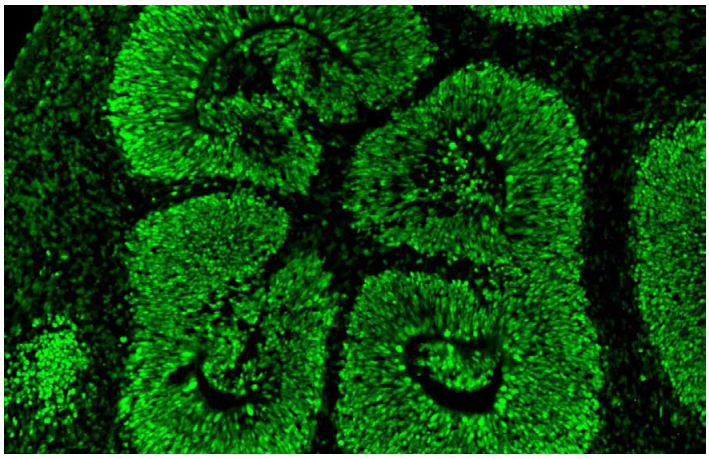
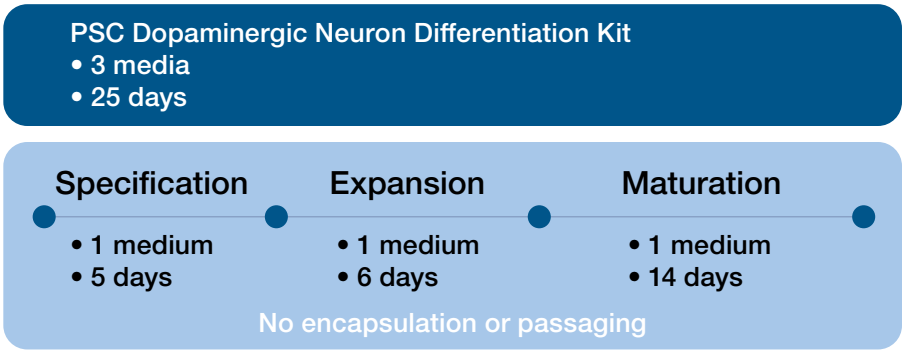
Generating midbrain organoids from pluripotent stem cell spheroids

Overview

The following protocol is for researchers who want to generate midbrain organoids from pluripotent stem cells (PSCs) grown in suspension culture. The stepwise procedure includes nucleation in Gibco™ StemScale™ PSC Suspension Medium (Cat. No. A4965001) followed by specification, expansion, and maturation using components of the Gibco™ PSC Dopaminergic Neuron Differentiation Kit (Figure 1).

By day 21, dopaminergic neurons should be detectable by staining for tyrosine hydroxylase.





Step	Nucleation	Specification	Expansion	Maturation
Duration	Overnight	Days 0–5	Days 5–11	Days 11+
Medium	StemScale PSC Suspension Medium	Floor Plate Specification Medium	Floor Plate Cell Expansion Medium	Dopaminergic Neuron Maintenance Medium
Format	Nunclon Sphera 96-well plate	Nunclon Sphera 96-well plate	Nunclon Sphera 96-well plate	Nunc Non-Treated Multidish or Nalgene Single-Use PETG Erlenmeyer Flask

Figure 1. Overview of the steps required for generating midbrain organoids.

Media preparation

Floor Plate Specification Medium	Volume	Cat. No.
Neurobasal Medium	95 mL	21103049
Floor Plate Specification Supplement (20X)	5 mL	A3146801

1. Thaw the Gibco™ Floor Plate Specification Supplement (20X) at 4°C overnight, or at room temperature (15–25°C) for 30 min.
2. Add 5 mL of 20X Floor Plate Specification Supplement to 95 mL of Gibco™ Neurobasal™ Medium, and mix well.
3. Store the complete Floor Plate Specification Medium at 4°C and use within 2 weeks. On the day of use, dispense the volume that is needed for that day and warm at 37°C; avoid repeated warming.

Floor Plate Cell Expansion Medium	Volume	Cat. No.
Floor Plate Cell Expansion Base Medium	490 mL	A3165801
Floor Plate Cell Expansion Supplement (50X)	10 mL	

1. Store Gibco™ Floor Plate Cell Expansion Base Medium at 4°C and Gibco™ Floor Plate Cell Expansion Supplement (50X) at –20°C until use. Do not prepare complete expansion medium until needed.
2. Thaw the Floor Plate Cell Expansion Supplement (50X) at 4°C overnight, or at room temperature (15–25°C) for 1 hour.
3. Remove 10 mL of medium from the bottle of Floor Plate Cell Expansion Base Medium and discard.
4. Add 10 mL of Floor Plate Cell Expansion Supplement (50X) to the remaining 490 mL of Floor Plate Cell Expansion Base Medium, and mix well.
5. Store the complete Floor Plate Cell Expansion Medium at 4°C and use within 2 weeks. On the day of use, dispense the volume that is needed for that day and warm at 37°C; avoid repeated warming.

Dopaminergic Neuron Maintenance Medium	Volume	Cat. No.
Neurobasal Plus Medium	474 mL	A3582901
Dopaminergic Neuron Maturation Supplement (50X)	10 mL	A3147401
B-27 Plus Supplement (50X)	10 mL	A3582801
GlutaMAX Supplement (100X)	1.25 mL (0.25X final)	35050061
Antibiotic-Antimycotic (100X)	5 mL	15240062

1. Store Gibco™ Dopaminergic Neuron Maturation Supplement (50X), B-27™ Plus Supplement (50X), and Antibiotic-Antimycotic (100X) at –20°C until use. Do not prepare complete maturation medium until needed.
2. Thaw the Dopaminergic Neuron Maturation Supplement (50X), B-27 Plus Supplement (50X), and Antibiotic-Antimycotic (100X) at 4°C overnight, or at room temperature (15–25°C) for 1 hour.
3. Add 10 mL of Dopaminergic Neuron Maturation Supplement (50X), 10 mL of B-27 Plus Supplement (50X), 1.25 mL of Gibco™ GlutaMAX™ Supplement (100X), and 5 mL of Antibiotic-Antimycotic (100X) solution to 474 mL of Gibco™ Neurobasal™ Plus Medium.
4. Store the complete Dopaminergic Neuron Maintenance Medium at 4°C and use within 2 weeks. On the day of use, dispense the volume that is needed for that day and warm at 37°C; avoid repeated warming.

Procedures

Prepare pluripotent stem cells (PSCs) for midbrain organoid generation

1. If PSCs are in Gibco™ Essential 8™ or StemFlex™ Medium, passage at least once in StemScale medium.
2. If PSCs are in StemScale medium, proceed to the nucleation step.

Nucleation (day –1)

1. Passage to single cells with Gibco™ StemPro™ Accutase™ Cell Dissociation Reagent.
2. Count live cells and dilute to 53.33 x 10^3 cells/mL in StemScale medium + 10 µM ROCK inhibitor (Y-27632).
3. Plate 150 µL/well (8 x 10^3 cells) in Thermo Scientific™ Nunclon™ Sphera™ 96-well U-bottom plates (Cat. No. 174929).
4. Incubate at 37°C, 5% CO_2 without rotation.

Specification (day 0): 2/3 fluid change to Floor Plate Specification Medium

1. Check that a single spheroid has formed in each well before continuing.
2. Carefully remove 100 µL from each well; check that spheroids are not discarded.
3. Add 100 µL/well of Floor Plate Specification Medium.
4. Incubate at 37°C, 5% CO_2 without rotation.
5. Be sure to thaw Gibco™ Geltrex™ matrix (Cat. No. A1413201) at 4°C to prepare for day 2.

ECM addition (day 2): 2/3 fluid change to Floor Plate Specification Medium with Geltrex matrix

1. Thaw an aliquot of 100% Geltrex matrix at 4°C.
2. Chill Floor Plate Specification Medium on ice (10 mL for full plate).
3. Add Geltrex matrix to cold Floor Plate Specification Medium to 3% (vol/vol) (e.g., 300 µL 100% Geltrex matrix in 10 mL of medium).
4. Keep Floor Plate Specification Medium with Geltrex matrix on ice.
5. Carefully remove 100 µL of medium from each well of the 96-well plate; check that spheroids are not discarded.
6. Add 100 µL/well of cold Floor Plate Specification Medium with Geltrex matrix.
7. Incubate at 37°C, 5% CO_2 without rotation.

Expansion (day 5): 2/3 fluid change to Floor Plate Cell Expansion Medium

1. Carefully remove 100 µL from each well; check that spheroids are not discarded.
2. Add 100 µL/well of Floor Plate Cell Expansion Medium.
3. Incubate at 37°C, 5% CO_2 without rotation.

Expansion (day 7 and day 9): 1/2 fluid change to Floor Plate Cell Expansion Medium

1. Exchange 75 µL/well of medium with Floor Plate Cell Expansion Medium.

Maturation (day 11): 1/2 fluid change to Dopaminergic Neuron Maintenance Medium

1. Remove 75 µL from each well of Floor Plate Cell Expansion Medium.
2. Add 75 µL/well of Dopaminergic Neuron Maintenance Medium.

Maturation (day 13): Transfer organoids to rotation culture

Organoids should have reached ~1 mm in diameter and be visible to the naked eye.

1. Prepare Thermo Scientific™ Nunc™ Non-Treated Multidishes (Cat. No. 150239) with 2 mL/well of Dopaminergic Neuron Maintenance Medium, or 125 mL sterile Thermo Scientific™ Nalgene™ Single-Use PETG Erlenmeyer Flasks (Cat. No. 4115-0125) with 20 mL of Dopaminergic Neuron Maintenance Medium.
2. Transfer organoids from the multidishes to a larger vessel using a sterile bulb pipette (e.g., Molecular Bioproducts, Cat. No. 19010009). Transfer of some medium with the organoids is acceptable.
3. Incubate at 37°C, 5% CO_2 at 90 rpm rotation.*

Maturation (day 15+): Rotation culture

1. Exchange fluid every other day by replacing 1/2 volume Dopaminergic Neuron Maintenance Medium.

* Midbrain organoids were cultured at 90 rpm on a Thermo Scientific™ CO_2 Resistant Shaker (Cat. No. 88881101). Other orbital shaker platforms may have a different radius and thus a different optimal speed. Choose a rotation speed fast enough to prevent aggregation while keeping organoids intact.

gibco

Desired outcome

By day 21, dopaminergic neurons should be detectable by staining for tyrosine hydroxylase (Figure 1). Organoids may start to turn brown from neuromelanin production around weeks 4–5, but this is likely cell line–dependent.

In summary, the PSC Dopaminergic Neuron Differentiation Kit can be adapted to generate midbrain organoids from PSCs nucleated in StemScale Medium.

- Organoids show dopaminergic neurons by 3 weeks, and dopaminergic activity beginning at 5 weeks, as detected by neuromelanin pigmentation and microelectrode array (MEA) assay.
- Organoids have a uniform diameter and shape by nucleation of embryoid bodies in U-bottom plates.
- Cell aggregation plus dilute extracellular matrix (ECM) speeds maturation of dopaminergic neurons.

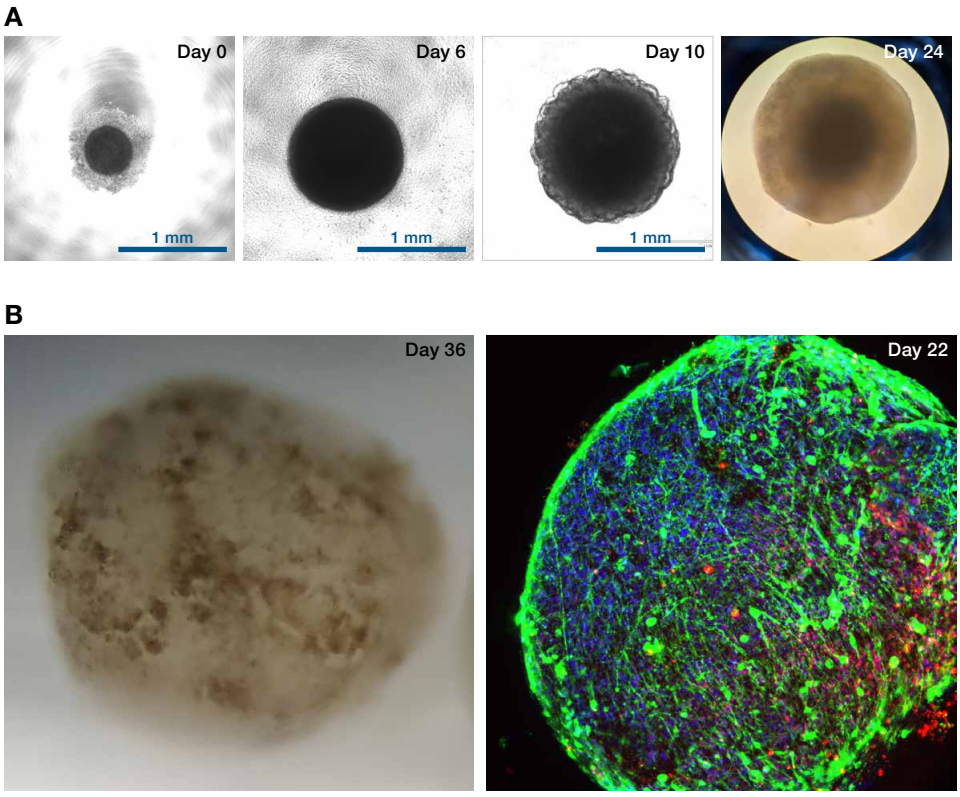


Figure 1. Representative images of organoids during differentiation. (A) Phase-contrast images of organoids on days 0, 6, 10, and 24. **(B)** Upon further maturation, organoids develop neuromelanin, a by-product of active dopaminergic neurons (day 36). Around differentiation day 22, organoids were composed of tyrosine hydroxylase–positive neurons (green) and of FOXA2-positive floor plate cells (red).

Learn more at **thermofisher.com/stemscale**

ThermoFisher SCIENTIFIC