hPSC-derived Midbrain Dopaminergic Neurons Generated in a Scalable 3-D Biomaterial

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Human pluripotent stem cell (hPSC)–derived midbrain dopaminergic (mDA) neurons may facilitate the development of therapies for Parkinson's disease via disease modeling, drug screening, and cell replacement therapy. However, large numbers of cells are typically needed for these applications, and 2-D culture–based approaches typically used for mDA differentiation are difficult to scale up and require a long time for mDA maturation. Here we present a protocol to rapidly generate functional mDA neurons in a fully defined, scalable, thermoresponsive 3-D biomaterial. Resource-efficient and accelerated differentiation of large numbers of mDA neurons may thus facilitate studying and treating PD. © 2018 by John Wiley & Sons, Inc.

Keywords: biomaterials • dopaminergic neurons • differentiation • human pluripotent stem cells • Parkinson's disease

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INTRODUCTION

Human pluripotent stem cells (hPSCs), with their potential for indefinite self-renewal and differentiation into all adult cell types, may be an unlimited cell source for the derivation of midbrain dopaminergic (mDA) neurons (Lindvall & Kokaia, 2009). These cells, which are specifically lost in Parkinson's disease (PD), have broad potential utility for a range of biomedical applications, including cell replacement therapy for PD, disease modeling, and drug screening. The 2-D cell culture platforms that are typically utilized for mDA differentiation are difficult to scale up and do not recapitulate a biomimetic 3-D niche that could better mimic natural development (Pampaloni, Reynaud, & Stelzer, 2007). Therefore, methods to efficiently generate large numbers of mDA neurons are needed.

In this protocol, we describe how to generate mDA neurons in 3-D. First, we discuss how to transition hPSCs cultured on conventional 2-D platforms into our 3-D hydrogel system. The adaptation phase is a critical step during which cells acclimate to the new 3-D environment. We then discuss how hPSCs may be rapidly expanded in 3-D, or maintained in 3-D culture for long periods of time if desired. Furthermore, we outline a protocol to differentiate hPSCs to midbrain dopaminergic neurons in 25 days. Finally, we provide support protocols describing how cells cultured in 3-D may be characterized using common techniques such as immunocytochemistry and qPCR. Overall, this protocol may be used to rapidly generate ~40% mDA neurons after 25 days of differentiation in the

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3-D hydrogel; cells may subsequently be easily harvested and used for cell replacement therapy, or matured further for disease modeling and drug screening.

BASIC PROTOCOL 1

hPSC TRANSITION FROM 2-D SURFACES TO 3-D HYDROGEL

This protocol describes how to transition hPSC cultures from conventional 2-D surfaces to a 3-D system based on a PNIPAAm-PEG polymer. Cells cultured on 2-D surfaces are harvested, dissociated to single cells, and encapsulated in 3-D hydrogels.

Materials

hPSCs cultured on Matrigel (see recipe)–coated surfaces [see, e.g., *UNIT 1C.18* (Miyazaki & Kawase, 2015); *UNIT 5B.6* (Santos, Kiskinis, Eggan, & Merkle, 2016)]
Pluripotency medium (see recipe)
10 mM Y-27632 (ROCK inhibitor; see recipe)
Mebiol (see recipe)
Phosphate-buffered saline (PBS; Corning, cat. no. 21-040-CV)
Accutase (Thermo Fisher Scientific, A11105-01); store at 4°C until expiration.
3-D pluripotency medium (see recipe)
0.4% (w/v) trypan blue

15-ml conical centrifuge tubes (e.g., Corning Falcon)
Hemacytometer or automated cell counter (e.g., Bio-Rad TC20)
Centrifuge
100-µl (P-100) and 1000-µl (P-1000) positive-displacement tips (pre-chill/store at –20°C)
Scissors

Additional reagents and equipment for growing hPSCs on Matrigel [see, e.g., *UNIT 1C.18* (Miyazaki & Kawase, 2015); *UNIT 5B.6* (Santos et al., 2016)]

1. Culture hPSCs on standard 2-D platforms, e.g., 12-well plate using Matrigel coating, with pluripotency medium [see, e.g., *UNIT 1C.18* (Miyazaki & Kawase, 2015); *UNIT 5B.6* (Santos, Kiskinis, Eggan, & Merkle, 2016)].

   We have found that hPSCs are maintained best in the 3-D hydrogels in E8 medium, and hPSCs should therefore be transitioned to E8-based pluripotency medium before transferring to 3-D. We recommend doing this gradually, by increasing the volume percent of E8-based pluripotency medium from 0% to 25%, 50%, 75%, and 100% over the course of 5 days of hPSC culture.

2. When cells have reached 70% to 90% confluence, add Y-27632 (ROCK inhibitor) at 10 µM 1 hr before cell harvest. Chill Mebiol, PBS, and a 15-ml centrifuge tube on ice.

   It is important to add Y-27632 at this stage to facilitate high-viability passage.

3. Aspirate medium, and gently wash cells once with 0.2 ml PBS/cm^2. Aspirate PBS.

4. Warm an aliquot of Accutase for 10 min at room temperature. Add 50 µl/cm^2 Accutase, and incubate the plate in a 37°C incubator for 3 min. Add a 5-fold excess of 3-D pluripotency medium with respect to the volume of Accutase used and pipet the mixture up and down 10 times with a 1000-µl (P-1000) pipet tip, followed by 10 times with a 200-µl (P-200) pipet tip.

   Cell clusters should not be visible if single-cell dissociation is achieved. The cell suspension will appear cloudy without visible particulates. Stop pipetting as soon as this is achieved, as excess pipetting may reduce cell viability.

5. Take a 50-µl sample of cell suspension and mix it 1:1 with 50 µl of trypan blue in a 1.5-ml microcentrifuge tube. Take 10 µl of this cell suspension into a hemacytometer and count the number of live cells to determine the concentration. Alternatively, use an automated cell counter such as a Bio-Rad TC20.

6. Based on the cell concentration calculated in step 5, transfer the desired number of cells (e.g., 100,000 cells for every 100 µl of gel to be seeded in 12-well plate wells) into the chilled 15-ml centrifuge tube. Spin down cells for 2.5 min at 250 × *g*, room temperature.

7. Aspirate all but 10 to 15 µl of the supernatant. Using a 20-µl (P-20) pipet tip, resuspend the pellet in the remaining 10 to 15 µl of supernatant.

   This ensures that the cells will be evenly distributed in the Mebiol gel.

   CAUTION: *A pellet of 100,000 cells is difficult to visualize. To reduce chances of accidentally aspirating the cell pellet, aspirate manually instead of using a vacuum pump. Fitting a 200-µl pipet tip onto the end of an aspirating pipet also provides more control during aspiration.*

8. Bring out the positive-displacement tips from the –20°C freezer. Using scissors, cut off 1 to 3 mm from the end of a 100-µl (P-100) tip. Rinse the blunted pipet tip by pipetting up and down three times in ice-cold PBS. If seeding more than one 100-µl gel, or a larger gel volume, use a 1000-µl (P-1000) positive-displacement pipet instead.

   Cutting the tip off facilitates subsequent pipetting of the viscous Mebiol. Pipetting in PBS lubricates the pipet tip, which facilitates handling of the gel.

9. For every gel to be seeded in the 12-well plate wells, pipet 100 µl of ice-cold Mebiol into the 10 to 15-µl cell suspension from step 7. Using a pipettor and the blunted, PBS-rinsed tip prepared in step 8, mix the Mebiol with the cell suspension by rapidly pipetting up and down 20 times. Avoid generating air bubbles.

   It is crucial to mix properly, as inhomogeneous distribution of cells in the gel may lead to aggregation and differentiation.

10. Using the P-100 positive displacement pipet and a blunted, PBS-rinsed tip prepared in step 8, seed 100 µl of the cell-Mebiol mix per well of a 12-well plate, and incubate in a 37°C incubator for 10 min.

    Leave a ~5 mm space between the gel and the wall of the well.

11. For every 100 µl gel, add 2 ml of the pre-warmed 3-D proliferation medium gently against the side of the well without disturbing the gel, and place the plate back in the 37°C incubator.

    Ensure that the medium is at 37°C. Adding cooler medium may result in the dissolution of the gel.

hPSC MAINTENANCE AND PROPAGATION IN 3-D

BASIC PROTOCOL 2

This protocol describes how to continuously culture hPSCs in 3-D Mebiol-based hydrogels. hPSCs are passaged as single cells about every 5 days as previously described (Lei & Schaffer, 2013) and fed every day with pre-warmed 3-D pluripotency medium.

Materials

Mebiol (see recipe)
Phosphate-buffered saline (PBS; Corning, cat. no. 21-040-CV)
hPSCs cultured in Mebiol (Basic Protocol 1)

Accutase (Thermo Fisher Scientific, A11105-01); store at 4°C until expiration.
3-D pluripotency medium (see recipe)

15-ml conical centrifuge tubes (e.g., Corning Falcon)
Centrifuge

1. Chill Mebiol, PBS, and 15-ml centrifuge tubes on ice.
2. Using a 5- or 10-ml serological pipet, add 2 ml of ice-cold PBS to each well with hydrogel-encapsulated hPSCs in the 12-well plate (see Basic Protocol 1). Using a 5-ml pipet, dissolve the gel by pipetting up 1 ml and triturating a 0.5-ml volume until the gel pieces are all dissolved. It may help to keep the 12-well plate on ice during this process to ensure the solution does not undergo gelation. Aspirate the dissolved gel and cell clusters into a chilled 15-ml centrifuge tube and place it back on ice.
3. Centrifuge the cell cluster suspension using a benchtop centrifuge 30 sec at 200 × *g*, room temperature. Aspirate off the supernatant.
4. Add 500 µl Accutase (for cells harvested from one to five 100-µl gels) and incubate in a 37°C water bath for 12 min.
5. Resuspend and dissociate clusters to single cells by pipetting up and down with a 1000-µl (P-1000) pipet tip 10 times and then pipetting seven times against the side of the centrifuge tube. Repeat this step with a 200-µl (P-200) pipet tip.
6. Add a 5-fold excess of 3-D pluripotency medium with respect to the volume of Accutase used. Repeat step 5.

   A single-cell suspension may be qualitatively assessed by the naked eye. Pipetting should continue until no more clusters are visible by naked eye. Pipetting too gently may result in dissociation to small aggregates instead of single cells, and pipetting more aggressively than this may reduce cell viability. Alternatively, a 50-µm cell strainer may be used to ensure that only single cells are re-encapsulated for further expansion, but this will increase the number of cells lost during passaging.

7. Follow steps 5 to 11 of Basic Protocol 1.
8. For daily feeding, which will be needed over the next 4 days, warm 3-D pluripotency medium to 37°C in the water bath. Then, take the plate out from the 37°C incubator, rapidly but gently aspirate conditioned medium, and add the fresh 37°C 3-D pluripotency medium gently down the edge of the well without disturbing the gel. Immediately replace the plate back into the 37°C incubator.
9. hPSCs typically require passaging every 5 days. Follow steps 1 to 8 above for continued expansion.

   hPSCs may be maintained in 3-D for at least 1 year of continuous passage without loss of pluripotency. However, pluripotency should be regularly tested using established protocols for immunocytochemistry, as well as embryoid body and teratoma formation assays (Fritz, Adil, Mao, & Schaffer, 2015).

BASIC PROTOCOL 3

hPSC DIFFERENTIATION INTO mDA NEURONS IN 3-D

This protocol describes the differentiation of mDA neurons in 3-D culture. We specifically refer to a 12-well plate cell-culture format, but this strategy may be scaled up or down as necessary. In particular, we describe the differentiation protocol we recently used to generate mDA neurons in 3-D (Adil et al., 2017); however, other culture media, from different protocols to generate mDA or other neurons, may also be compatible with our 3-D gel platform. Also, we describe cell harvest from the hydrogels at the end of the desired differentiation period for analysis or subsequent mDA usage.

Materials

hPSCs cultured in 3-D hydrogels for at least 2 passages (Basic Protocol 2) in 12-well plates
Differentiation media for different days of differentiation (see Table 2D.21.1)
Phosphate-buffered saline (PBS; Corning, cat. no. 21-040-CV)
Accutase (Thermo Fisher Scientific, A11105-01); store at 4°C until expiration.
Wash medium (see recipe)
0.25% trypsin (Corning, cat. no. 25-053-CI)
Hanks' balanced salt solution (HBSS; optional) with and without 0.05% (w/v) DNase I

15- and 50-ml conical centrifuge tubes (e.g., Corning Falcon)
Centrifuge
Hemacytometer or automated cell counter (e.g., Bio-Rad TC20)
Laminin-coated plates (see recipe)

Additional reagents and equipment for seeding gels and Mebiol treatment (Basic Protocol 1, steps 7 to 10)

1. hPSCs should be cultured in 3-D hydrogels for at least 2 passages before initiating differentiation. This allows cells to adapt to the 3-D environment. Start differentiation at least 5 days after the third single-cell passaging in 3-D as described in Basic Protocol 2.

   Different hPSC lines may need different time periods for adaptation. Once adapted, >90% of seeded single cells will survive and grow into larger, 50- to 100-μm clusters, in 4 to 5 days.

2. On Day 0 of differentiation, aspirate the 3-D pluripotency medium and add 2 ml of Day 0 differentiation medium per well of a 12-well plate. For the desired period of differentiation, change medium every day according to the differentiation conditions described in Table 2D.21.1.

   mDA neurons may be differentiated in hydrogels for at least 25 days. For highly proliferative cell lines (e.g., undergoing 10-fold expansion in less than 3 to 4 days in 3-D culture), mDA progenitor spheres undergoing differentiation may be harvested and reseeded as smaller clusters into new hydrogels at Day 11 of differentiation, following the steps outlined below. This will avoid cell starvation and cell-sphere necrosis.

   Once the targeted differentiation stage is reached, harvest cells from the hydrogels for analysis or additional experiments.

3. Chill PBS and 15-ml centrifuge tubes on ice.

4. Place the 12-well plate with cell-encapsulated hydrogels on ice. Add 2 ml of ice-cold PBS to each 100-μl gel. Using a 5-ml pipet, dissolve the gel by pipetting up 1 ml and triturating a 0.5-ml volume until the entire gel is dissolved. Transfer the dissolved gel and cell clusters into a chilled 15-ml centrifuge tube and place back on ice.

5. Pellet cell clusters by centrifuging 30 sec at 200 × *g*, room temperature.

   At this stage, cells may be lysed if needed for subsequent analysis, which is described below in Support Protocol 3.

6. Aspirate off the supernatant and add 1 ml of ice-cold PBS per 100 μl gel. Linearly increase the volume of PBS if cells are harvested from additional wells. However, if more than 10 ml of PBS are needed, split the cell pellet after resuspension into multiple 15-ml centrifuge tubes. Alternatively, use a 50-ml centrifuge tube, but if more than 20 ml of PBS is needed, use multiple 50-ml centrifuge tubes.

Table 2D.21.1 Media Compositions for mDA Differentiation in 3-D

Factors		LDN193189[a]	SB431542[a]	Purmorphamine[a]	FGF8[a]	SHH[a]	CHIR9902[a]	BDNF[a]	L-Ascorbic acid[a]	GDNF[a]	DAPT[a]	TGFB3[a]	cAMP[a]
Working concentration		100 nM	10 μM	2 μM	100 ng/ml	100 ng/ml	3 μM	20 ng/ml	0.2 mM	20 ng/ml	10 μM	1 ng/ml	0.5 mM
Stock		100 μM-1mM	10-100 mM	2-20 mM	100 μg/ml	100 μg/ml	6 mM	100 μg/ml	0.2 M	100 μg/ml	10-100 mM	20 μg/ml	200 mM
Day	Base medium[b]	Medium conditions											
0	N2B27-1	+	+										
1	N2B27-1	+	+	+	+	+							
2	N2B27-1	+	+	+	+	+							
3	N2B27-1	+	+	+	+	+	+						
4	N2B27-2	+	+	+	+	+	+						
5	N2B27-2	+		+	+	+	+						
6	N2B27-2	+		+	+	+	+						
7	N2B27-2	+					+						
8	N2B27-2	+					+						
9	N2B27-2	+					+						
10	N2B27-2	+					+						
11	B27						+	+	+	+	+	+	+
12	B27						+	+	+	+	+	+	+

continued

Table 2D.21.1 Media Compositions for mDA Differentiation in 3-D, *continued*

Factors		LDN193189[a]	SB431542[a]	Purmorphamine[a]	FGF8[a]	SHH[a]	CHIR9902[a]	BDNF[a]	L-Ascorbic acid[a]	GDNF[a]	DAPT[a]	TGFB3[a]	cAMP[a]
Working concentration		100 nM	10 μM	2 μM	100 ng/ml	100 ng/ml	3 μM	20 ng/ml	0.2 mM	20 ng/ml	10 μM	1 ng/ml	0.5 mM
Stock		100 μM-1mM	10-100 mM	2-20 mM	100 μg/ml	100 μg/ml	6 mM	100 μg/ml	0.2 M	100 μg/ml	10-100 mM	20 μg/ml	200 mM
Day	Base medium[b]	Medium conditions											
13	B27							+	+	+	+	+	+
14	B27							+	+	+	+	+	+
15	B27							+	+	+	+	+	+
16	B27							+	+	+	+	+	+
17	B27							+	+	+	+	+	+
18	B27							+	+	+	+	+	+
19	B27							+	+	+	+	+	+
20	B27							+	+	+	+	+	+
21	B27							+	+	+	+	+	+
22	B27							+	+	+	+	+	+
23	B27							+	+	+	+	+	+
24	B27							+	+	+	+	+	+
25	B27							+	+	+	+	+	+

[a]See stock solution recipe in Reagents and Solution.
[b]See media recipes (B27 and N2B27-2) in Reagents and Solutions.

These volume recommendations ensure that a P-1000 pipet tip may be used to break up the clusters efficiently.

7. Using a pipettor and P-1000 pipet tip, triturate the aggregates gently by pipetting up and down 10 to 30 times, and break up mDA neuron spheres into smaller clusters.

   According to our experience, some hPSC lines may generate more cohesive mDA neuron spheres. If pipetting does not result in smaller cell clusters, or excessive pipetting appears to reduce cell viability, the pellet may instead be resuspended in 500 µl of Accutase (i.e., for cells harvested from one to five 100- µl gels; use 1000 µl for cells harvested from six to ten 100-µl gels, and so on) and, after a 12-min incubation period at 37°C, spheres can be broken into small clusters by triturating five to ten times with a pipettor and P-1000 pipet tip.

   Prolonged Accutase treatment followed by excessive pipet mixing may reduce cell viability. Reduce incubation time with Accutase, and pipet-mix fewer times if mDA viability decreases.

8. Add a 5-fold excess of wash medium with respect to volume of Accutase used. Take a 20-µl aliquot, add a 5-fold excess of trypsin in a microcentrifuge tube, and incubate at 37°C for 5 min. Triturate to single cells, and count a 10-µl sample using a hemacytometer or an automated cell counter.

9. At this stage, cells may be re-seeded onto a new platform, or further prepped for transplantation, as described below.

   a. For re-encapsulation in Mebiol at Day 11, pellet the entire cell suspension for 1 min at 200 × *g*, room temperature, use a P-1000 pipet to gently break cell clusters into smaller spheres, and re-seed three new gels for every 100-µl gel harvested via steps 7 to 10 in Basic Protocol 1 (i.e., 1:3 passage in 3-D). Add 2 ml of stage-specific differentiation medium, pre-warmed to 37°C, for every 100-µl gel seeded. Immediately replace plate in the 37°C incubator.
   b. For re-seeding onto 2-D laminin-coated plates for immunocytochemistry (see Support Protocol 1), electrophysiology, or long-term maturation, based on the counts from step 8, pellet 100,000 cells per cm^2 to be seeded by centrifuging the appropriate volume of cell suspension for 2.5 min at 250 × *g*, room temperature. Resuspend cells in 0.25 ml of stage-appropriate differentiation medium per cm^2 to be seeded, aspirate off laminin from laminin-coated plates, add cell suspension at 0.25 ml/cm^2, and incubate at 37°C.
   c. For transplantation into animals, cells should be pelleted by centrifuging the appropriate volume of cell suspension 2.5 min at 250 × *g*, room temperature, and re-suspended at 50,000 to 125,000 cells/µl in wash medium or in HBSS, with or without 0.05% w/v DNase I. Usually, 100,000 to 250,000 cells may be stereotaxically transplanted into the rodent striatum following established protocols (Adil et al., 2017; Kirkeby et al., 2016; Kriks et al., 2011).

SUPPORT PROTOCOL 1

IMMUNOCYTOCHEMISTRY

This protocol describes how to analyze cultures using immunocytochemistry. Cells harvested from 3-D hydrogels and re-seeded on laminin-coated plates are fixed, stained with the appropriate antibodies, and imaged using a fluorescence microscope.

Materials

Cells harvested from 3-D hydrogels and re-seeded on laminin-coated plates (Basic Protocol 3, step 9b)
Phosphate-buffered saline (PBS; Corning, cat. no. 21-040-CV)
4% (w/v) paraformaldehyde (PFA; see recipe)

Table 2D.21.2 Antibodies to Verify Differentiation to Midbrain Dopaminergic Neurons

Antibodies	Company	Cat. No.	Host	Dilution
FOXA2	Santa Cruz	sc-101060	Mouse	1:500
LMX1A	Millipore	MAB10533	Rabbit	1:500
TUJ1	Invitrogen	480011	Mouse	1:500
TH	Pel-freeze	P40101	Rabbit	1:500
OCT4	Santa Cruz	sc-5279	Mouse	1:200
NANOG	Santa Cruz	sc-33759	Rabbit	1:200
MSX1	Hybridoma bank	4G1-C	Mouse	1:100
PAX6	Biolegend	PRB-278P	Rabbit	1:300
Alexa 647 Donkey anti Ms	Jackson Immunoresearch	A31571	Donkey	1:1000
Alexa 555 Donkey anti Rb	Jackson Immunoresearch	A31572	Donkey	1:1000
Alexa 488 Donkey anti Ch	Jackson Immunoresearch	703-545-155	Donkey	1:1000

Primary blocking buffer (see recipe)
Primary and secondary antibodies [refer to Table 2D.21.2 for catalog numbers and concentrations]
0.1% (v/v) PBST (0.1% v/v Triton X-100 in PBS) and 0.2 % (v/v) PBST (0.2% v/v Triton X-100 in PBS)
2% bovine serum albumin (BSA) in PBS
5 mg/ml DAPI (see recipe)
Fluorescence microscope

1. To wash cells, gently pipet 0.2 ml/cm^2 PBS along the edge of the well onto cells on 2-D surfaces. Incubate for 1 min at room temperature, and gently aspirate off PBS.
2. Add 0.2 ml/cm^2 of 4% PFA, and incubate at room temperature for 15 min.

   CAUTION: *PFA is toxic.*
3. Aspirate off 4% PFA, and wash cells three times with PBS as described in step 1. Leave PBS in the well on the final wash.

   At this point, cells may be left in PBS and plate may be wrapped in Parafilm (to prevent drying) and stored at 4°C.
4. Aspirate off PBS and incubate cells with 0.2 ml/cm^2 primary blocking buffer for 1 hr at room temperature with gentle rocking on an orbital shaker.
5. Aspirate off blocking buffer, add the appropriate concentration (according to Table 2D.21.2) of primary antibody diluted in primary blocking buffer at 0.12 ml/cm^2, and incubate overnight with gentle rocking at 4°C.
6. On the next day, aspirate off primary antibodies. Add 0.2% (v/v) PBST at 0.2 ml/cm^2, and incubate at room temperature with gentle rocking for 5 min. Aspirate off PBST, and wash twice with 0.2 ml/cm^2 of 0.1% (v/v) PBST with gentle rocking at room temperature for 5 min each. Aspirate off PBST.
7. Add 0.12 ml/cm^2 of 1:1000 dilution of secondary antibody in PBS containing 2% (w/v) BSA, and incubate at room temperature with gentle rocking for 2 hr. Then, 30 min before the end of incubation, add DAPI (nuclear stain) directly to the wells at 1:1000 dilution.

8. Aspirate off secondary antibodies. Wash cells three times with PBS as described in step 1. Leave PBS in the well on the last wash.

   At this point, cells may be left in PBS, the plate may be wrapped in Parafilm (to prevent drying) and aluminum foil (to protect fluorescently labeled antibodies from light), and stored at 4°C.

9. Image cells using a fluorescence microscope.

   The fraction of cells expressing markers of interest may be quantified by manually counting positively stained cells using ImageJ, or using automated counting in Cell Profiler, for example.

SUPPORT PROTOCOL 2

GENE EXPRESSION ANALYSIS

This protocol describes how to extract RNA from cells and perform a quantitative gene expression analysis.

Materials

Cells harvested from 3-D hydrogels (Basic Protocol 3, step 6 or 9b)
Qiagen RNAeasy Mini kit (Qiagen, cat. no. 74104)
iScript cDNA synthesis kit (Bio-, cat. no. 170-8891)
SYBR-based qPCR master mix (e.g., ThermoFisher Scientific, cat. no. 4309155)
Primers (Table 2D.21.3)

96-well qPCR plate
Thermal cycler

1. Extract RNA from cells using an RNeasy Mini kit according to the manufacturer's instructions.
2. Convert 1 μg RNA to 20 μl cDNA using iScript Reverse Transcriptase Kit (Bio-Rad) according to the manufacturer's instructions.
3. Dilute 20 μl of the cDNA 10-fold to 200 μl using molecular-biology-grade water or elution buffer from the Qiagen kit.
4. Add 1 μl of diluted cDNA to 19 μl of SYBR-based qPCR master mix containing the appropriate primers (Table 2D.21.3). For each sample, prepare 20-μl reactions, and load into a 96-well qPCR plate in triplicate. Include the corresponding undifferentiated hPSCs before Day 0 of differentiation to calculate the fold changes in gene expression levels.
5. Run the qPCR analysis using a thermal cycling scheme as follows:

1 cycle:	10 min	95°C
40 cycles:	10 sec	95°C
	30 sec	55°C

   As an alternative to running qPCR, PCR may be used to qualitatively assess gene expression levels in differentiating mDA progenitors/neurons compared to undifferentiated hPSCs.

6. Normalize C_t counts to appropriate housekeeping genes. Analyze fold changes in gene expression with respect to expression levels in undifferentiated hPSCs.

Table 2D.21.3 Primers to Verify Differentiation to Midbrain Dopaminergic Neurons

Gene	Forward primer (F)	Reverse primer (R)	Amplicon size	Tm F; Tm R
OCT4	CACCATCTGTCGCTTCGAGG	AGGGTCTCCGATTTGCATATCT	132	62.6; 60.7
NANOG	AAGGTCCCGGTCAAGAAACAG	CTTCTGCGTCACACCATTGC	237	62; 61.9
PAX6	AACGATAACATACCAAGCGTGT	GGTCTGCCCGTTCAACATC	120	60; 60.8
FOXA2	GGAGCAGCTACTATGCAGAGC	CGTGTTCATGCCGTTCATCC	83	62.3; 61.7
OTX2	CATGCAGAGGTCCTATCCCAT	AAGCTGGGGACTGATTGAGAT	200	60.8; 60.6
PITX3	CCTACGAGGAGGTGTACCCC	CCCACGTTGACCGAGTTGA	112	62.6; 61.9
DAT	TTTCTCCTGTCCGTCATTGGC	AGCCCACACCTTTCAGTATGG	223	62.4; 61.8
TH	GGGCTGTGTAAGCAGAACG	AAGGCCCGAATCTCAGGCT	107	60.7; 63
NURR1	ACCACTCTTCGGGAGAATACA	GGCATTTGGTACAAGCAAGGT	175	60; 61.1
GIRK2	CACATCAGCCGAGATCGGAC	GGTAGCGATAGGTCTCCCTCA	103	60.3; 61.2
TUJ1	GGCCAAGGGTCACTACACG	GCAGTCGCAGTTTTCACACTC	85	62.3; 62
LMX1A	ACGTCCGAGAACCATCTTGAC	CACCACCGTTTGTCTGAGC	248	61.8; 61
EN1	GAGCGCAGGGCACCAAATA	AATAACGTGTGCAGTACACCC	138	62.7; 60.3
SHH	CTCGCTGCTGGTATGCTCG	ATCGCTCGGAGTTTCTGGAGA	176	62.8; 62.7
CORIN	CCTCCTCCGGTTCCTATTGC	CCAAAGGTTCACTCCCATTTGA	131	61.7; 60.4
GAPDH	GGAGCGAGATCCCTCCAAAT	GGCTGTTGTCATACTTCTCATGG	197	61.6; 60.9

REAGENTS AND SOLUTIONS

Use deionized, distilled water or equivalent in recipes and protocol steps.

3-D pluripotency medium

To pluripotency medium (see recipe), add 10 μM Y-27632 (from 10 mM Y-27632 stock; see recipe). Store at 4°C for up to 2 days.

L-Ascorbic acid

Reconstitute L-ascorbic acid (Sigma, cat. no. A4403) at 0.2 M in PBS (Corning, cat. no. 21-040-CV). Store in aliquots at –20°C for up to 1 year. Store thawed aliquots at 4°C for up to 1 week.

B27 medium

Purchase 50× B27 supplement (Thermo Fisher Scientific, cat. no. 12587010). Store in aliquots at –20°C. Keep thawed aliquots at 4°C for up to 2 weeks. Do not freeze/thaw aliquots.

Purchase neurobasal medium (Thermo Fisher Scientific, cat. no. 21103-049). Store at 4°C.

To prepare B27 medium: To neurobasal medium (see recipe), add each of the following at the indicated final concentrations: 2 mM glutamine (Thermo Fisher Scientific, cat. no. 35050-061), 0.5% (v/v) penicillin/streptomycin (Gibco, cat. no. 15140-122), and 1:50 dilution of 50× B27 supplement (see recipe). Store at 4°C for up to 1 week.

BDNF

Reconstitute BDNF powder (Peprotech, cat. no. 450-02) at 100 μg/ml in sterile PBS (Corning, cat. no. 21-040-CV). Store in aliquots at –20°C for up to 12 months. Store thawed aliquots at 4°C for up to 1 week. Do not freeze/thaw aliquots.

cAMP, dibutyryl

Reconstitute dibutyryl cAMP powder (Santa Cruz Biotechnology, sc-201567B) at 0.5 mM in DMSO. Store in aliquots at –20°C. Store thawed aliquots at 4°C for up to 1 week.

CHIR99021

Reconstitute CHIR99021 powder (Stemgent, cat. no. 04-0004) at 6 mM in DMSO. Store in aliquots at –20°C for up to 6 months. Freeze/thaw aliquots up to two times.

DAPI

Reconstitute DAPI (Sigma, cat. no. D1306) at 5 mg/ml and store at –20°C. Aliquots may be stored for at least 6 months.

DAPI is a mutagen, handle with caution.

DAPT

Reconstitute DAPT powder (Selleckchem, S2215) at 100 mM in DMSO. Store in aliquots at –20°C. Store thawed aliquots at 4°C for up to 1 week.

FGF8

Reconstitute FGF8 powder (Peprotech, cat. no. 100-25) at 100 μg/ml in sterile PBS (Corning, cat. no. 21-040-CV). Store in aliquots at –20°C for up to 12 months. Store thawed aliquots at 4°C for up to 1 week. Do not freeze/thaw aliquots.

GDNF

Reconstitute GDNF powder (Peprotech, 450-10) at 100 μg/ml in sterile PBS (Corning, cat. no. 21-040-CV). Store in aliquots at –20°C for up to 12 months. Store thawed aliquots at 4°C for up to 1 week. Do not freeze/thaw aliquots.

Laminin, 1 mg/ml

Purchase laminin (Thermo Fisher Scientific, cat. no. 23017-015). Store at –80°C until expiration. Reconstitute at 1 mg/ml in sterile PBS (Corning, cat. no. 21-040-CV) and store at –20°C for up to 2 weeks.

Laminin-coated plates

To each well to be coated, add 0.15 ml/cm^2 of poly-L-ornithine (Sigma, cat. no. P4957-50 ml) and incubate at room temperature for 30 min. Pipet up poly-L-ornithine and return to stock bottle. Dry plates in the hood for 5 to 10 min. To each well to be coated, add 0.15 ml/cm^2 of 20 μg/ml laminin (see recipe for 1 mg/ml stock) and incubate at 37°C for 2 hr before use. Alternatively, coated plates may be wrapped in Parafilm and stored at –20°C for up to a month.

LDN193189

Reconstitute LDN193189 powder (Stemgent, cat. no. 04-0074) at 1 mM in DMSO. Store in aliquots at –20°C for 6 months. Store thawed aliquots at 4°C for up to 1 week.

Matrigel

Store Matrigel (Corning, cat. no. 354277) in 50- to 150-μl aliquots at –80°C. For use, thaw on ice and add 80 μl of Knockout DMEM (Thermo Fisher Scientific, 10829-018) per μl of Matrigel. Store reconstituted aliquots at 4°C for up to 2 weeks.

Mebiol

Add 80 μl Essential 8 medium (Thermo Fisher Scientific, cat. no. A1517001) per g of Mebiol (PNIPAAm-PEG thermoresponsive polymer; Cosmobio, cat. no. MBP-PMW20), and dissolve the polymer for 3 to 4 days at 4°C. Use a serological pipet pre-chilled at –20°C to aliquot the reconstituted Mebiol into 10- or 50-ml centrifuge tubes for storage at 4°C protected from light. Reconstituted Mebiol is stable at 4°C for at least 3 months.

N2 supplement, 100×

Purchase 100× N2 supplement (Life Technologies, cat. no. 17502-048). Store in aliquots at –20°C until expiration. Keep thawed aliquots at 4°C for 2 weeks. Do not freeze/thaw aliquots.

N2B27-1 medium

To a 1:1 mixture of DMEM/F12 medium (ThermoFisher Scientific, cat. no. 11039021) and neurobasal medium (see recipe), add each of the following at the indicated final concentrations: 2 mM glutamine (Thermo Fisher Scientific, cat. no. 35050-061), 0.5% (v/v) penicillin/streptomycin (Gibco, cat. no. 15140-122), 1:100 of 100× N2 (see recipe), and 1:50 of 50× B27 supplement (Thermo Fisher Scientific, cat. no. 12587010). Store at 4°C for up to 1 week.

N2B27-2 medium

To a 1:1 mixture of DMEM/F12 medium (ThermoFisher Scientific, cat. no. 11039021) and neurobasal medium (see recipe), add each of the following at the indicated final concentrations: 2 mM glutamine (Thermo Fisher Scientific, cat. no. 35050-061), 0.5% (v/v) penicillin/streptomycin (Gibco, cat. no. 15140-122). 1:200

of 100× N2 supplement (see recipe), and 1:100 of 50× B27 supplement (see recipe). Store at 4°C for up to 1 week.

Paraformaldehyde (PFA), 4% (w/v)

Warm PBS (Corning, cat. no. 21-040-CV) to 60°C on a stirring hot plate in a fume hood. Weigh out PFA (Sigma, cat. no. P6148) at a weighing station; PFA is a potential carcinogen, be cautious when handling it, and use a respirator or face mask. Slowly add 4 g PFA to 100 ml of PBS. Add pellets of sodium hydroxide (NaOH) until PFA solution turns clear. Adjust pH to 7.4 using 12 M hydrochloric acid (HCl). NaOH and HCl are corrosive; use caution when handling.

Pluripotency medium

Add 50× Essential 8 (E8) supplement (Thermo Fisher Scientific, cat. no. A1517001) to E8 medium (Thermo Fisher Scientific, A1517001). Store at 4°C for up to 2 days.

Primary blocking buffer

Prepare 5% (v/v) donkey serum (Sigma Aldrich, cat. no. D9663-10ml), 2% (w/v) bovine serum albumin (Sigma Aldrich, cat. no. A4503), and 0.1% (v/v) Triton X-100 in PBS (Corning, cat. no. 21-040-CV). Store at 4°C for up to 1 month.

Purmorphamine

Reconstitute purmorphamine powder (Stemgent, cat. no. 04-0009) at 20 mM in DMSO and protect from light. Store in aliquots at –20°C for up to 6 months. Store thawed aliquots at 4°C for up to 1 week.

SB431542

Reconstitute SB431542 powder (Selleckchem, S1067) at 100 mM in DMSO. Store in aliquots at –80°C up to 2 years. Store thawed aliquots at 4°C for up to 1 week.

SHH

Reconstitute SHH powder (R&D systems, cat. no. 464-SH-025) at 100 μg/ml in sterile PBS (Corning, cat. no. 21-040-CV). Store in aliquots at –20°C for up to 12 months. Store thawed aliquots at 4°C for up to 1 week.

TGF-β3

Reconstitute TGF-β3 powder (R&D systems, 243-B3/CF) at 20 μg/ml in sterile 4 mM HCl. Store in aliquots at –20 °C for up to 3 months. Store thawed aliquots at 4°C for up to 1 month.

Wash medium

To neurobasal medium (see recipe), add each of the following at the indicated final concentrations: 0.5% (v/v) penicillin/streptomycin (Gibco, cat. no. 15140-122), and 1:50 of 50× B27 supplement (see recipe). Store at 4°C for up to 1 week.

Y-27632

Reconstitute Y-27632 (Selleckchem, 50-863-6) at 10 mM in sterile PBS (Corning, cat. no. 21-040-CV) and protect from light. Store in aliquots at –20°C for up to 6 months. Store thawed aliquots at 4°C for up to 1 week. Do not freeze/thaw aliquots.

COMMENTARY

Background Information

Human pluripotent stem cells (hPSCs) are, in principle, an unlimited source of cells for a range of biomedical applications, including cell replacement therapies, drug screening, and disease modeling. However, the applications typically require large-scale production of specific differentiated cell types (Adil & Schaffer, 2017), and the 2-D culture platforms typically used for hPSC expansion

Table 2D.21.4 Troubleshooting Guide for Generating hPSC-derived Midbrain Dopaminergic Neurons in a Scalable 3-D Biomaterial

Protocol	Problem	Probable cause	Potential solution
Basic Protocol 1,2	Cells are not pluripotent	Clusters are overgrowing and aggregating	During culture, ensure that hPSC clusters do not start to overgrow and aggregate. If cells start to aggregate, passage immediately as single cells.
Basic Protocol 1,2	Cell clusters grow heterogeneously	Clusters are not fully dissociated to single cells during passage	Duration of Accutase treatment and amount of pipet mixing may need to be adjusted for different cell types. If cells are not broken up into single cells, incubate longer with Accutase, and pipet more times.
Basic Protocol 1,2,3	Low viability of cells in 3-D hydrogels	Passaging conditions are too harsh	Duration of Accutase treatment and amount of pipet mixing may need to be adjusted for different cell types. If viability of cells is reduced, incubate for shorter amounts of time with Accutase, and pipet fewer times.
Basic Protocol 1,2,3	Mebiol gels prematurely during passage	Mebiol is warming up	Prevent Mebiol container and positive-displacement pipet tips from warming up. Take the pipet tips out of the –20°C freezer only when needed, to prevent them from warming up unnecessarily. If doing multiple wells, make sure tip is not warming up. To be safe, use a fresh tip for each gel.
Basic Protocol 1,2,3	Gel with encapsulated cells aspirated/lost during feeding	Mebiol spread out on the bottom; medium not warm enough	When placing the cell-hydrogel mix in the well, take care that it does not touch the well edge, as this will make medium aspiration on subsequent days more difficult. Always keep an eye on the gel when aspirating, to avoid accidental gel aspiration.
		Mebiol cooling down during feeding	Avoid unnecessary wait times with the gel outside 37°C incubatator
		Medium not warm enough	Always feed with medium at 37°C
Basic Protocol 1,2,3	Cell yield low after harvest	Cells not adapted to 3-D	Passage for longer in 3-D before starting differentiation
		Mebiol not dissolved fully	Ensure that the PBS to be added is ice-cold, and that the collection tube has been pre-chilled for at least 10 min on ice. Avoid unnecessary wait steps outside the ice bucket during centrifugation, as Mebiol might re-solidify and interfere with cell harvest.
Support Protocol 1	Cells lift off during fixing and staining	High mechanical stress during staining steps	Neurons, especially late-stage neurons, may become fragile and non-adherent after fixing. To avoid this, be very gentle when adding and removing solutions for all steps in the support protocol. Aspirate manually instead of using a vacuum pump. Do not rock at high speed, as this may detach fragile neuronal cells.

and differentiation are difficult to scale up and do not emulate the 3-D microenvironment of natural tissues. Previously, we used a scalable, thermoreversible 3-D hydrogel (Mebiol) to continuously culture and efficiently expand hPSCs for long periods of time while maintaining pluripotency and normal karyotype (Lei & Schaffer, 2013). Based on our recent results (Adil et al., 2017), here we discuss how this platform can be tuned and harnessed for the scalable production of midbrain dopaminergic neurons, which are the type of cells primarily affected in Parkinson's disease. At the end of the desired period of differentiation, cells can be easily harvested from the thermoresponsive gels by cooling, gel dilution, and liquefaction, followed by centrifugation. Many markers of midbrain dopaminergic identity were expressed at a higher level at an earlier time for cells differentiated within the 3-D hydrogel (Adil et al., 2017), in comparison to cells differentiated on a 2-D Matrigel-coated surface. This apparent accelerated differentiation was further confirmed by increased electrophysiological activity, measured using voltage-sensitive dyes, as recently described (Kulkarni et al., 2016) in 3-D-generated neurons. Furthermore, this protocol yields mDA neurons with high levels of EN1 and FOXA2 expression, which are important for long-term cell survival and maintenance of mDA phenotype (Kirkeby et al., 2016). In summary, accelerated, scalable production of functional mDA neurons may facilitate cell replacement therapy, drug screening, and disease modeling in PD.

Critical Parameters and Troubleshooting

For successful implementation of this protocol, several steps, including methods for single-cell passage and handling the thermoresponsive hydrogel, require special attention. Additionally, we have experienced lot-to-lot variability with Mebiol. If possible, experiments should be performed using the same lot of PNIPAAm-PEG.

Tabulated in Table 2D.21.4 are common problems in specific phases of the protocol, probable causes, and potential troubleshooting steps.

Anticipated Results

5 to 10 million cells, with 40% expressing TH and >80% $FOXA2^+/LMX1A^+$ demonstrating a floorplate-derived midbrain phenotype (Kriks et al., 2011), may be generated in 25 days from 100,000 hPSCs in a 100-µl gel in one well of a 12-well plate (Adil et al., 2017). Using this protocol, we have successfully differentiated H1 hESCs, H9 hESCs, WIBR3 hESCs, and 8FLVY6C2 hiPSCs (derived from healthy human fibroblasts; Lan et al., 2013) to mDA neurons (Adil et al., 2017). Expression of additional markers relevant to midbrain dopaminergic neurons, such as GIRK2, EN1, PITX3, or NURR1, may also be detected (Adil et al., 2017). After differentiation in the 3-D hydrogel for 25 days, followed by maturation on 2-D laminin for 20 more days, 80% of the cells fire action potentials, and 25% of the cells can fire action potentials characteristic of mDA neurons. Cells may be striatally transplanted into rodents, and, in rats, cells transplanted survive for at least 4 months and maintain their mDA phenotype (Adil et al., 2017).

Time Considerations

Cells are typically passaged every 4 to 5 days during hPSC culture. After transition from 2-D to 3-D culture, cells should be maintained in 3-D for two passages (10 days) before initiating differentiation. Using the protocol described here, mDA progenitors can be differentiated within 11 days, and mDA neurons can be generated within 25 days (Adil et al., 2017).

Conflict of Interest

M.M.A. and D.V.S. are inventors on patent applications related to biomaterials platforms for stem cell expansion and differentiation.

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