Chapter 11

A Synergistic Engineering Approach to Build Human Brain Spheroids

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Abstract

Self-assembling brain spheroids derived from human stem cells closely emulate the tangled connectivity of the human brain, recapitulate aspects of organized tissue structure, and are relatively easy to manipulate compared to other existing three-dimensional (3D) cellular models. However, current platforms generate heterogeneously sized and short-lived spheroids, which do not robustly and reproducibly model human brain development and diseases. Here, we present a method to generate large-scale arrays of homogeneously sized 3D brain spheroids derived from human-induced pluripotent stem cells (hiPSCs) or immortalized neural progenitor cells to recapitulate Alzheimer's disease (AD) pathology in vitro. When embedded in extracellular matrix, these brain spheroids develop extensive outward projection of neurites and form networks, which are mediated by thick bundles of dendrites. This array facilitates cost-effective, high-throughput drug screening and mechanistic studies to better understand human brain development and neurodegenerative conditions, such as AD.

Key words Brain spheroids, 3D cell culture, Alzheimer's disease, Disease modeling, High-throughput, Drug screening

1 Introduction

Neuronal differentiation in three-dimensional (3D) cell culture allows precise spatial and temporal control, facilitates synapse formation, neurite outgrowth, and emulates network connectivity of the brain compared to conventional 2D cultures [1, 2]. Besides emulating the 3D connectivity of the brain, 3D cell cultures prevent rapid diffusion of pathogenic proteins, which is key to modeling neurodegenerative proteinopathies, such as AD [3]. In brief, 3D cell culture systems are broadly classified into three categories (Fig. 1); (a) microfluidic-based microsystems, also known as organs-on-chips or microphysiological systems (MPS), emulate organ-level pathophysiology on a microscale [4]; (b) matrix-based models, rely on artificial extracellular matrices that promote neural

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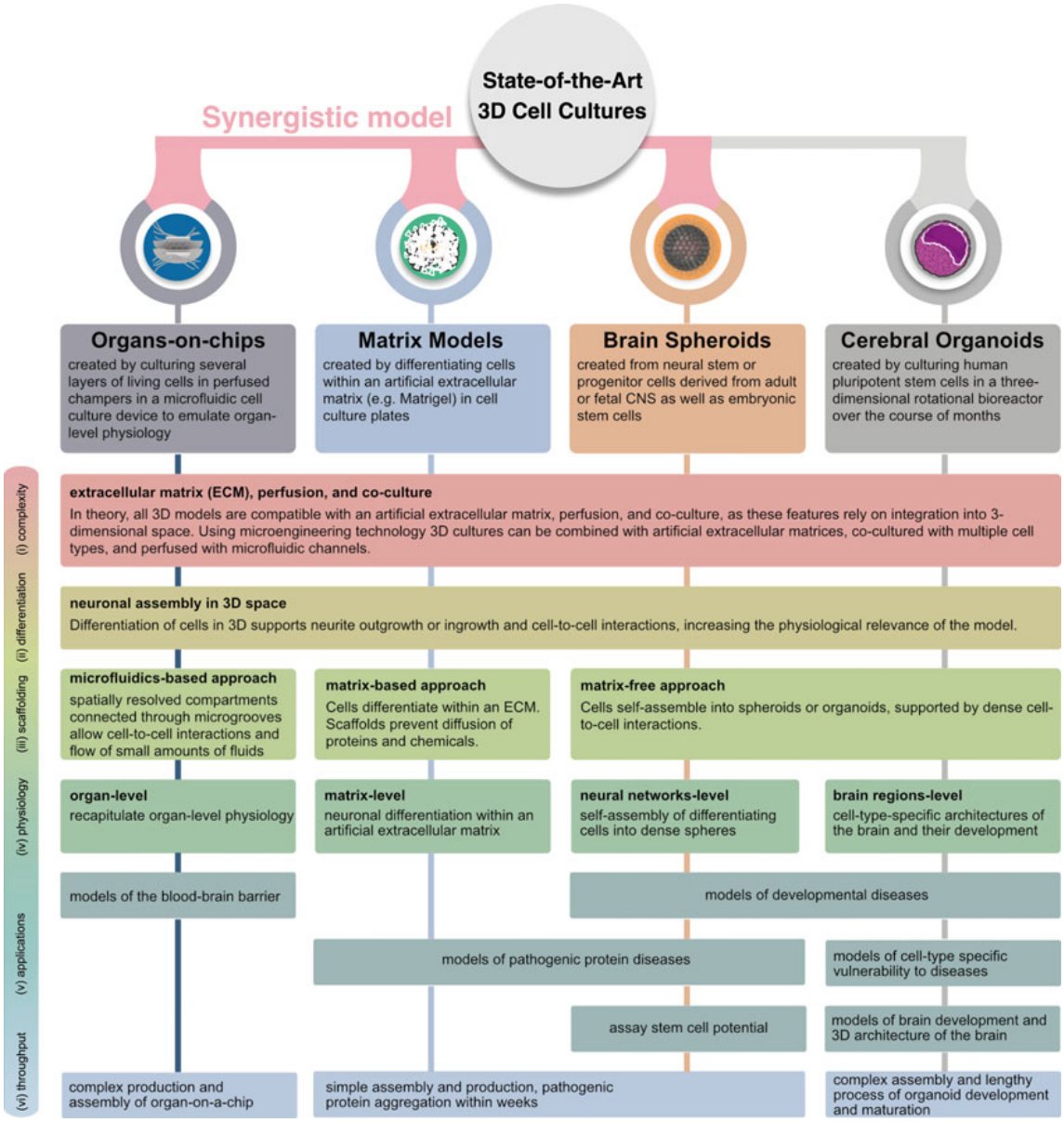

Fig. 1 Overview of state-of-the-art 3D cell culture systems and their main features. Our 3D brain spheroids array combines multiple features, including microfluidics technology, extracellular matrices, and the self-assembly of human stem cells

differentiation in 3D space [5]; and (c) matrix-free systems, including cerebral organoids and brain spheroids, rely on self-assembly of human stem cells into 3D spheroids [6–9]. A number of studies have begun to examine 3D cell culture models as a necessary alternative to AD rodent models and 2D cell cultures [10, 11]. For example, Choi et al. showed that genetically engineered human stem cells, embedded within an artificial extracellular matrix (i.e., Matrigel), recapitulated the two main pathological hallmarks of AD, including amyloid plaques and tauopathy

[11]. This has not been possible in 2D cell cultures and animal models, mainly due to diffusion of pathogenic proteins and differences in genetic background, respectively [12–14]. While an attempt to model AD pathology was also made using human-induced pluripotent stem cell (hiPSC)-derived brain spheroids, these spheroids were highly heterogenous in size and lacked robust pathology [14]. However, very recent progress toward hiPSC-derived organoids has provided substantial insights into the cellular basis of AD [15]. This is well-exemplified in the work undertaken by Lin *et al.*, which found an important contribution of the APOE4 AD risk allele to impaired glial clearance of AD pathology using an hiPSC-derived organoids model [16]. For further details on modeling neurological diseases in brain spheroids and organoids, readers are referred to recent reviews [7, 17].

Although brain spheroids constitute promising platforms to study brain development and diseases in vitro, these models are still in their infancy. Multiple limiting factors constrain robust disease modeling in existing systems, including (a) size heterogeneity, which limits reproducibility and data consolidation between studies, (b) inward projection of neurites, (c) short life spans of brain spheroids, likely due to the lack of pro-survival signals from a physiologically relevant matrix environment, and (d) diffusion of pathogenic proteins in the absence of an extracellular matrix. To overcome these limitations, we have merged microfluidic technology, brain spheroids derived from human stem cells, and an artificial extracellular matrix, to create a single synergistic model that combines multiple key advantages from recent advances in 3D systems (Fig. 1) [6]: Our microengineered array (a) consistently generates uniformly sized brain spheroids, (b) mimics the brain microenvironment via an extracellular matrix, which promotes outward projection of neurites and increases brain spheroid lifespan, (c) is compatible with hiPSCs and genetically engineered human neural progenitor cells (hNPCs) that generate high levels of aggregated pathogenic amyloid-β (Aβ) to model AD pathology, (d) facilitates high-throughput biochemical and imaging readouts as well as automated drug screening, (e) is straightforward to manipulate experimentally, and (f) enables monitoring of individual brain spheroids during long-term cell culture.

The brain spheroids array comprises 1536 microwells of 500 μm in diameter, which physically constrain uniformly sized spheroids derived from hiPSCs or hNPCs. After self-assembly, the brain spheroids are embedded within an extracellular matrix, which promotes extensive outward projections of neurites and neural network formation. The array is highly customizable to other neurobiological questions of interest. Spheroid size is easy to modify by altering microwell diameters, while the extent of network formation is controlled by changing the microwell depth. The formation of extensive neural networks unlocks numerous possibilities for

basic mechanistic studies, including disease pathology propagation and compound toxicity. The extracellular matrix limits diffusion of pathogenic proteins, which generates aggregated protein pathology associated with numerous neurodegenerative diseases, including AD. Using this novel array platform, we robustly recapitulated hallmarks of AD in uniformly sized brain spheroids derived from hNPCs that overexpress familial AD (fAD) genes with multiple disease mutations [6]. We also found reduced production of Aβ species by treating the fAD brain spheroids with β-secretase inhibitor, a commonly used inhibitor of APP processing and a primary candidate in AD therapeutics. Overall, this unique synergistic model constitutes a reliable platform for modeling specific disease phenotypes and cost-effective, high-throughput drug screening for new interventions. Moreover, the array is a promising substrate for the design of next-generation spheroid platforms to answer a range of niche neurobiological questions.

2 Materials

2.1 Array Platform

1. MakerBot Replicator 2 desktop 3D printer and ABS material.
2. AutoCAD and MakerBot Printing software.
3. SU8–100 photoresist (MicroChem Co.).
4. SU8 developer, BTS-220 (Doe & Ingalls).
5. Silicon wafer 6-in. (NOVA Electronic Materials).
6. High reflective chrome photomask (Front Range Photomask LLC).
7. Polydimethylsiloxane (PDMS)-Sylgard 184 Silicon (Ellsworth Adhesives).
8. Glass slides (Nexterion, Applied Microarrays, Inc.).
9. MTP 96-well superstructures and sealing film (Nexterion, Applied Microarrays, Inc.).
10. Plate lid (Nexterion, Applied Microarrays, Inc.).

2.2 Cell Culture

Carry out all procedures under a sterile biosafety cell culture hood.

1. 20 μg/mL human recombinant epidermal growth factor (EGF) stock solution: Filter 10 mM acetic acid through a 0.2-μm membrane. Add 2.0 mL of the filtered acetic acid to 2.0 mg of lyophilized EGF (Sigma-Aldrich; 1 mg/mL) and mix thoroughly. Make up a final concentration of 20 μg/mL by further diluting with 0.2 μm filtered 0.1% (wt/vol) bovine serum albumin (BSA) solution. Prepare 1.0 mL aliquots and store at −80 °C (*see* **Note 1**).

2. 25 μg/mL human recombinant basic fibroblast growth factor (bFGF) stock solution: Filter 10 mM Tris (pH 7.6) through a 0.2 μm membrane. Add 2.0 mL of the filtered Tris to 50 μg of lyophilized bFGF (Stemgent), mix thoroughly, and make 0.2 mL aliquots. Store aliquots at −80 °C (*see* **Note 1**).
3. ReN-cell proliferation medium: 484.5 mL DMEM/F12 medium, 0.5 mL heparin, 10 mL B27, 5.0 mL 100× penicillin/streptomycin, 0.5 mL amphotericin B, 0.5 mL EGF (20 μg/mL, Sigma-Aldrich), 0.4 mL bFGF (25 μg/mL, Stemgent).
4. hiPSC proliferation medium: 99 mL ENStem—A neural proliferation medium, 1.0 mL EmbryoMax L-Glutamine solution (100×, 200 mM), 1.0 mL 100× EmbyroMax ES Cell-Qualified Penicillin—Streptomycin solution (EMD Millipore).
5. ReN-cell differentiation medium: 484.5 mL DMEM/F12 medium, 0.5 mL of heparin, 10 mL of B27, 5.0 mL 100× penicillin/streptomycin, and 0.5 mL amphotericin B.
6. ENStem.
7. IRES-mediated polycistronic lentiviral vectors CSCW-GFP (control), CSCW-APPSL-GFP (AD), and CSCW-APPSL-PS1Δe9-mCherry (AD) (all constructs are available through the corresponding author). For more details on the constructs, readers are referred to ref. [5].
8. Human iPSC-derived neural progenitor cells (EMD Millipore).
9. Matrigel (Corning).
10. Matrigel-coated vented T25 cell culture flasks: Add 3 mL of Matrigel:DMEM-F12 medium (1:100 dilution) to the bottom of each vented T25 flask, shake gently to cover the surface, and incubate at 37 °C for at least 1 h. Aspirate the media and store flasks at 4 °C until use (*see* **Note 2**).
11. Accutase (Life Technologies).
12. Phosphate-buffered saline (PBS).
13. ReN-cell neural stem cell freezing medium (EMD Millipore).
14. Cell counting machine, cell counting slides, and Trypan Blue (Invitrogen).

2.3 Immunohistochemistry

1. Blocking solution: Add 2.5 g of BSA (Sigma-Aldrich), 5.63 g of glycine, and 0.25 g of gelatin to 200 mL of Tris-buffered saline (TBS-T, Boston BioProducts). Incubate the solution at 55 °C for ~10 min for gelatin to dissolve. Add 10 mL of donkey serum (Sigma-Aldrich) and TBS-T to a final volume of 250 mL. Filter the solution through a 0.4-μm filter unit and store at 4 °C.

2. Primary and secondary antibodies:
   (a) Anti-MAP 2 (EMD Millipore, AB5543, 1:500).
   (b) Anti-Tuj1 (Sigma, T2200, 1:100).
   (c) Anti-DCX (Abcam, Ab135349, 1:200).
   (d) AT-8 (ThermoFisher Scientific, MN1020, 1:30).
   (e) β-amyloid (1–42 specific) (Cell Signaling Technology, D9A3A, 1:200).
   (f) Alexa Fluor 488 Secondary antibody (Abcam, Ab150077, 1:200).
   (g) Alexa Fluor 568 Secondary antibody (Life Technology, A-11041, 1:400).
   (h) Cy5 Secondary antibody (Jackson ImmunoResearch, 715–175–150, 1:200).
   (i) Cy5 Secondary antibody (Jackson ImmunoResearch, 711–175–152, 1:200).
3. 4% Paraformaldehyde.
4. Nikon Eclipse Ti microscope.
5. ImageJ software.

2.4 Scanning Electron Microscopy

1. Aluminum Mounts.
2. Conductive Adhesive Tabs (double-sided adhesion).
3. SEM Pin Stub Mount Gripper 45° angle (TED PELLA, INC.).
4. Universal Specimen Mount Holder.
5. Glutaraldehyde 2.5% in 0.1 M Phosphate Buffer.
6. Hexamethyldisilazane.
7. Osmium tetroxide solution 4% in H_2O.
8. Ultra55 Field Emission Scanning Electron Microscope.

2.5 ELISA

1. Human amyloid-β ELISA Kit (Wako Pure Chemicals).
2. MesoScale Discovery 96-well Mouse Pro-Inflammatory V-PLEX Assay (Meso Scale Discovery).
3. Synergy 2 ELISA plate reader (BioTek Instruments).
4. Meso QuickPlex SQ 120 (Meso Scale Discovery).

3 Methods

3.1 Fabricating the Brain Spheroids Array

All designs can be generated using AutoCad software. All designs in this chapter are available through https://github.com/jorfilab/brainspheroids. An overview of the fabrication process is shown in Fig. 2a.

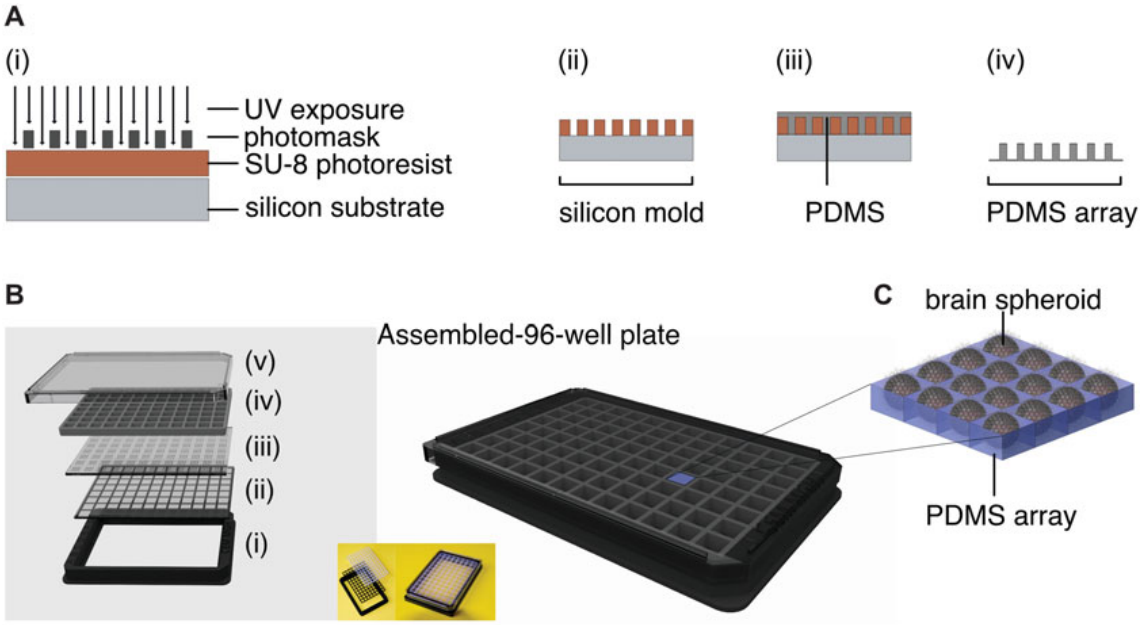

Fig. 2 Microfabrication process of the 3D brain spheroids array. (**a**) Generation of the PDMS brain spheroids array using soft lithography technique. (*i*) Expose masked SU8–100 photoresist to UV light. (*ii*) Remove the unexposed photoresist. (*iii*) Cast PDMS over silicon mold. (*iv*) Remove cured PDMS from mold. (**b**) The assembled 96-well array has five components: (*i*) a 3D designed and printed 96-well frame, (*ii*) a high-quality glass with transmittance of over 92% and high optical clarity for fluorescence wavelengths, (*iii*) a microfabricated PDMS microwell array of 1536 microwells, (*iv*) a 96-well silicon superstructure that groups 16 microwells into a single superstructure well, and (*v*) a plate lid. Adapted with permission [6]. Copyright 2018, Nature Publishing Group. (**c**) A single superstructure well in a 96-well plate holds 16 brain spheroids, each cultured in a 500-μm diameter microwell in the PDMS array

3.1.1 Master Fabrication

1. Design the microfluidic spheroids array using AutoCad software and print it onto the chrome photomask.
2. Dehydrate a silicon wafer in an oven at 250 °C for 30 min. Allow the silicon wafer to cool down to room temperature before proceeding to the next step.
3. Plasma-treat the silicon wafer using an oxygen plasma machine (March Plasma System) for 3 min at 100 watts.
4. After plasma treatment, blow-dry the silicon wafer with nitrogen gas, place it onto the spinner chuck, and turn on the vacuum.
5. Cover the silicon surface with ~5 mL of SU8–100 and spin the silicon wafer at 600 rpm for 5 s, followed by spinning at 1650 rpm for 30 s. This spinning protocol produces a photoresist thickness of ~200 μm.
6. Place the coated silicon wafer onto a hot plate. Soft-bake the wafer at 70 °C for 25 min, followed by baking at 100 °C for 80 min. Allow the coated silicon wafer to cool down to room temperature before proceeding to the following step.

7. Repeat **steps 5** and **6** three times to achieve ~600 μm photoresist thickness suitable for the 500 μm brain spheroids array (*see* **Note 3**).
8. Place the coated silicon wafer onto the exposure stage, facing the UV lamp. Place the photomask with the microwell design onto the exposure stage. Open the shutter of the UV lamp and expose the photoresist for 80 s.
9. Place the exposed silicon wafer onto a hot plate, with the SU8–100 coating facing upwards. Post-bake the silicon wafer at 70 °C for 1 min, followed by baking at 100 °C for 16 min. Allow the coated silicon wafer to cool down to room temperature.
10. Develop the exposed silicon wafer using the developer solution for ~1 h, followed by developing in a fresh developer for another 2 min. Rinse the silicon wafer with fresh developer and blow-dry using nitrogen gas.
11. Place the silicon wafer onto the exposure stage with the SU8–100-coated surface facing the UV lamp. Place a clear photomask or glass on top of the silicon wafer. Post-expose the photoresist for another 65 s in case of uncompleted reactions initiated during the first exposure.
12. Place the flood-exposed silicon wafer onto a hot plate with the coated layer on top. Post-bake the wafer at 70 °C for 2 min, followed by baking at 100 °C for 6 min. Allow the coated silicon wafer to cool down to room temperature and store for later use.

3.1.2 PDMS Mold Fabrication

1. Prepare 10:1 PDMS by combining 40 g of Sylgard 184 base with 4 g of curing agent in a plastic dish. Mix thoroughly and degas the mixture for 2–3 h inside a desiccator connected to a vacuum to remove all air bubbles generated during mixing.
2. Gently pour the PDMS mixture over the silicon wafer mold to avoid generation of new air bubbles.
3. Bake the PDMS-coated silicon wafer in an oven at 75 °C for 12 h.
4. Cut out the PDMS spheroids array from the silicon wafer substrate using a sharp blade.
5. Place the PDMS spheroids array block (containing 1536 microwells, 500 μm in diameter) on a clean tray inside the oxygen plasma machine with the microwells facing down. Place the glass slide onto the same tray and expose both surfaces to plasma for 70 s at 50 watts.
6. Invert the PDMS spheroids array block and bond it to the glass slide. Bake the bonded PDMS-glass slide in a 70 °C hot plate for 20 min.

3.1.3 Brain Spheroids Array Assembly

1. Print the 96-well frame plate using MakerBot Replicator 2 desktop 3D printer and ABS material following the vendor protocol.
2. Assemble the 96-well plate as shown in Fig. 2b. Note that each superstructure well supports the formation of 16 brain spheroids (Fig. 2c).
3. Store the plate in a clean environment for future use (*see* **Note 4**).

3.2 Generating the Genetically Engineered AD hNPCs

1. Package lentiviral vectors. Transfect ReN cells with CSCW constructs (*see* **Note 5**). Enrich the transfected cells for top 2% GFP/mCherry-expressing cells using fluorescence-activated cell sorting (FACS).
2. Generate clonal control and fAD ReN cell lines by FACS-assisted 96-well single-cell cloning into Matrigel-coated 96-well plates (Fig. 3).
3. Sequentially expand the cells in 6-well plates, T25, and T75 flasks. Change the proliferation medium twice a week.

   Once cells reach confluency, aspirate media, wash cells with PBS to remove dead residual cells, incubate cells in Accutase (0.5 mL for T25 and 1.5 mL for T75) at 37 °C until cells are dissociated from the plate, and neutralize the reaction with an equal volume of proliferation medium. Centrifuge cells at 1500 rpm for 3 min. Re-suspend cell pellets in corresponding freezing medium and transfer cells into appropriate freezing vials to store in liquid nitrogen for later use.

3.3 Maintaining ReN Cells and hiPSCs

Carry out all procedures under a sterile biosafety cell culture hood.

1. Quickly thaw control, AD ReN cells, and hiPSCs by placing frozen stocks into a bead bath at 37 °C. Equilibrate thawed cells into proliferation medium by slowly adding a small volume of appropriate proliferation medium directly to the cells. Pipette up and down and transfer the entire solution to a new

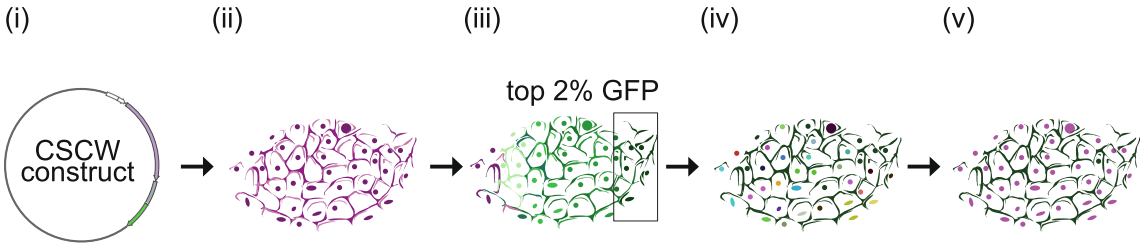

Fig. 3 Workflow to generate single-clonal fAD-ReN cell lines. (*i*) fAD mutations are introduced into the CSCWW-APP-(PS1)-GFP construct by site-directed mutagenesis. (*ii*) hNPCs are transfected with the construct. (*iii*) Transfected hNPCs are FACS-enriched by GFP signal. (*iv*) The sorted cells are heterogeneous for genomic integration of the construct. (*v*) Single-cell-FACS enrichment generates single-clonal hNPCs

tube. Centrifuge cells at 1500 rpm for 3 min, aspirate the supernatant, and re-suspend the cell pellets in 4–7 mL pre-warmed proliferation medium.

Transfer the cells to a matrigel-coated vented T25 flask and place in a cell culture incubator at 37 °C and 5% CO_2 (*see* **Notes 6** and 7).

2. Maintain cells at 37 °C in proliferation medium with full biweekly medium changes until the cells become confluent. Proliferation medium may be changed more frequently if cells are dividing slowly.

3.4 Generating Brain Spheroids from Stem Cells

Carry out all procedures in a sterile biosafety cell culture hood to avoid contamination.

1. Once cells have reached 90–100% confluency, aspirate medium, wash cells with PBS, and incubate cells with Accutase (0.5 mL for T25 and 1.5 mL for T75) at 37 °C until cells are dissociated from the flask. Then, neutralize Accutase with an equal volume of proliferation medium. Centrifuge the cell mixture and resuspend the cell pellets in 5 mL of fresh proliferation medium. Mix cells thoroughly by pipetting up and down so that cells are evenly dispersed and cell counting is accurate.
2. Dilute the cells 1:10 by adding 10 μL of resuspended cells to 90 μL of medium and mix thoroughly. Further dilute the cells 1:1 with Trypan Blue, by mixing 15 μL of cells with 15 μL of Trypan Blue and mix thoroughly. Dispense 10 μL of the solution into each side of the cell counting chip. Count the cells in each side of the chip using an automated cell counter, average the values, and adjust for the dilution (*see* **Note 8**). Centrifuge the cells at 1500 rpm for 3 min. Resuspend the cells in appropriate volume of proliferation medium to achieve a final concentration of 12×10^6 cells/mL (*see* **Note 9**).
3. If the cell count is insufficient, cells must be expanded further. To passage the cells for further proliferation. Following **step 1**, resuspend the cell pellets in proliferation medium and transfer them into three Matrigel-coated T25 flasks. Keep changing medium until the cells become confluent. Count the cells as described in **step 2** and proceed with the following steps.
4. Plate 100 μL of the cells from **step 2** into each suprastructure well of the brain spheroids array (Fig. 4, **Step 1**).
5. After 20 min, add 200 μL of proliferation medium against the wall of each suprastructure well. Aspirate the medium to remove floating cells from the wells and add fresh 200 μL of proliferation medium to each well (Fig. 4, **Step 2**).

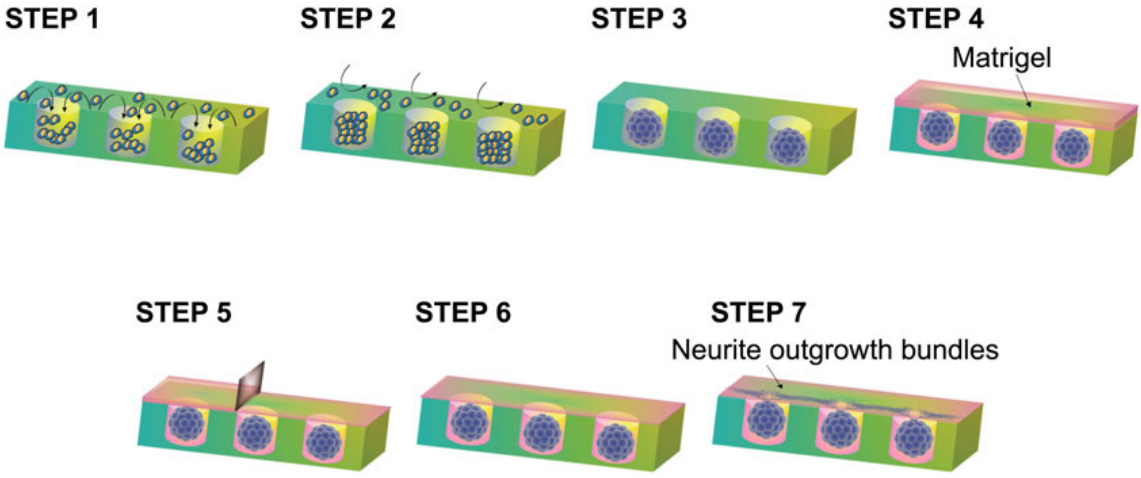

Fig. 4 Workflow for plating stem cells in the 3D array platform. **Step 1**. Plate stem cells into the array. **Step 2.** Once cells aggregate, remove floating cells. **Step 3.** Allow 3D spheroids to form during incubation. **Step 4.** Cover array with extracellular matrix. **Step 5.** Remove excess matrix. **Steps 6** and **7**. Allow spheroids to differentiate and form neurite projections during incubation

6. Incubate the arrays at 37 °C and 5% CO_2 atmosphere (Fig. 4, **Step 3**). Change the proliferation medium after 24 h and incubate the arrays at 37 °C and 5% CO_2 atmosphere for another 24 h to generate fully formed brain spheroids.
7. After 48 h, aspirate the entire proliferation medium and apply 100 μL of 1:4 Matrigel:differentiation medium (for ReN-derived spheroids) or 1:1 proliferation:differentiation medium (for hiPSC-derived spheroids) to each well to cover the whole surface area (Fig. 4, **Step 4**). Incubate at 37 °C for 2 h to allow Matrigel to solidify (*see* **Note 10**).
8. Remove excess Matrigel from the surface of the brain spheroids array by gently scraping a gel loading tip tilted at 90° across the surface of the PDMS array (Fig. 4, **Step 5**). Replace the media with 200 μL fresh pre-warmed differentiation medium.
9. Maintain the cell culture up to 8 weeks, changing half of the differentiation media every 4 days.
10. OPTIONAL: For applications that require the removal of the brain spheroids from the arrays, following **step 7**, place the array in a 6-well plate tilted at 45° and spray proliferation medium over the array using a 1-mL tip. Repeat this step three times to extract all the spheroids from the arrays. Images can be captured using the Z-stacking function on an inverted Nikon Eclipse Ti microscope, to allow visualization of full brain spheroids after removal (*see* Fig. 5a). Process images for quantification of spheroid diameters using ImageJ software (Fig. 5b).

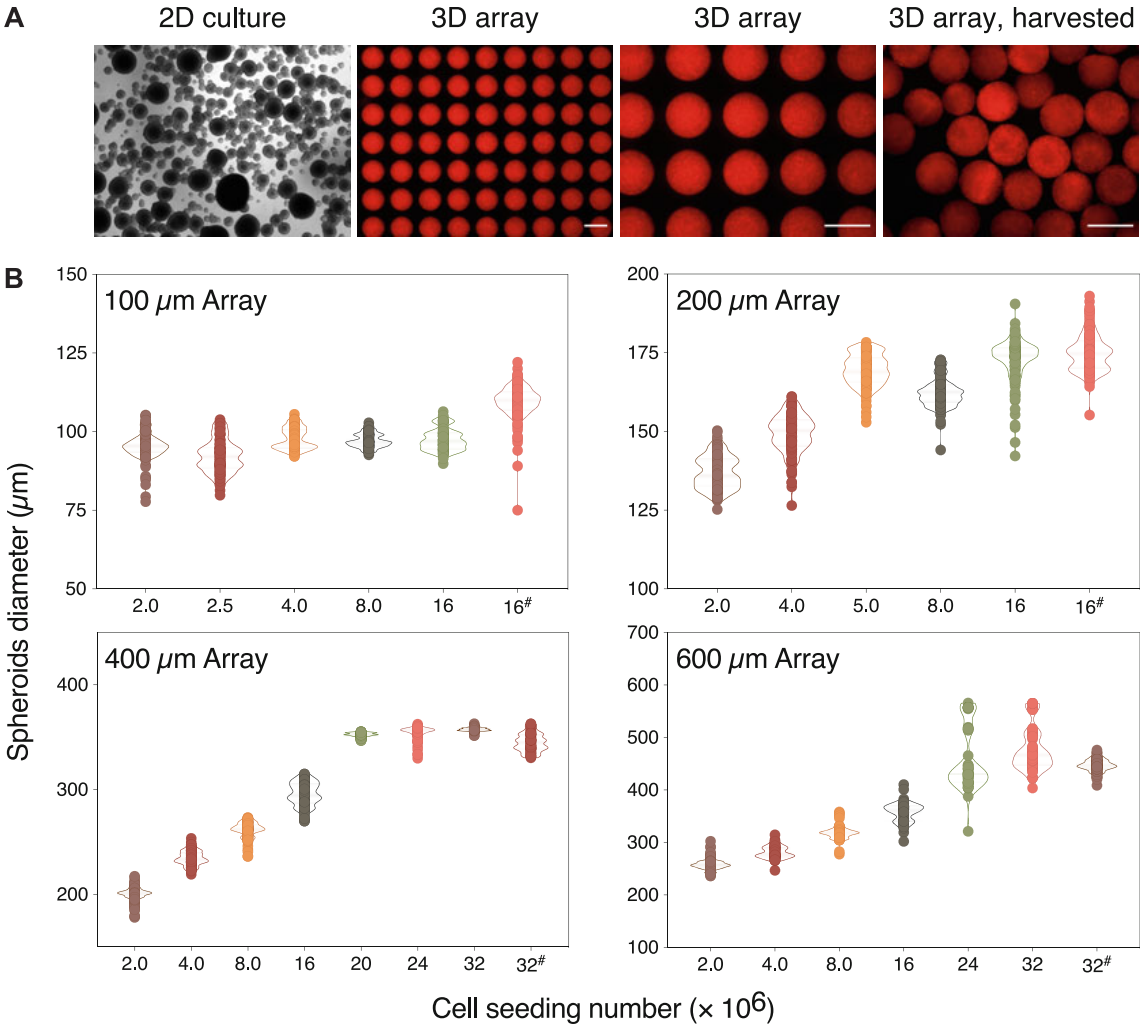

Fig. 5 Generation and recovery of uniformly sized brain spheroids in high-throughput arrays. (**a**) Confocal images show plated and harvested homogenously sized brain spheroids generated in the 3D array platform compared to conventional 2D platforms. Scale bars indicate 400 μm. (**b**) Violin plots show the relationship between cell seeding concentration, microwell diameter, and spheroid diameter after a 24-h formation period in the 3D array platform. $n > 100$ spheroids for each condition

3.5 Application I: Imaging Brain Spheroids Morphology

1. Rinse plates with PBS and fix in 4% paraformaldehyde (PFA) overnight at 4 °C. Wash plates three times with 1× Tris Buffered Saline with Tween 20 (TBS-T). Keep cells overnight at 4 °C in blocking solution. Then, permeabilize the cellular membrane of the cells with 0.1% Triton X-100 for 45–60 min at room temperature. Next, wash the cells three times with 1× TBS-T, leaving each wash in for 15 min. Incubate the brain spheroids with primary antibodies in blocking solution at 4 °C overnight, followed by incubation with species-specific secondary antibodies at 4 °C overnight in the dark. Alternatively, after secondary antibody staining and washing three times with 1× TBS-T, cell nuclei can be stained with Hoechst 3342 (1:2000 dilution) for 20 min at room temperature. Following staining

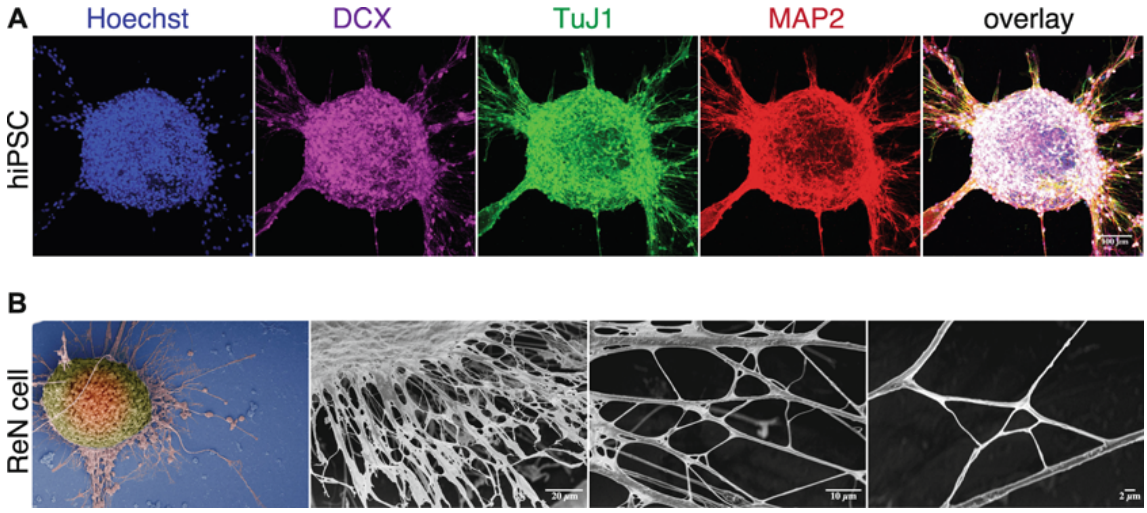

Fig. 6 Uniformly sized brain spheroids generated in the 3D array form extensive neurite networks. (**a**) Representative confocal images show expression of neuronal markers (MAP-2, microtubule-associated protein 2; TuJ1, ß-tubulin III; and DCX, doublecortin) and nuclei (Hoechst) in hiPSC-derived spheroids at 2-weeks differentiation in the 3D array platform. (**b**) Scanning electron microscopy images for control and fAD ReN cells differentiated as spheroids in the 3D array platform for 8 weeks. Outward neurite projections enable formation of spheroid networks (right three panels). Reproduced with permission [6]. Copyright 2018, Nature Publishing Group

of the brain spheroids, a series of images can be captured using the Z-stacking function on an inverted Nikon Eclipse T*i* microscope, to allow visualization of full brain spheroids (Fig. 6a) (*see* **Note 11**). Process images using ImageJ software.

2. To visualize brain spheroids using electron microscopy, fix the differentiated spheroids in 2.5% glutaraldehyde for 30 min, wash three times with PBS, and then post-fix in aqueous 1% OsO_4 in a certified chemical hood with personal protective equipment for 60 min (*see* **Note 12**). Then, wash the brain spheroids with PBS three times, leaving each wash in for 15 min. Dehydrate through a graded series of ethanol diluted in PBS (30–90% v/v) for 15 min each, followed by washing with absolute ethanol three times before drying in hexamethyldisilazane solution. Air-dry under safety hood overnight. Mount the arrays with fixed brain spheroids onto aluminum stubs, sputter-coat with 5 nm of platinum/palladium, and image in an Ultra55 Field Emission Scanning Electron Microscope at 10 kV with In lens SE detector (Fig. 6b).

3.6 Application II: Monitoring Pathophysiology

1. Immunohistochemical detection of Aβ and tau pathology: Follow **step 1** in Subheading 3.5 for fixing, permeabilizing, and blocking the brain spheroids. Next, choose appropriate anti-Aβ and anti-phosphorylated tau primary and secondary antibodies (*see* Subheading 2.3, **item 2**) to immunostaining the brain spheroids for Aβ and tau pathology (Fig. 7a, b).

2. Quantify Aβ isoform levels using Human Amyloid-β ELISA Kit: Collect the conditioned media from differentiated ReN-brain spheroids at the timepoint of interest and dilute 1:2 in the provided dilution buffer. Use a Synergy 2 ELISA plate reader to quantify Aβ40 and Aβ42 fluorescent signals (Fig. 7c).

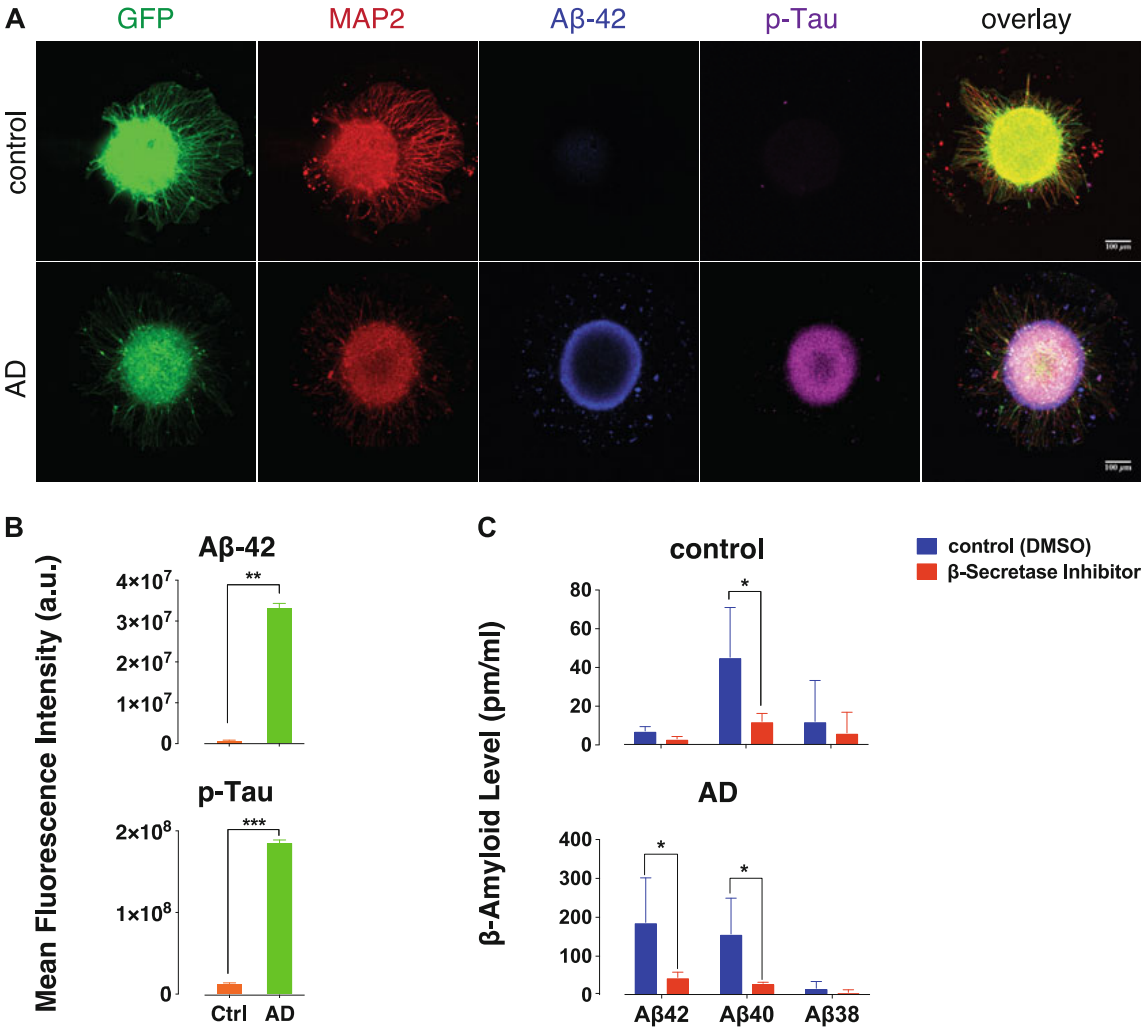

Fig. 7 Modeling AD pathology in the 3D brain spheroids array. (**a**) fAD ReN-cell-derived spheroids were differentiated in the array for 8 weeks and stained for Aβ and phospho-Tau (p-Tau). Pathological hallmarks show distinct localization throughout the spheroids, with Aβ aggregated along the perimeter of the spheroids and p-Tau present throughout the spheroids. (**b**) MAP 2, Aβ42, and p-Tau levels quantified by immunofluorescence in control and AD ReN-cell spheroids. (**c**) Aβ42, Aβ40, and Aβ38 levels in the media quantified by ELISA for control and AD ReN cells differentiated for 7 weeks in the array platform and treated with β-secretase inhibitor. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$; ANOVA followed by a post hoc Dunnett's test; means ± SEM; $n = 6$ per each sample. Reproduced with permission [6]. Copyright 2018, Nature Publishing Group

3. Quantify Aβ isoform levels using MesoScale Discovery 96-well Mouse Pro-Inflammatory V-PLEX Assay: Add 150 μL of provided diluent to the plate, which is coated with an array of Aβ capture antibodies. Incubate the plate at room temperature while shaking for 1 h. Wash the plate with the washing buffer. Add 25 μL of detection antibody solution and 25 μL of the prepared samples to the plate according to the manufacturer's protocol. Incubate the plate at room temperature for 2 h with vigorous shaking. Wash the plate in washing buffer and add 150 μL of 2× Read Buffer T. Immediately read the fluorescent signal on a Meso QuickPlex SQ 120.
4. Aβ levels in the media can be quantified by ELISA at various timepoints to monitor the effect of treatment (*see* Subheading 3.7, **Application III**) with active compounds on levels of secreted pathogenic proteins in the media (Fig. 7c).

3.7 Application III: High-Throughput Drug Screening of AD Brain Spheroids with Automated Readouts

1. Plate control and fAD ReN-cells into the brain spheroids array platform at a concentration of 12×10^6 cells/mL in proliferation medium.
2. After 20 min, add 200 μL of proliferation medium against the wall of each suprastructure well. Aspirate the medium to remove floating cells from the wells and add fresh 200 μL of proliferation medium.
3. Incubate the arrays at 37 °C and 5% CO_2 atmosphere for 48 h to allow full spheroids formation.
4. Add drugs of choice to the proliferation medium to achieve the desired concentration. Treat the brain spheroids with different drugs at varying concentrations 2 days after plating (Fig. 8). Make fresh working stock for each media change.
5. Repeat the drug treatment after 4 days.
6. Maintain the brain spheroids for 7 days at 37 °C and 5% CO_2 atmosphere. After 7 days, rinse the brain spheroids with PBS and fix in 4% PFA overnight at 4 °C. Wash three times in PBS for 15 min each, and leave 200 μL PBS in each well.
7. For high-throughput drug screening, set up an automated imaging-job on the Nikon Eclipse T*i* microscope. Since the x and y dimensions of each microwell are known and the brain spheroids are homogenous in size, confocal images of the 1536 spheroids (16 brain spheroids per well in a 96-well plate) can be acquired in an automated high-throughput manner. Toxicity readouts, including brain spheroid diameters and neurite extensions, can also be quantified as part of this automated process through postprocessing, using ImageJ software (Fig. 9).

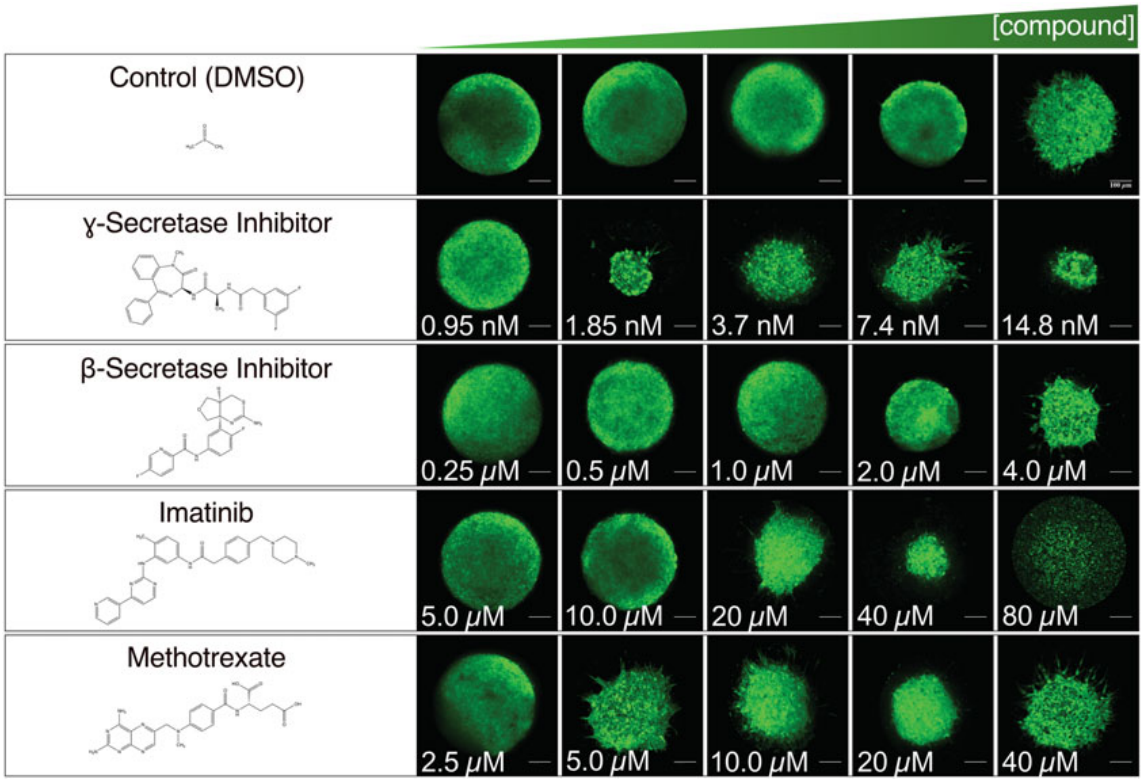

Fig. 8 Monitoring the effect of active compounds on AD ReN-cell-derived spheroids cultured in the 3D array platform. Confocal images of representative brain spheroids after a 7-day drug treatment with DMSO, ɣ-secretase inhibitor (Compound E), β-secretase inhibitor (LY2886721), Imatinib, or Methotrexate at five different concentrations. Reproduced with permission [6]. Copyright 2018, Nature Publishing Group

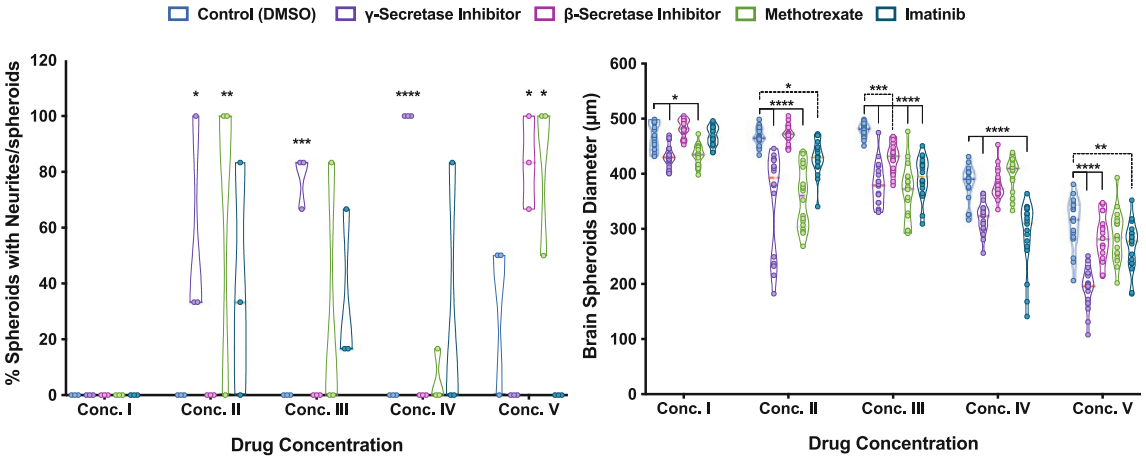

Fig. 9 Quantifying the effect of active compounds on AD ReN-derived spheroids cultured in the 3D array platform. Quantification of neurite number and brain spheroids diameter after 7-day drug treatments from Fig. 8. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$; ANOVA followed by a post hoc Dunnett's test; means $\pm$ SEM; $n = 6$ per each sample. Adapted with permission [6]. Copyright 2018, Nature Publishing Group

4 Notes

1. Stock aliquots can be stored for up to 1 year at −80 °C. Once thawed, store aliquots at 4 °C and use within 2–3 weeks to avoid activity loss associated with multiple freeze–thaw cycles.
2. Take out Matrigel stock from −80 °C freezer and store at 4 °C 1 day prior to experiment to give the gel plenty of time to thaw. Make fresh working solution each time. Precool pipettes in cold DMEM-F12 medium prior to taking up Matrigel to prevent the gel from solidifying. Keep Matrigel and Matrigel: DMEMF12 solution on ice throughout coating. Make sure the Matrigel:DMEMF12 solution covers the base of each T25 flask evenly prior to incubating. Matrigel-coated flasks can be stored for up to 2 months at 4 °C.
3. The spheroids array design can be customized using AutoCAD software according to specific needs and interests. For example, one can modify the spheroids array diameter, spheroid–spheroid distance, number of spheroids per well, or add connections between the spheroids. Repeat **steps 5** and **6** (Subheading 3.1.1) once to achieve ~200 μm photoresist thickness that is suitable for the 100 and 200 μm brain spheroids arrays or twice to achieve ~400 μm photoresist thickness that is suitable for the 400 μm brain spheroids array.
4. Place the assembled 96-well brain spheroids plate without a lid onto a tray inside the oxygen plasma machine and expose the array surface to plasma for 70 s at 50 watts before adding the cell suspension to the array.
5. Cells were transfected with the CSCW-APPSL-GFP construct, encoding full-length β-amyloid precursor protein (APP695) with the London (V717F), Swedish (K670N/M671L) mutations, and GFP reporter gene, the CSCW-APPSL-PS1Δe9-mCherry construct, additionally encoding the PS1 gene with the Δe9 mutation, or a control CSCW-GFP control construct as described in ref. [11].
6. Thaw cells quickly in the water bath (37 °C) until no crystals remain. Add proliferation medium to the thawed cells in small increments, while gently shaking the vial to disperse the medium and avoid cell membrane rupture. Keep the cells on UV-sterilized ice whenever possible.
7. Excess cells may be frozen down. Alternatively, the cell pellet may be split into multiple flasks. Ensure that flasks are not overpopulated with cells as they may reach confluency too quickly. Once cells settle, they should occupy about one-third of the surface area so that cells have plenty of room to proliferate.

Under these conditions, ReN cells usually take 2–3 days to become confluent. A single 95% confluent T25 flask yields ~2–3 $\times$ 10^6 cells.

8. Trypan Blue stains dead cells, allowing the cell counter to generate a "live," "dead," and "total" cell count. When using Trypan Blue, average the live cell counts from both readings. Please note that the cell counter may be set to automatically account for the 1:1 Trypan Blue dilution. If the counts are discrepant by $>10^6$ cells, mix cells thoroughly before recounting.
9. To optimize the cell density in brain spheroids arrays with different microwell diameters, resuspend the cells in an appropriate volume of proliferation medium to achieve a final concentration of 2, 2.5, 4, 8, 16, 20, 24, or 32 $\times$ 10^6 cells/mL.
10. This is a key step that prevents brain spheroids from diffusing out of the microwells.
11. The excitation wavelengths of mCherry and anti-chicken Alexa Fluor 568 antibodies (MAP 2 staining) partially overlap. However, the major differences in signal strength allows differentiation between the MAP 2 signal versus mCherry.
12. Osmium tetroxide (OsO_4) is an oxidizing and highly toxic material. OsO_4 solution must be handled in a certified chemical hood with personal protective equipment (PPE) including lab coat, protective gloves, and eye/face protection. Read thoroughly the material safety data sheet (MSDS) before using the OsO_4 solution. To dispose the leftover OsO_4 solution, neutralize it by twice its volume of vegetable oil (e.g., corn oil) by pouring the oil into the OsO_4 solution and wait for the oil to completely turn black.

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