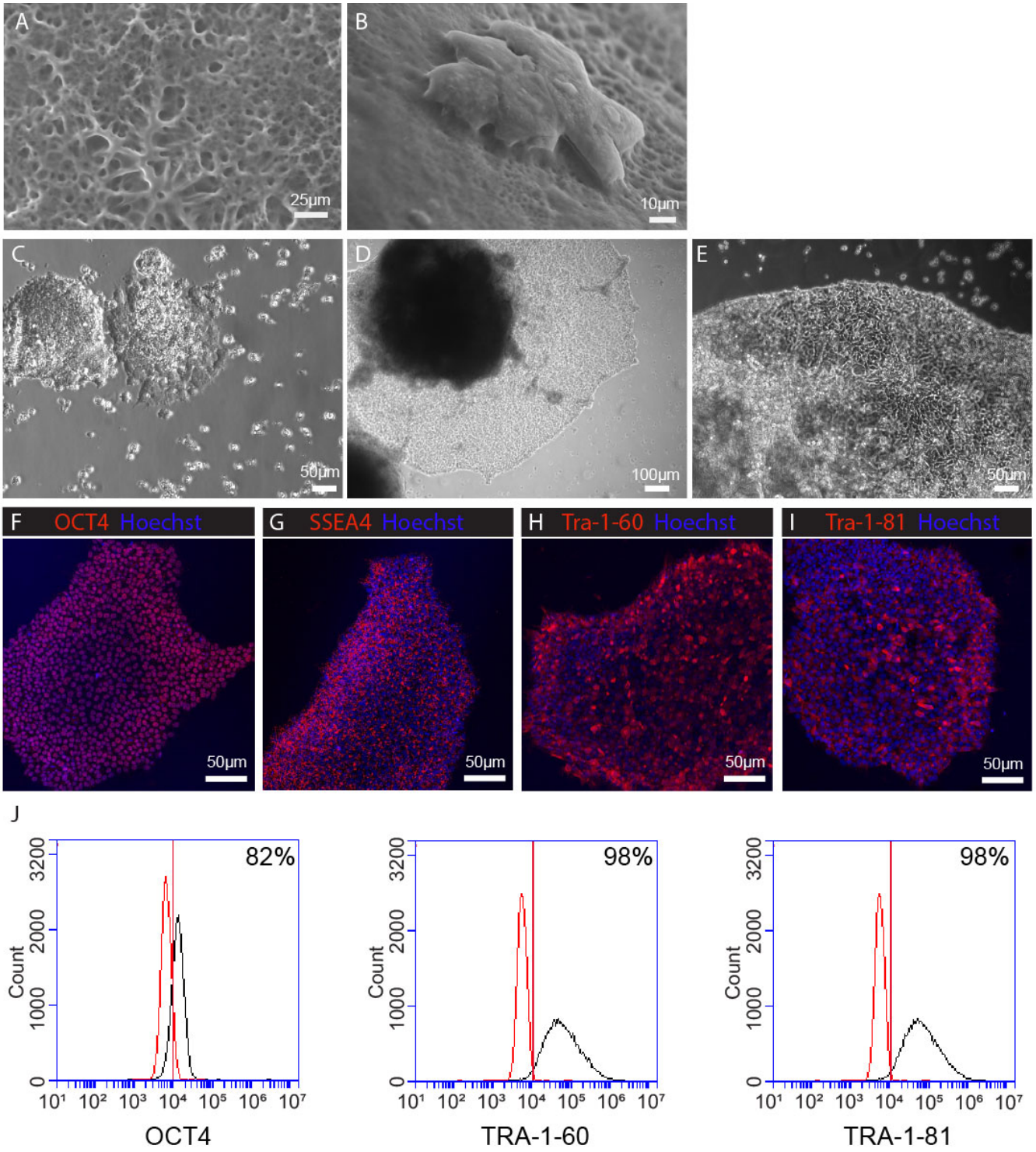

Figure 2. Attachment of human iPSC colonies and formation of neural derivatives on GelMA hydrogel. (**A**): SEM showing surface porosity of 5% GelMA. (**B**): SEM showing attachment of iPSCs on surface of GelMA. (**C**–**E**): Bright-field microscopy images showing flattened morphology of iPSC colonies at day 4 expansion, characteristic flattened morphology of an iPSC colony after day 21 expansion, and characteristic cuboidal and high nuclear-cytoplasmic ratio morphology of iPSCs seen at higher magnification, respectively. (**F**–**I**): Immunophenotyping of iPSCs at day 21 expansion showing expression of pluripotency markers: OCT4, SSEA4, TRA-1-60, and TRA-1-81, respectively. (**J**): Flow cytometry of iPSCs confirming expression of OCT4, TRA-1-60, and TRA-1-81. Red histograms indicate the isotype controls.

3.2. iPSC Culture and Differentiation on GelMA Hydrogels

iPSC culture on GelMA hydrogel discs and multiwell arrays was performed using commercially available mTeSR™1 cell culture medium, without conventional cell growth substrates, such as Matrigel™ or fibroblast feeder cells. Cells were propagated and sub-cultured as iPSC-colonies, with neural induction to intermediate neural progenitor cells (NPCs) performed over 7 days using off-the-shelf neural induction media, followed by differentiation to organoids over 4 weeks in differentiation medium, with further maturation and maintenance in the same culture medium thereafter (Figure 1).

More specifically, GelMA hydrogel iPSC cultures exhibited characteristic stem cell colonies by day 4 after seeding (Figure 2C), which expanded with cell proliferation and merged with neighboring colonies without discernable differentiation. Ongoing culture in mTeSR1 maintenance media resulted in flattening of the colonies (indicative of cell adhesion) as predominantly monolayers of cells by day 21 (Figure 2D,E). Immunophenotyping showed cells expressed iPSC markers OCT4, SSEA4, TRA-1-60, and TRA-1-81 (Figure 2F–J).

3.3. Generation of Brain Organoids

The generation of organoids (n > 100) on GelMA hydrogel was reproducibly demonstrated using multiple different (biologically independent) iPSC lines (ATCC-BXS0116: 6 independent experiments, each including >12 technical replicates; JMC1i-SS9: 3 independent experiments, each including >12 technical replicates), with organoids able to be maintained in culture for more than 3 years (Figure 3H). Initial neural induction resulted in iPSCs differentiating to NPCs, with cell cultures reflecting antecedent iPSC colonies (Figure 3A), and successively forming compact neurospheres with peripheral microspikes or more developed elongated extensions (Figure 3B,C). By day 7 of induction, cultures exhibited radially organized neural rosettes, manifesting for neurospheres as radiating zones of NPCs boarding deeper hollows (Figure 3D,E).

At day 10 of neural differentiation (17 days after the commencement of neural induction), isolated differentiation at the margin of colonies or neuropheres (Figure 3F, outline box) was distinctly neural in appearance (Figure 3F inset). Neurons initially expressed TUJ1 by day 14 of differentiation (Figure 3G), were radially assembled to form polarized neural extensions from tight clusters of cells with neurites projecting long distances from their site of origin and exhibiting varicosities/boutons (Figure 3G inset). Boutons formed along the length of neurites and terminal bulbs at the end of neurites (Figure 3G inset). Succeeding neurons expressed mature neuronal microtubule–associated protein 2 (MAP2; by day 28 of differentiation) and self-organised to form 3D organoids (Figure 3H–M). While organoids that formed on the surface of GelMA discs were less constrained in terms of shape and size (Figure 3H), those formed in 3D multiwell arrays tended to be more uniformly sized and shaped inside wells (Figure 3J). Accordingly, array-based organoids tended to be orbicular (Figure 3K). Furthermore, while multiple organoids propagated on individual hydrogel discs, single organoids formed within wells of arrays (Figure 3J,K). For both configurations, however, organoids exhibited protrusions from which neurites appeared as individual and bundles of radiating processes that projected out to infiltrate the underlying and/or surrounding hydrogel matrix and to connect neighboring organoids to form networks of organoids. Specifically, for organoids propagated in multiwell arrays, neurites and bundles protruded through the adjoining walls of hydrogel wells (Figure 3L), while for disc-based systems, neurite bundles extended laterally and free-floating through culture medium from one organoid to another (Figure 3M).

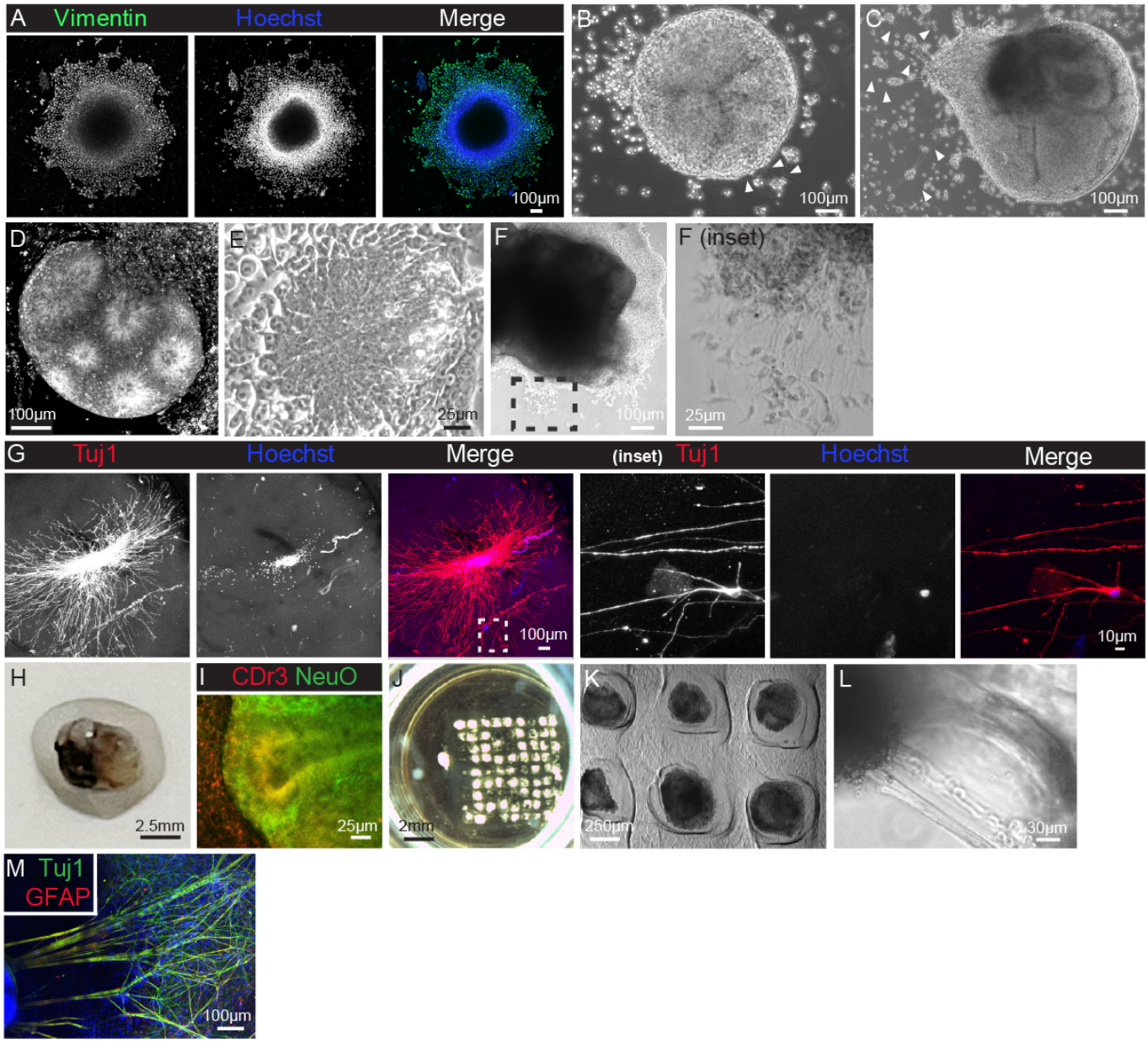

Figure 3. Induction of brain organoids from iPSCs on GelMA hydrogel, with monolayer colonies transitioning to 3D neural spheroids and subsequent differentiation to organoids. (**A**): iPSC-derived NPC cultures expressing vimentin and reflecting antecedent iPSC colonies. (**B**,**C**): Early-stage neural spheroids (neurospheres) with microspikes or elongated neural extensions (arrow heads), respectively. (**D**): Live-cell (CDr3) labelling (grey scale) of NPCs in a neurosphere on day 7 of neural induction with characteristic radial neural rosettes. (**E**): A bright-field microscopy image of a neural rosette on day 7 of neural induction. (**F**): Neural outgrowths at the margin of a colony following 7 days of differentiation (14 days after initiating neural induction). (**G**): Neural differentiation on day 14 (21 days after initiating neural induction) with characteristic radially extended neural processes and Tuj1 expressing neurons. (**H**): Photograph of a 3.5-year-old organoid in a droplet of PBS, with black/brown-pigmentation reminiscent of neuromelanin. (**I**): An early-stage 33 day old organoid (4 days iPSC expansion + 7 days neural induction + 22 days neural differentiation) with live-cell neuronal (NeuO) and NPC (CDr3) labelling revealing early cell-specific patterning and structural heterogeneity. (**J**,**K**): 59 day old organoids (4 days iPSC expansion + 7 days neural induction + 48 days neural differentiation) formed inside wells of 3D printed GelMA-based multiwell arrays. (**L**): Neurites and neurite bundles of an 84 day old organoid (4 days iPSC expansion + 7 days neural

induction + 69 days neural differentiation) protruding through the hydrogel wall of a GelMA-based multiwell array to neighbouring organoids. (**M**): Neurites and neurite bundles stained with Tuj1 and partially colocalised with GFAP, emanated from organoids propagated on hydrogel discs and projected through culture medium (i.e., free-floating) to neighbouring organoids. Pseudocoloured images as indicated by colour of text. Scale bars as indicated.

3.4. Brain Organoids Exhibit Cellular and Regional Heterogeneity

By day 42 of differentiation, organoids were large (up to 3 mm diameter) heterogeneous masses of densely packed cell soma with prolific neurite formation throughout the organoids, including radiating neurites and neurite bundles from zones of neural progenitor cells that arranged to form tuboid structures (Figure 4A, Movies S1 and S2). Presynaptic vesicle glycoprotein synaptophysin was apparent as small high-density puncta adjacent to neurites and cell bodies, and clearly demarcated an outer apical region of the organoids (Figure 4A, Movies S1 and S2). Synaptophysin colocalised with MAP2-labelled bundles of radiating processes, suggestive of axonal tracts (Figure 4A–C, Movies S1 and S2). Significantly, taken together with the aforementioned neurite bundles, the regional divisions of organoids resembled cortical plate or rudimentary grey matter tissue containing cell bodies, dendrites, and axon terminals of neurons, with underlying tissue inclusive of bundles or axon tracts, analogous to cortical and deep brain tissue, respectively. Molecular characterisation of strong FOXG1 and PAX6 expression, together with cortical plate (TBR1) marker expression more specifically evidenced dorsal forebrain identity, while neural stem/progenitor cell marker nestin (NES) indicated persistent neurogenesis, and reelin (RELN) expression was suggestive of cortical tissue-like neuronal layer formation (Figure 4D). Immunohistochemistry also demonstrated discrete neural cell types of GABA-expressing neurons (Figure 4E), together with punctate localisation of glutamic acid decarboxylase (GAD67; Figure 4E) and vesicular glutamate transporter (v-Glut; Figure 4F), and doublecortin (DCX) expressing cortical neurons (Figure 4F).

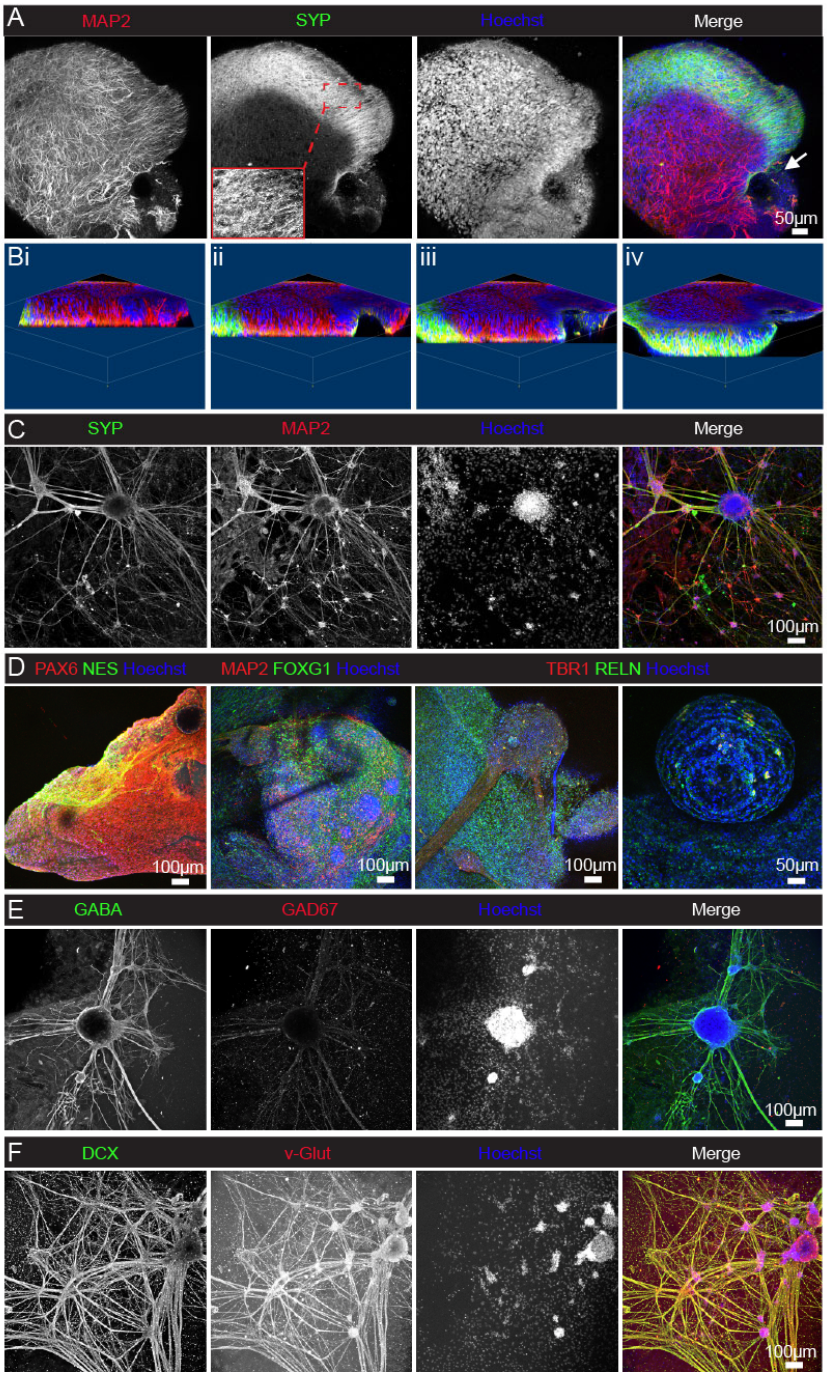

Figure 4. Cellular and regional heterogeneity of 53-day old brain organoids. (**A**): A 53-day old organoid (4 days iPSC expansion + 7 days neural induction + 42 days neural differentiation) with a tuboid structure (arrow), abundant MAP2-labelled processes, and demarked synaptophysin expression (with synaptic puncta evident in the magnified inset). (**B**): 3D visualization of orthoslice images at (i) 280 µm, (ii) 440 µm, (iii) 540 µm, and (iv) 760 µm from edge of organoid depicted in (**A**). (**C**): MAP2 partially colocalised with synaptophysin expression, recurrently signifying regions of neuronal networking within organoids and between networks of organoids connected by long-projecting neurite bundles. (**D**): PAX6 and FOXG1 expression, together with cortical plate (TBR1) marker expression more specifically evidencing dorsal forebrain identity, with RELN and NES expression indicative of cortical tissue-like neuronal layer formation and a persistent neural progenitor cell population, respectively. (**E**,**F**): GABA, GAD67, v-Glut, and cortical neuronal marker DCX expression of organoid networks. Pseudocoloured images as indicated by colour of text. Scale bars as indicated.

3.5. Recapitulation of Archetypal Brain Neuronal Network Activity

Critically, neurons formed networks with synaptic contacts, initially shown to be active by recurrent increases in extracellular calcium concentration (Figure 5A) in response to disinhibition of cells by gama-aminobutyric acid (GABA) receptor-A antagonist bicuculline (Figure 5B). Interrogating intercellular communication within the organoids using microelectrode arrays (MEAs) demonstrated network formation and function (Figure 5C,D). Recordings from 4 electrodes illustrated neuronal activity, with glutamate induced synchronous bursting activity measured across the organoid surface. The synchronous glutamate-induced bursting activity decreased in amplitude over 90 s, while some single-cell spiking activity increased in amplitude and spike rate beyond this, likely related to a delay in glutamate reaching cells deeper within the organoid.

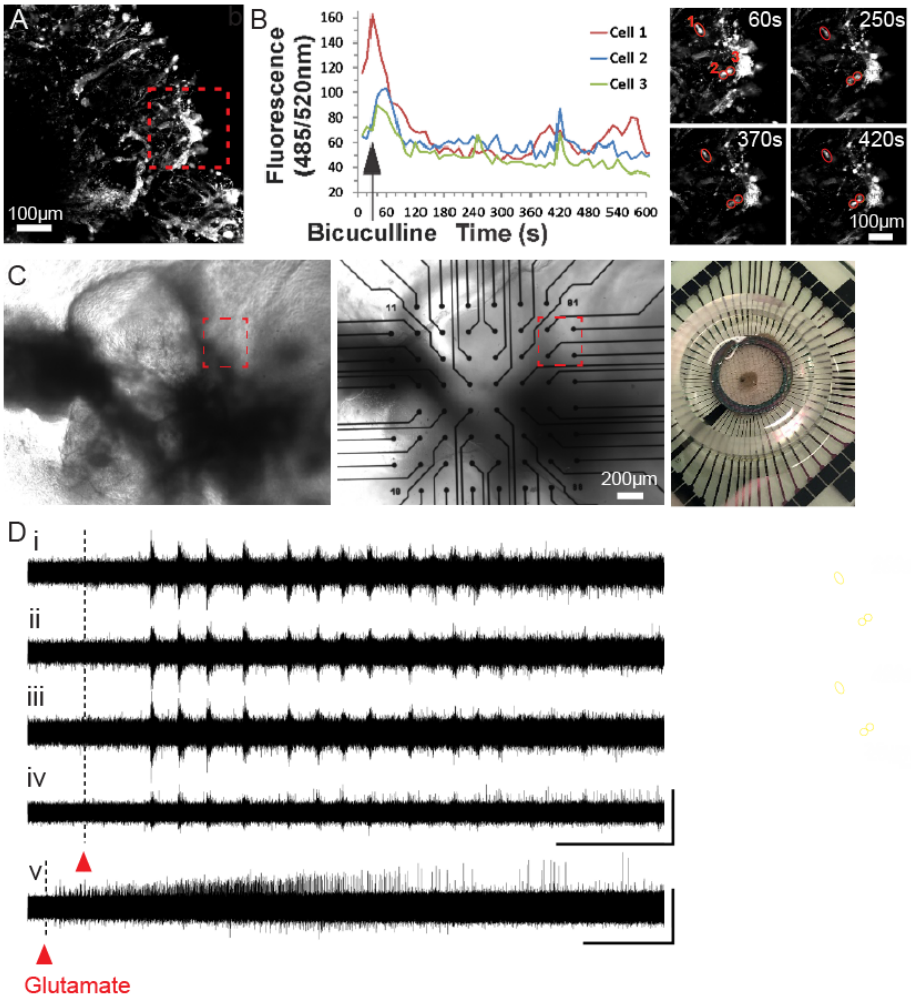

Figure 5. Neuronal network function of brain organoids. (**A**): Cells labelled with fluorescent calcium indicator, Fluo-4. Inset indicates the region of cells evaluated for calcium flux. (**B**): Time course of bicuculline-induced calcium flux for individual neurons 1–3 that are denoted in the corresponding photomicrographs. (**C**): Bright-field photomicrographs of a 55-day old (4 days iPSC expansion + 7 days neural induction + 44 days neural differentiation) organoid on an MEA showing both the region of extracellular electrophysiological recording and the configuration of electrodes. (**D**): Electrophysiological recordings from 4 electrodes (i–iv) across a single organoid surface, with synchronous bursting across the organoid surface in response to glutamate addition. The lower trace (v) repeats trace (iv) over a longer time course and shows the single cell spiking activity increasing in amplitude following initial glutamate response. Timing of glutamate addition is indicated by dashed line. Scale bars (i–iv) 50 µV vertical, 20 s horizontal, and (v) 50 µV vertical, 100 s horizontal.

4. Discussion

We have developed a defined and uncomplicated method of generating human brain organoids from PSCs that will hopefully facilitate access to this technology for in vitro modelling and translational application. By virtue of its simplicity, and GelMA being a clinically compliant cell growth substrate, the method is amenable to cGLPs and cGMPs, for quality assured research and creating tissues for pharmaceuticals development, and clinical compliance for cell therapeutics.

The organoids produced using the method comprise of densely packed cell soma that self-organize to form hollow neural tube-like structures, and neurons with prolific neurite outgrowth that form functional 3D networks with synaptic contacts. Consistent with recent reports of optimal substrate stiffness for iPSC survival, induction of neuronal cell fate, neurite extension, and innervation, GelMA was mechanically tuned for a softer gel, resembling the physiological stiffness of brain tissue [12,13]. Also, being 3D printable enabled the quick and precise fabrication of GelMA hydrogel-multiwell arrays for more scalable production of organoids.

Chen et al. similarly described the use of microwell arrays for unguided Matrigel™-free organoid formation [14]. However, unlike our simple and easy to prepare hydrogel arrays that are ready to derive organoids immediately after printing, Chen et al.'s platform involves preparing molded polydimethylsiloxane (PDMS) arrays using 3D printed 'reverse molds' for 'soft lithography', followed by surface coating with "cell-repulsive" methoxy polyethylene glycol silane (mPEG) substrate for ensuing organoid derivation [14].

GelMA is a versatile, clinically compatible, semisynthetic matrix derived from collagen and used extensively for biomedical applications [15]. Its bioactivity can be explained by the presence of cell-attaching and MMP-reactive peptide motifs, enabling cell proliferation and migration [10]. Therefore, by incorporating the intrinsic bioactivity of natural matrices with the fidelity of synthetic biomaterials, GelMA's physicochemical properties are modifiable for a variety of cell supporting applications, presently demonstrated to encompass iPSC support and differentiation.

Like Matrigel™, GelMA acts as an ECM, and combined with specialist culture media promotes self-organization and self-patterning in the absence of additional specific exogenous patterning factors. However, unlike Matrigel™-based or other Matrigel™-free culture systems (e.g., using abovementioned mPEG substrate [14], decellularized porcine brain hydrogel matrix [16], or protonated chitosan blended sodium hyaluronan hydrogel [17]), stem cell colonization on GelMA hydrogel can be immediately followed by neural induction and differentiation, including polarization and migration of iPSC-NPCs and neurons. Accordingly, unlike other methods, there is no need to manipulate cultures by, for example, detachment and transfer of iPSCs to low attachment culture surfaces [14], centrifugation for cell aggregation [17], and/or transfer of embryoid bodies to organoid-induction substrates, or for embedding in ECM [16,17].

Following neural induction and differentiation of iPSCs on GelMA, succeeding neural aggregates increase in size and pattern with ongoing culture to form organoids, with the outgrowth of 3D neuroepithelial buds successively forming other organoids. Interestingly, the regional heterogeneity of organoids, including rudimentary cortical grey matter-like, and deeper white matter-like, tissues, together with the long-projecting neurite bundles, mirror earlier reports of gray and white matter architecture and cortical axon tracts derived from human embryonic stem cell-derived brain organoids [18]. Also, while earlier point-of-reference methods describe dorsal and ventral forebrain-specific organoids [19,20], or midbrain-specific organoids [21], ours displayed dorsal-forebrain identity in the absence of extrinsic patterning factors. Although not presently tested, it is likely that modified patterning through, for example, ventralizing treatment could be attained with our system [19]. Finally, the 3D neuronal networking presently evaluated using calcium imaging and MEAs is indicative of the utility of the organoids as models for network formation and function that mechanistically recapitulates archetypal neuronal network activity within the brain [11], plasticity, and homeostatic mechanisms, as well as disruption with neural

networking disorders, and application for drug and toxicity testing. More specifically, the response of organoids to exogenously delivered compounds, such as GABA(A) receptor antagonist bicuculline and glutamate, provide proof-of-concept of quantifying drug effects.

5. Limitations, Conclusions and Future Directions

In conclusion, we have demonstrated a novel, defined, direct, and relatively simple approach to culturing and differentiating iPSCs using GelMA hydrogel for the uninterrupted generation of human brain organoids. Our method is adaptable for custom-building hydrogel configurations, such as discrete discs using uncomplicated casting and molding, or more sophisticated multiwell arrays by 3D printing for more scalable and standardized organoid production. By recapitulating the basic form and function of early human central nervous tissue, we plan to employ the GelMA platform for modelling neural development and disease. In the longer term, as organized collections of defined and clinically amenable human cells that are compatible with the immune system and biochemistry of their primary cell source, the organoids have the potential to be developed and applied for personalised cell-based replacement and regenerative therapy, and early-phase drug discovery. Our plans for disease modelling include brain tumour modelling by incorporating patient-derived tumours into organoids [22–24]. More specifically, we are establishing co-cultures of patient derived glioma cells and brain organoids to better model tumour formation within the brain microenvironment. The models will enable important multi-dimensional and multi-directional communication between neoplastic and non-neoplastic cells, and provide extracellular cues that effect glioma cell phenotypes, tumour formation, and responsivity to conventional and novel therapeutics. By doing so we hope to attain clinically relevant findings previously unattainable with traditional pre-clinical (e.g., 2D/planar cell culture and animal) modelling.

Finally, it is important to acknowledge that, like all models, our described organoid system is not without limitations since the organoids do not recapitulate all features of brain biology. As such, future research will strive to further develop the tissues by, for example, including cells found in the brain with non-neural origins such as vascular cells and immune cells, enhancing arealization, and increasing the size of individual organoids as a fundamental limiting factor for recapitulating late stages of human brain development [25].

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/organoids2010002/s1, Movie S1: 3D visualization of brain organoid orthoslices showing MAP2 (red), SYP (green) and Hoechst (blue) labelling. Movie S2: 3D visualization of brain organoid optical slices rotated through 360° on the *y*-axis and showing MAP2 (red), SYP (green) and Hoechst (blue) labelling.

Author Contributions: Conceptualization, E.T.-C. and J.M.C.; Data curation, E.T.-C., B.Z., J.B. and J.M.C.; Formal analysis, E.T.-C., B.Z., J.B. and J.M.C.; Funding acquisition, G.G.W. and J.M.C.; Investigation, E.T.-C. and J.M.C.; Methodology, E.T.-C. and J.M.C.; Project administration, E.T.-C. and J.M.C.; Resources, J.M.C.; Supervision, E.T.-C. and J.M.C.; Validation, S.L.H.; Visualization, J.M.C.; Writing—original draft, J.M.C.; Writing—review & editing, E.T.-C., S.L.H., B.Z., J.B. and G.G.W.; Writing—Revisions & editing, J.M.C. and E.T.-C. All authors have read and agreed to the published version of the manuscript.

Funding: The work was funded by the Australian Research Council (ARC) Centre of Excellence Scheme (CE140100012) and the Arto Hardy Family.

Institutional Review Board Statement: The study was approved by the University of Wollongong Institutional Review Committee (IBC; protocol number: GT14/04) and Human Research Ethics Committee (HE14/049).

Informed Consent Statement: Not applicable.

Data Availability Statement: The datasets generated for the current study are available from the corresponding author on request.

Acknowledgments: The authors wish to acknowledge the generous support of the Arto Hardy Family, as well as funding from the Australian Research Council (ARC) Centre of Excellence Scheme (CE140100012), the use of facilities at the University of Wollongong Electron Microscopy Centre, and support of the Australian National Fabrication Facility (ANFF)—Materials Node for the provision of GelMA. Figure 1 and the Graphical Abstract for this manuscript were created with BioRender.com.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Lancaster, M.A.; Knoblich, J.A. Generation of cerebral organoids from human pluripotent stem cells. *Nat. Protoc.* **2014**, *9*, 2329–2340. [CrossRef] [PubMed]
2. Lancaster, M.A.; Renner, M.; Martin, C.A.; Wenzel, D.; Bicknell, L.S.; Hurles, M.E.; Homfray, T.; Penninger, J.M.; Jackson, A.P.; Knoblich, J.A. Cerebral organoids model human brain development and microcephaly. *Nature* **2013**, *501*, 373–379. [CrossRef] [PubMed]
3. Aurand, E.R.; Lampe, K.J.; Bjugstad, K.B. Defining and designing polymers and hydrogels for neural tissue engineering. *Neurosci. Res.* **2012**, *72*, 199–213. [CrossRef] [PubMed]
4. Tong, Z.; Solanki, A.; Hamilos, H.; Levy, O.; Wen, K.; Yin, Z.; Karp, J.M. Application of biomaterials to advance induced pluripotent stem cell research and therapy. *EMBO J.* **2015**, *34*, 987–1008. [CrossRef]
5. Gu, Q.; Tomaskovic-Crook, E.; Wallace, G.G.; Crook, J.M. 3D Bioprinting human induced pluripotent stem cell constructs for in situ cell proliferation and successive multi-lineage differentiation. *Adv. Healthc. Mater.* **2017**, *6*, 1700175. [CrossRef]
6. Thomson, J.A.; Itskovitz-Eldor, J.; Shapiro, S.S.; Waknitz, M.A.; Swiergiel, J.J.; Marshall, V.S.; Jones, J.M. Embryonic stem cell lines derived from human blastocysts. *Science* **1998**, *282*, 1145–1147. [CrossRef]
7. Xu, C.; Inokuma, M.S.; Denham, J.; Golds, K.; Kundu, P.; Gold, J.D.; Carpenter, M.K. Feeder-free growth of undifferentiated human embryonic stem cells. *Nat. Biotechnol.* **2001**, *19*, 971–974. [CrossRef]
8. Ludwig, T.E.; Levenstein, M.E.; Jones, J.M.; Berggren, W.T.; Mitchen, E.R.; Frane, J.L.; Crandall, L.J.; Daigh, C.A.; Conard, K.R.; Piekarczyk, M.S.; et al. Derivation of human embryonic stem cells in defined conditions. *Nat. Biotechnol.* **2006**, *24*, 185–187. [CrossRef]
9. Polykandriotis, E.; Arkudas, A.; Horch, R.E.; Kneser, U.; Mitchell, G. To matrigel or not to matrigel. *Am. J. Pathol.* **2008**, *172*, 1441–1442. [CrossRef]
10. Nichol, J.W.; Koshy, S.T.; Bae, H.; Hwang, C.M.; Yamanlar, S.; Khademhosseini, A. Cell-laden microengineered gelatin methacrylate hydrogels. *Biomaterials* **2010**, *31*, 5536–5544. [CrossRef]
11. Bourke, J.L.; Quigley, A.F.; Duchi, S.; O'Connell, C.D.; Crook, J.M.; Wallace, G.G.; Cook, M.; Kapsa, R.M.I. Three-dimensional neural cultures produce networks that mimic native brain activity. *J. Tissue Eng. Regen. Med.* **2018**, *12*, 490–493. [CrossRef] [PubMed]
12. Conway, A.; Schaffer, D.V. Biophysical regulation of stem cell behavior within the niche. *Stem Cell Res. Ther.* **2012**, *3*, 50. [CrossRef] [PubMed]
13. Keung, A.J.; Asuri, P.; Kumar, S.; Schaffer, D.V. Soft microenvironments promote the early neurogenic differentiation but not self-renewal of human pluripotent stem cells. *Integr. Biol.* **2012**, *4*, 1049–1058. [CrossRef]
14. Chen, C.; Rengarajan, V.; Kjar, A.; Huang, Y. A matrigel-free method to generate matured human cerebral organoids using 3D-Printed microwell arrays. *Bioact. Mater.* **2020**, *6*, 1130–1139. [CrossRef] [PubMed]
15. Yue, K.; Trujillo-de Santiago, G.; Alvarez, M.M.; Tamayol, A.; Annabi, N.; Khademhosseini, A. Synthesis, properties, and biomedical applications of gelatin methacryloyl (GelMA) hydrogels. *Biomaterials* **2015**, *73*, 254–271. [CrossRef]
16. Simsa, R.; Rothenbucher, T.; Gurbuz, H.; Ghosheh, N.; Emneus, J.; Jenndahl, L.; Kaplan, D.; Bergh, N.; Serrano, A.M.; Fogelstrand, P. Brain organoid formation on decellularized porcine brain ECM hydrogels. *PLoS ONE* **2021**, *16*, e0245685. [CrossRef]
17. Lindborg, B.A.; Brekke, J.H.; Vegoe, A.L.; Ulrich, C.B.; Haider, K.T.; Subramaniam, S.; Venhuizen, S.L.; Eide, C.R.; Orchard, P.J.; Chen, W.; et al. Rapid induction of cerebral organoids from human induced pluripotent stem cells using a chemically defined hydrogel and defined cell culture medium. *Stem Cells Transl. Med.* **2016**, *5*, 970–979. [CrossRef]
18. Cullen, D.K.; Gordián-Vélez, W.J.; Struzyna, L.A.; Jgamadze, D.; Lim, J.; Wofford, K.L.; Browne, K.D.; Chen, H.I. Bundled three-dimensional human axon tracts derived from brain organoids. *iScience* **2019**, *21*, 57–67. [CrossRef]
19. Bagley, J.A.; Reumann, D.; Bian, S.; Lévi-Strauss, J.; Knoblich, J.A. Fused cerebral organoids model interactions between brain regions. *Nat. Methods* **2017**, *14*, 743–750. [CrossRef]
20. Qian, X.; Nguyen, H.N.; Song, M.M.; Hadiono, C.; Ogden, S.C.; Hammack, C.; Yao, B.; Hamersky, G.R.; Jacob, F.; Zhong, C.; et al. Brain-region-specific organoids using mini-bioreactors for modeling ZIKV exposure. *Cell* **2016**, *165*, 1238–1254. [CrossRef]
21. Jo, J.; Xiao, Y.; Sun, A.X.; Cukuroglu, E.; Tran, H.D.; Göke, J.; Tan, Z.Y.; Saw, T.Y.; Tan, C.-P.; Lokman, H.; et al. Midbrain-like organoids from human pluripotent stem cells contain functional dopaminergic and neuromelanin-producing neurons. *Cell Stem Cell* **2016**, *19*, 248–257. [CrossRef] [PubMed]
22. Ogawa, J.; Pao, G.M.; Shokhirev, M.N.; Verma, I.M. Glioblastoma Model Using Human Cerebral Organoids. *Cell Rep.* **2018**, *23*, 1220–1229. [CrossRef] [PubMed]

23. Krieger, T.G.; Tirier, S.M.; Park, J.; Jechow, K.; Eisemann, T.; Peterziel, H.; Angel, P.; Eils, R.; Conrad, C. Modeling glioblastoma invasion using human brain organoids and single-cell transcriptomics. *Neuro-Oncology* **2020**, *22*, 1138–1149. [CrossRef] [PubMed]
24. Goranci-Buzhala, G.; Mariappan, A.; Gabriel, E.; Ramani, A.; Ricci-Vitiani, L.; Buccarelli, M.; D'Alessandris, Q.G.; Pallini, R.; Gopalakrishnan, J. Rapid and Efficient Invasion Assay of Glioblastoma in Human Brain Organoids. *Cell Rep.* **2020**, *31*, 107738. [CrossRef]
25. Qian, X.; Song, H.; Ming, G.L. Brain organoids: Advances, applications, and challenges. *Development* **2019**, *146*, dev166074. [CrossRef]