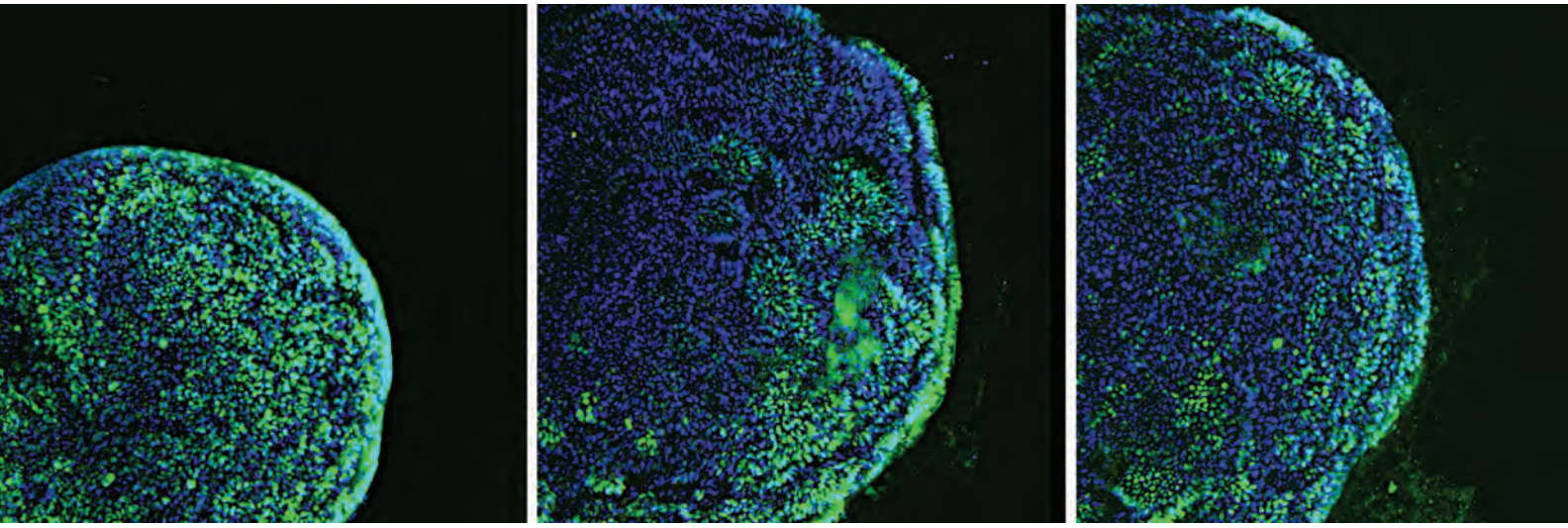


3D midbrain organoid model development

Floor plate differentiation of iPSC-derived dopaminergic neurons in 3D culture.

The drug development path—from hit discovery and lead advancement to the clinical space and commercialization—relies on accurate experimental data that are predictive of outcomes in downstream preclinical and clinical settings. Increasing the complexity of disease models with 3D organoids in the early stages of drug discovery is an approach that provides a more relevant cellular context, potentially more accurate physiological data in orthogonal experiments, and improved chances of eliminating false-positive hits during high-throughput screening. To realize these benefits, however, complex disease models must exhibit a similar cellular makeup and function as the organ under investigation. The development of 3D organoids that better represent the corresponding organ complexity enables researchers to better scale experiments, make data-driven decisions, and systemize those processes to speed discovery. →

Figure (above). ECM addition during floor plate specification supports prominent rosette-like structures in early organoids. See details in Figure 3 caption.

Highlighted here are some of the first steps in generating a functional 3D ventral midbrain neural organoid for modeling Parkinson's disease (PD). Achieving an organoid model of the brain—with the correct cell types that interact with each other and develop toward mature functionality—depends on many carefully defined parameters. These include physical factors such as those defined by basement membrane matrices, culture conditions, and spatial properties, as well as experimental factors such as how to best leverage the capabilities that advanced gene editing and imaging technologies provide. In the case of neurological diseases such as PD, research is hindered by a lack of access to diseased tissue. To model PD, we have developed a method for differentiating human induced pluripotent stem cells (iPSCs) to midbrain dopaminergic (DA) neurons, while also incorporating CRISPR technology to engineer iPSC-derived organoids such that they harbor either the PD-linked α-synuclein A30P mutation or its unaltered wild-type counterpart.

Comparison of 2D and 3D floor plate specification

Optimizing the conditions for organoid growth and structure is not a straightforward task, but with the right tools and technologies, *in vitro* disease models can be reproducibly generated at large scale. The Gibco™ PSC Dopaminergic Neuron Differentiation Kit enables the specification of iPSCs to midbrain floor plate cells. This kit is a set of three components (Floor Plate Specification Supplement, Floor Plate Cell Expansion Base and Supplement, and Dopaminergic Neuron Maturation Supplement) optimized for 2D midbrain floor plate specification, expansion, and maturation. In this discussion we demonstrate the use of this kit with and without an extracellular matrix (ECM) to specify floor plate cells and further differentiate DA neurons in 3D suspension culture. The schematic in Figure 1 describes the parallel floor plate derivation and DA maturation processes for 2D and 3D cultures.

The initial 3D workflow was kept as similar as possible to the optimized 2D schedule of passages and medium changes. In the 3D scenario, human iPSC spheroids in rotating suspension culture were dissociated and seeded into low-attachment 96-well, U-bottom (96U-well) microplates for floor plate specification in static suspension, then changed into expansion medium and maturation medium while in suspension without further passaging. As shown in Figure 1, the time requirements for specification and expansion in 3D were significantly reduced, with equivalent expression of floor plate markers. As expected, iPSCs that underwent 3D neural differentiation—based on their self-organization of progeny cells into organoids with brain-like structures and function—exhibited phenotypes not observed in 2D culture.

Organoid culture in the absence and presence of extracellular matrices

Attempts to improve the complexity of brain-like organoids are often accompanied by decreases in throughput and reproducibility, both of which impact research results and the scalability of disease models for drug discovery. As described in Figure 2, a comparison of four different 3D organoid culture methods was performed to assess early spheroid morphologies during differentiation (i.e., in rotating suspension versus U-well microplates; and without ECM, encapsulated in ECM, or suspended in ECM). To this end, cultures were grown in the absence of

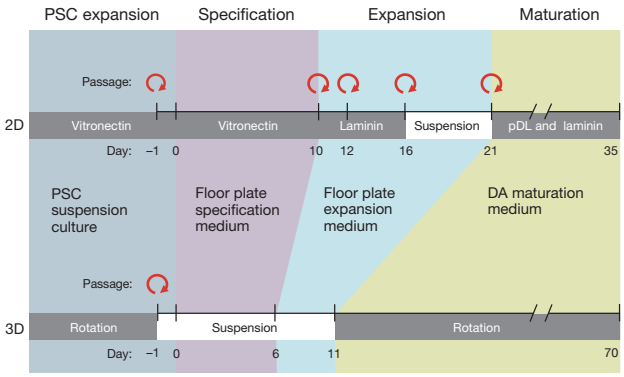
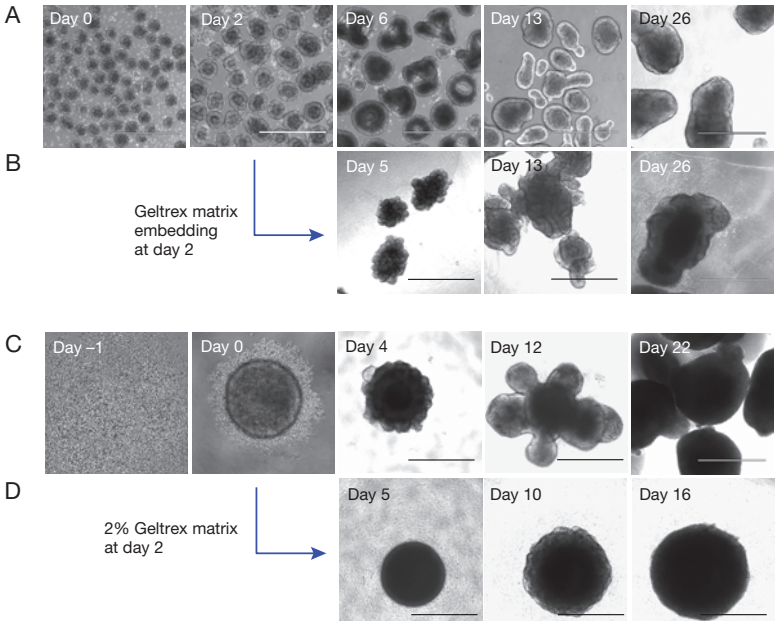


Figure 1. Comparison of 2D and 3D floor plate derivation processes using three different media prepared with the components provided in the PSC Dopaminergic Neuron Differentiation Kit. Starting with a human iPSC culture growing in Gibco™ Essential 8™ Medium (Cat. No. A1517001), we used the Gibco™ PSC Dopaminergic Neuron Differentiation Kit (Cat. No. A3147701) for the 2D and 3D floor plate derivation process. The top workflow shows 2D floor plate specification of an attached human iPSC culture, followed by multiple passages in expansion medium until day 21. Floor plate cells are then passaged onto poly-D-lysine (pDL) and laminin for differentiation of DA neurons up to day 35. The bottom workflow shows 3D midbrain organoid formation, beginning with human iPSCs in rotating suspension culture. These cells are dissociated and seeded into suspension culture for 3D floor plate specification. The floor plate specification medium is sequentially replaced with floor plate expansion medium and DA maturation medium while in suspension, without further passaging. As compared with 2D cultures, the duration of specification and expansion for 3D cultures can be shortened while maintaining equivalent expression of floor plate markers.



Comparison of methods shown in Figures 2A–D.

Method	Rotation (First 2 weeks)	ECM	Geltrex matrix
A	+	–	–
B	+	+	Encapsulate
C	–	–	–
D	–	+	Dilute

Figure 2. Organoid formation is boosted by ECM. The numbering of days corresponds to the 3D workflow shown in Figure 1. Floor plate specification and expansion of human iPSCs in rotating suspension **(A)** without ECM addition or **(B)** with ECM encapsulation at day 2 of floor plate specification in Gibco™ Geltrex™ LDEV-Free Reduced Growth Factor Basement Membrane Matrix (Cat. No. A1413201) diluted 1:1 in DMEM/F-12. **(C)** Floor plate specification and expansion of human iPSCs in static suspension culture in Thermo Scientific™ Nunclon™ Sphera™ 96U-Well Microplates (Cat. No. 174925) without ECM; organoids were transferred to rotating culture for maturation. **(D)** Floor plate specification and expansion of human iPSCs in static suspension culture in U-well microplates using floor plate specification medium supplemented with 2% Geltrex matrix at day 2; all other medium changes matched those in (C). Overall, we found that ECM encapsulation or a U-well microplate changes the morphology and complexity of midbrain floor plate organoids. Scale bar = 1,000 µm.

ECM and in the presence of 50% Gibco™ Geltrex™ LDEV-Free Reduced Growth Factor Basement Membrane Matrix (encapsulated in ECM) or a dilute ECM (suspended in 2% Geltrex matrix), and evaluated based on growth patterns, morphology, and maturation to understand the effects of different growth environments on the physical properties of the organoids.

Culture conditions that included ECM encapsulation or U-well microplates changed the morphology and complexity of midbrain floor plate organoids. Importantly, static suspension cultures grown in U-well microplates with addition of dilute ECM could reproduce some of the known benefits of ECM encapsulation, such as organoid complexity and faster neuronal maturation, without the difficulty and low throughput of encapsulation methods (method D, Figure 2). Surprisingly, U-well microplates increased the outgrowth of neural epithelia and yielded complex organoids, and the combination of U-well microplates and 2% Geltrex matrix produced a regular shape to the complex organoids (method D).

Confirmation of organoid morphology using high-content imaging and analysis

New instruments for imaging the whole brain, coupled with fluorescent gene reporters and reagents for optical clearing of tissue, can help shed light on neurodegenerative disease states [1]. The Thermo Scientific™ CellInsight™ CX7 LZR High-Content Analysis (HCA) Platform provides a powerful combination of fluorescence microscopy, image processing, automated cell measurements, and informatics tools to characterize the physical and biochemical properties of 3D organoids using a broad range of multiwell plate formats. When paired with onstage incubation, robotic plate handling, and the Thermo Scientific™ HCS Studio™ Cell Analysis Software, the CellInsight CX7 LZR platform can help to make 3D organoid research more scalable by taking advantage of rapid acquisition of z-stacks from multicellular structures.

To best understand the architectural effects that ECM has on floor plate specification in microwell-grown organoids, organoid culture →

methods C and D (Figure 2) were compared using HCA. Day 7 organoids grown without ECM or with dilute laminin or Geltrex matrix were cleared using the Invitrogen™ CytoVista™ 3D Cell Culture Clearing Reagent, immunostained for N-cadherin and counterstained with DAPI nuclear stain, and then imaged on the CellInsight CX7 LZR platform (Figure 3). N-cadherin (neural cadherin) is a transmembrane protein found to play a role in neural crest development, cell-to-cell adhesion, differentiation, and signaling [2]. N-cadherin antibody and DAPI staining showed that the addition of ECM increased the appearance of rosette-like structures on the surface of day 7 organoids.

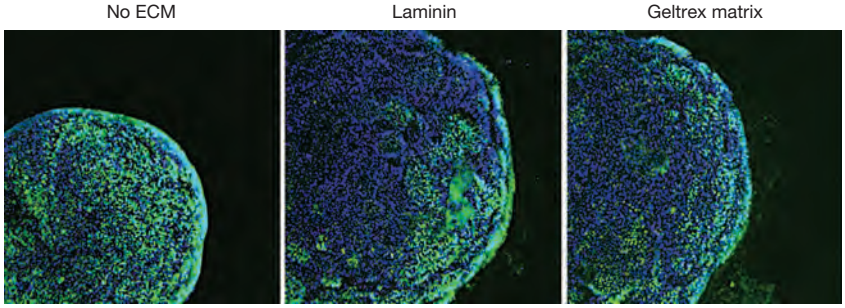


Figure 3. ECM addition during floor plate specification supports prominent rosette-like structures in early organoids. After specification by method C (Figure 2), static U-well microplate organoids (no ECM, left panel) were supplemented at day 2 with 200 µg/mL laminin (middle panel) or 2% Gibco™ Geltrex™ LDEV-Free Reduced Growth Factor Basement Membrane Matrix (Cat. No. A1413201) (right panel). Organoids were fixed on day 7 and labeled using Invitrogen™ N-cadherin monoclonal antibody (clone 3B9, Cat. No. 33-3900) in conjunction with Invitrogen™ Alexa Fluor™ 488 donkey anti–mouse IgG antibody (green, Cat. No. A21202) and DAPI (blue, Cat. No. D1306). Shown are maximal intensity projections of 8 µm optical sections, captured on the Thermo Scientific™ CellInsight™ CX7 LZR High-Content Analysis Platform (Cat. No. CX7A1110LZR).

Modeling Parkinson's disease with 3D organoid models

PD is characterized by the selective loss of DA neurons in the substantia nigra of the midbrain. This loss of DA neurons is observed in postmortem tissue along with Lewy bodies, which contain aggregates of phosphorylated α-synuclein protein. Lewy body formation in neurons has been described as the causative factor in DA neuron degeneration and the progressive loss of motor function associated with PD [3-5, also see *BioProbes 80* "Elucidate the underlying mechanisms of Parkinson's disease and other neurological disorders"]. Although only a minority of patients have a family history of PD, a growing number of genetic risk loci have been linked to sporadic cases and appear to influence susceptibility to environmental triggers.

In vitro models that reproduce the genetic basis of human disease can now be obtained by reprogramming patient cells to create iPSCs. Additionally, advances in gene editing technologies have led to the ability to create iPSC lines with any disease-relevant genome alteration. To apply these promising steps toward a reproducible 3D PD model, PD-associated single-nucleotide polymorphisms (SNPs) were engineered into iPSCs by CRISPR gene editing. Genes were edited in a stable Cas9-expressing iPSC line for high efficiency of cleavage and

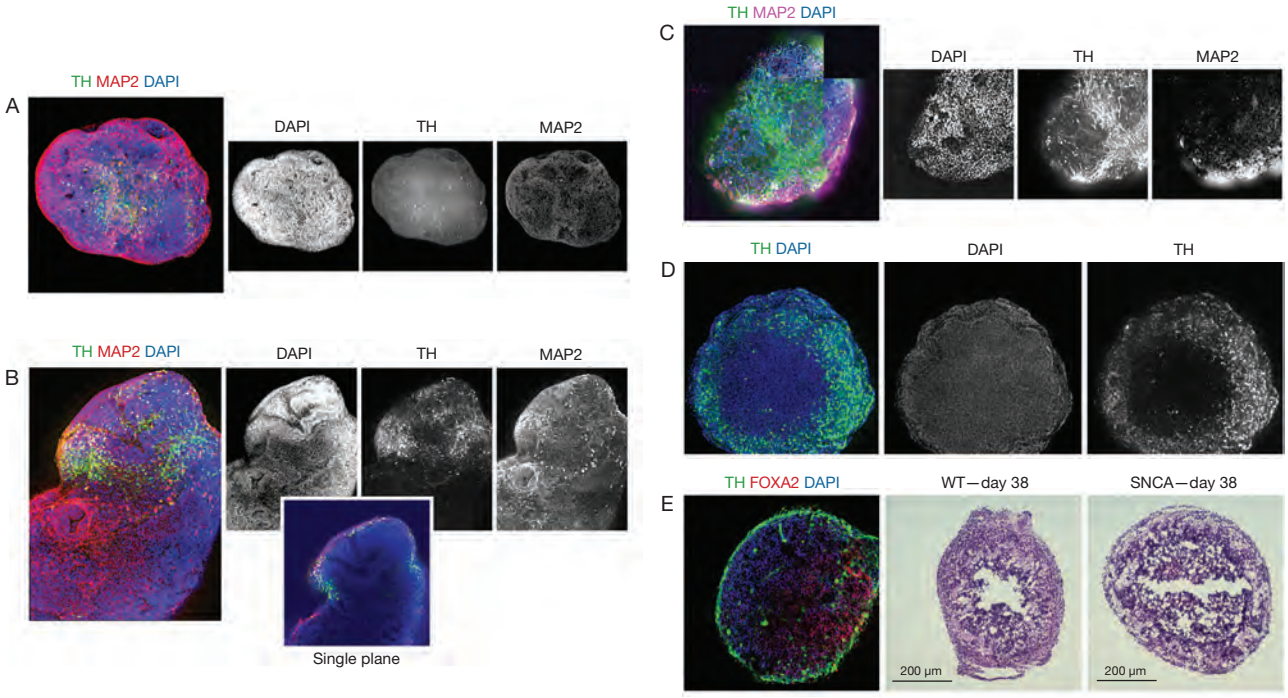


Figure 4. ECM encapsulation and U-well microplates increase organoid complexity and DA neuron yield. (A) Rotating suspension organoid (method A, Figure 2) at day 19 (early maturation): maximal intensity projection of Invitrogen™ MAP2 (Cat. No. PA5-17646) and tyrosine hydroxylase (TH) (Cat. No. MA1-24654) antibody staining and DAPI nuclear staining, as imaged on the Thermo Scientific™ CellInsight™ CX7 LZR High-Content Analysis Platform (Cat. No. CX7A1110LZR). **(B)** Encapsulated organoid (method B, Figure 2) at day 19: maximal intensity projection of MAP2 and TH antibody and DAPI staining. **(C)** Static U-well microplate organoid (method C, Figure 2) at day 19: maximal intensity projection of MAP2 and TH antibody and DAPI staining. **(D)** 23-day-old static U-well microplate organoid specified in the presence of 2% Gibco™ Geltrex™ LDEV-Free Reduced Growth Factor Basement Membrane Matrix (Cat. No. A1413201) at day 2 (method D, Figure 2): maximal intensity projection of TH antibody and DAPI staining. Midbrain organoids formed with ECM or in U-well microplates have a more complex epithelial morphology and earlier outgrowth of DA neurons. **(E)** 3-week-old static U-well microplate organoid specified in 2% Geltrex matrix: single optical section of TH and Invitrogen™ FOXA2 (Cat. No. 701698) antibody **(Cat. No. 701698)**and DAPI staining (left) and two images of hematoxylin/eosin–stained organoid sections (middle, right) from a SNCA wild-type (WT) or SNCA A30P mutant CRISPR iPSC line, which reveal thick bands of cells at the outside of the organoids surrounding a dense core of degrading cells. For immunodetection, primary antibodies were detected with Invitrogen™ Alexa Fluor™ 488 donkey anti–mouse IgG (Cat. No. A21202), Invitrogen™ Alexa Fluor™ 594 donkey anti–rabbit IgG (Cat. No. A21207), or Invitrogen™ Alexa Fluor™ 647 donkey anti–rabbit IgG (Cat. No. A32795) secondary antibody.

homology-driven repair. CRISPR editing was followed by one round of isolation by fluorescence-activated cell sorting (FACS), after which single cells showed robust clonal survival and growth when plated onto Gibco™ rhLaminin-521 in Gibco™ StemFlex™ Medium with Gibco™ RevitaCell™ Supplement. The precision of the mutations and clonality of the Cas9-expressing mutant and wild-type (WT) cell lines were verified by next-generation sequencing (NGS). One of the engineered SNPs creates the PD-linked A30P mutation in α-synuclein (SNCA). These SNCA mutant and WT control iPSC lines were differentiated toward midbrain organoids in U-well microplates with 2% Geltrex matrix in solution.

Early differentiation of the 3D cultures is marked by morphological change and expression of microtubule-associated protein 2 (MAP2) in neurons at the organoid surface. A portion of these are tyrosine hydroxylase (TH)-positive DA neurons (Figure 4). Comparison of organoid culture methods A, B, C, and D (Figure 2) demonstrates that midbrain organoids formed in free suspension without ECM (method A, Figure 4A) have a simple architecture with a single layer of neuronal cell bodies, whereas encapsulation in Geltrex matrix (method B, Figure 4B) increases neuroepithelial folding and the outgrowth of DA neurons. These effects are partially mimicked by organoid formation in low-attachment U-well microplates without (method C, Figure 4C) or with (method D, Figures 4D and 4E) a suspension of low-concentration Geltrex matrix.

Growth in this dilute ECM suspension (method D), however, outperforms encapsulation (method B) in promoting the maturation of midbrain organoids. The reddish-brown pigment neuromelanin is a byproduct of dopamine synthesis that gives dark coloration →

to the substantia nigra *in vivo* [6]. Midbrain organoids that have been encapsulated in ECM are dotted with pigment after many weeks of differentiation (Figure 5); organoids formed in U-well microplates with diffuse Geltrex matrix reach this milestone in about half the time, beginning to show neuromelanin pigmentation within 5 weeks.

Importantly, we generated midbrain organoids from WT and SNCA A30P iPSC lines using U-well microplates with dilute ECM and saw similarly complex epithelial morphology, earlier outgrowth of DA neurons, and evidence of rapid dopamine synthesis (Figures 4 and 5). Given the advantages in ease of use and faster maturation, we have chosen to continue midbrain organoid formation in U-well microplates with diffuse Geltrex matrix for our downstream functional and neurotoxicity studies.

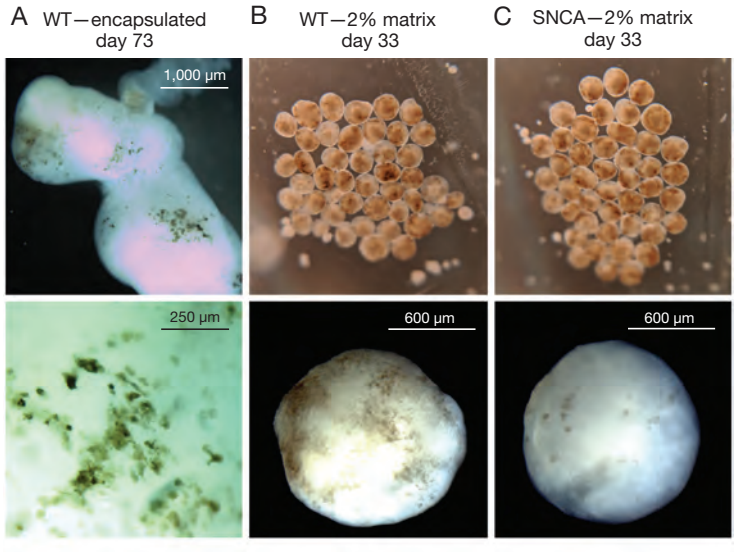


Figure 5. ECM promotes maturation of floor plate organoids. (A) Encapsulated organoid (method B, Figure 2) was imaged at day 73 without stains or dyes. The reddish-brown color suggests the presence of neuromelanin, a pigment that is a byproduct of dopamine synthesis [6]. **(B,C)** 23-day-old static U-well microplate organoid specified in the presence of 2% Gibco™ Geltrex™ LDEV-Free Reduced Growth Factor Basement Membrane Matrix (Cat. No. A1413201) at day 2 (method D, Figure 2) **(B)** from a SNCA wild-type (WT) CRISPR iPSC line or **(C)** from a SNCA A30P mutant CRISPR iPSC line. Earlier detection of neuromelanin in U-well microplate organoids with dilute Geltrex matrix suggests more rapid maturation of DA neurons.

Detecting action potentials in neural organoids using multielectrode arrays

Multielectrode arrays (MEAs) measure extracellular field potentials and are useful for studying neural circuit connectivity in monolayer cultures or organoids [7]. To detect spontaneous network activity in our midbrain model, we allowed single organoids to attach to an MEA with 16 electrodes, monitoring a surface area of 1.2 mm^2. Midbrain organoids generated by our method displayed spontaneous coordinated activity across the recording area in as little as 5 weeks of total differentiation time (Figure 6). *In vivo*, action potentials in the DA neurons of the substantia nigra are inhibited by excess dopamine [8]. Dopamine addition silenced the coordinated bursts we detected in midbrain organoids, confirming that these action potentials are driven by DA neurons.

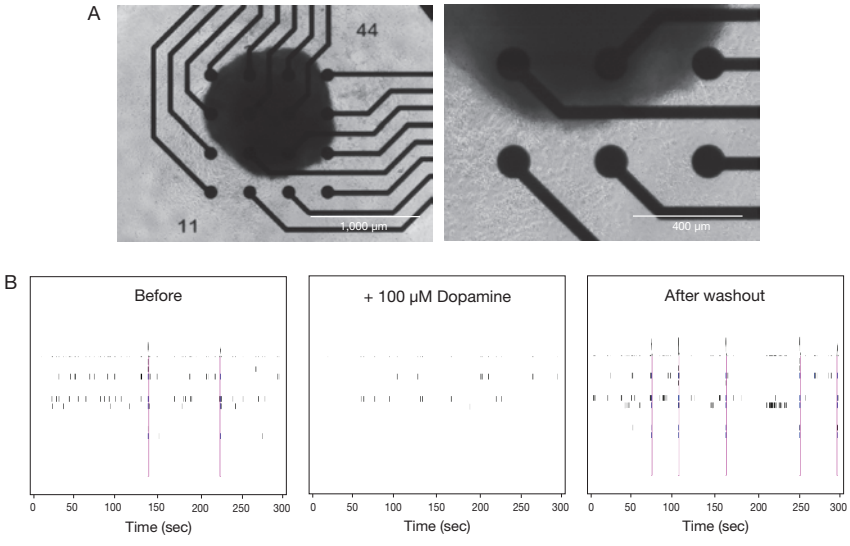


Figure 6. Floor plate organoids mature functionally. (A) Brightfield images show a static U-well microplate organoid specified in the presence of 2% Gibco™ Geltrex™ LDEV-Free Reduced Growth Factor Basement Membrane Matrix (Cat. No. A1413201) at day 2 (method D, Figure 2), cultured in suspension until day 21, and then allowed to attach in culture on a poly-D-lysine (pDL) and laminin–coated multielectrode array (MEA) for 14 days. **(B)** Raster plots of MEA recordings were derived from the plated U-well microplate organoid in (A). Each plot shows 300 sec of activity, first in maturation medium, second after addition of 100 µM dopamine, and third following washout of the dopamine. Vertical pink bars indicate detected network bursts. Midbrain organoids formed in U-well microplates with dilute Geltrex matrix can produce coordinated DA neuron activity in as little as 5 weeks.

In short, we have generated midbrain organoids from iPSCs by modifying the 2D protocol of the PSC Dopaminergic Neuron Differentiation Kit. This user-friendly method hastens functional maturation of DA neurons and makes promising steps toward a reproducible disease model for PD.

Advancing neuroscience research with 3D cell models

3D *in vitro* models of the brain and its disease states have become the focus of neuroscience research, due in part to the disappointing responsiveness of 2D culture models, but also to recent technological improvements that allow neuronal structures and functions to be observed in dense cell assemblies. Here we show our initial progress in generating organoids that show relatively rapid differentiation of DA neurons and capture several developmental events of the substantia nigra. We have engineered PD-relevant mutations into human iPSCs with CRISPR technology and are applying the derived midbrain organoids to model critical PD events. Ultimately, the use of iPSCs to build 3D brain models promises to advance our understanding of human disease mechanisms that are important to the development of therapeutic drugs and the implementation of cell and gene therapies. For more information on organoids, spheroids, and 3D cell culture, visit **thermofisher.com/3dculture**. ■

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Product	Quantity	Cat. No.
Imaging instruments and reagents		
CellInsight™ CX7 LZR High Content Analysis Platform	1 each	CX7A1110LZR
CellInsight™ CX7 LZR High Content Analysis Platform and Store Standard Edition (SE) Software	1 each	CX7B1112LZR
CellInsight™ CX7 LZR High Content Analysis Platform with Store Standard Edition (SE) Software and Orbitor™ RS Plate Mover	1 each	CX7C1115LZR
CytoVista™ 3D Cell Culture Clearing Reagent	10 mL 30 mL 100 mL	V11326 V11315 V11316
HCS Studio™ 2.0 Cell Analysis Software	1 each	SX000041A
Onstage Incubator for CellInsight™ CX7 HCA Platform	1 each	NX7LIVE001
Culture reagents		
DAPI (4′,6-Diamidino-2-Phenylindole, Dihydrochloride)	10 mg	D1306
DMEM/F-12	500 mL 10 x 500 mL	11320033 11320082
Essential 8™ Medium	500 mL	A1517001
Geltrex™ LDEV-Free Reduced Growth Factor Basement Membrane Matrix	1 mL 5 mL	A1413201 A1413202
Laminin Mouse Protein, Natural	1 mg	23017015
Nunclon™ Sphera™ Microplates, 96U-Well Plate	1 case of 8	174925
Poly-D-Lysine	100 mL	A3890401
PSC Dopaminergic Neuron Differentiation Kit	1 kit	A3147701
rhLaminin-521	100 µg 1 mg	A29248 A29249
StemFlex™ Medium	500 mL	A3349401
RevitaCell™ Supplement (100X)	5 mL	A2644501
Antibodies		
FOXA2 Recombinant Rabbit Monoclonal Antibody (clone 9H5L7)	100 µg	701698
MAP2 Polyclonal Antibody	100 µL	PA5-17646
N-Cadherin Monoclonal Antibody (clone 3B9)	100 µg	33-3900
Tyrosine Hydroxylase (TH) Monoclonal Antibody (clone 185)	50 µL	MA1-24654