METHOD ARTICLE



Generation of human midbrain organoids from induced pluripotent stem cells [version 1; peer review: 1 approved, 3

approved with reservations]

Nguyen-Vi Mohamed⁽¹⁾, Meghna Mathur, Ronan V. da Silva, Lenore K. Beitel⁽¹⁾, Edward A. Fon, Thomas M. Durcan⁽¹⁾

Neurology and Neurosurgery, Montreal Neurological Institute and Hospital, Montreal, Quebec, H3A2B4, Canada

 First published: 03 Apr 2019, 3:1 https://doi.org/10.12688/mniopenres.12816.1
 Latest published: 11 Feb 2021, 3:1 https://doi.org/10.12688/mniopenres.12816.2

Abstract

The development of brain organoids represents a major technological advance in the stem cell field, a novel bridge between traditional 2D cultures and *in vivo* animal models. In particular, the development of midbrain organoids containing functional dopaminergic neurons producing neuromelanin granules, a by-product of dopamine synthesis, represents a potential new model for Parkinson's disease. To generate human midbrain organoids, we introduce specific inductive cues, at defined timepoints, during the 3D culture process to drive the stem cells towards a midbrain fate. In this method paper, we describe a standardized protocol to generate human midbrain organoids (hMOs) from induced pluripotent stem cells (iPSCs). This protocol was developed to demonstrate how human iPSCs can be successfully differentiated into numerous, high quality midbrain organoids in one batch. We also describe adaptations for cryosectioning of fixed organoids for subsequent histological analysis.

Keywords

Organoid, midbrain, iPSC, cryosections, neuromelanin, dopaminergic neurons

Open Peer Review

| Approval S | tatus ষ | / / / | ? | |
|--|------------------|------------------|------|------------------|
| | 1 | 2 | 3 | 4 |
| version 2 (revision) 11 Feb 2021 | view | view | | |
| version 1 03 Apr 2019 | ? view | ? view | view | ? view |

- Scott D. Ryan ^D, University of Guelph (U of G), Guelph, Canada
- 2. **Olga Corti**, Brain and Spine Institute (ICM), Paris, France

Philippe Ravassard, Brain and Spine Institute (ICM), Paris, France

- 3. **Mark E. Hester**, The Research Institute at Nationwide Children's Hospital, Columbus, USA
- Birgitt Schuele, Stanford University School of Medicine, Stanford, USA

Any reports and responses or comments on the article can be found at the end of the article.

Corresponding author: Thomas M. Durcan (thomas.durcan@mcgill.ca)

Author roles: Mohamed NV: Conceptualization, Data Curation, Formal Analysis, Funding Acquisition, Investigation, Methodology, Validation, Writing – Original Draft Preparation, Writing – Review & Editing; **Mathur M**: Conceptualization, Methodology, Writing – Original Draft Preparation, Writing – Review & Editing; **da Silva RV**: Methodology, Writing – Original Draft Preparation; **Beitel LK**: Conceptualization, Writing – Original Draft Preparation, Writing – Review & Editing; **Fon EA**: Funding Acquisition, Resources, Supervision, Writing – Review & Editing; **Durcan TM**: Conceptualization, Funding Acquisition, Supervision, Writing – Original Draft Preparation, Writing – Review & Editing

Competing interests: No competing interests were disclosed.

Grant information: NVM is supported by Jeanne Timmins Costello, Fonds de Recherche du Québéc-Santé and Parkinson Canada fellowships. EAF is supported by a Foundation Grant from the Canadian Institutes of Health Research, the Consortium Québécois sur la Découverte du Médicament, Fonds de Recherche du Québéc-Santé Quebec Parkinson Network and the Michael J. Fox Foundation. TMD is supported by a Parkinson's Canada New Investigator Award, and through funding of the brain organoid program at the MNI by the Van Berkom-Saucier Foundation.

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Copyright: © 2019 Mohamed NV *et al*. This is an open access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

How to cite this article: Mohamed NV, Mathur M, da Silva RV *et al.* Generation of human midbrain organoids from induced pluripotent stem cells [version 1; peer review: 1 approved, 3 approved with reservations] MNI Open Research 2019, 3:1 https://doi.org/10.12688/mniopenres.12816.1

First published: 03 Apr 2019, 3:1 https://doi.org/10.12688/mniopenres.12816.1

Background

Parkinson's disease (PD) is a neurodegenerative disorder, affecting more than 1% of the population over 65 years of age. The majority of cases are idiopathic, while about 10% have been linked to genetic mutations. Classical hallmarks of PD are the loss of dopaminergic (DA) neurons in the substantia nigra pars compacta, accompanied by the presence of neuronal inclusions called "Lewy bodies". Several cellular pathways have been implicated in PD pathogenesis, including mitochondrial dysfunction¹⁻³, perturbed neuronal activity^{4,5} and dysregulated protein homeostasis due to lysosomal, autophagy and proteasomal defects⁶⁻⁹. However, there is no treatment to halt the progression of the disease. To date, treatment of PD is limited to symptom management. It is therefore necessary to refine the models we use in fundamental research to understand the pathophysiology of PD and to develop more effective therapeutic strategies.

In 2006, Drs. Kazutoshi Takahashi and Shinya Yamanaka described the reprogramming of somatic cells into induced pluripotent stem cells (iPSCs)10. Since their discovery, this technology has opened up many new research avenues, including for PD. With their self-renewal abilities and potential to be differentiated into disease-relevant cells from all three developmental lineages, iPSCs provide a unique tool to study PD within a human neuron, without the difficulties in obtaining neurons from a human brain¹¹. iPSCs can be directly reprogrammed from skin, blood or urine of an individual without raising the ethical concerns previously triggered by the use of fetal stem cells. In 2009, Soldner et al. were the first to describe the generation of an iPSC cell-line from a patient with sporadic PD, and the subsequent differentiation of these cells into dopaminergic (DA) neurons¹². Taking advantage of iPSC technology, many studies have started to investigate the pathological mechanisms of PD in iPSC-derived DA neurons from patients, compared to neurons from healthy individuals¹³⁻³³.

Recently, the development of organoids has become a major technological advance in the stem cell field and represents a bridge between traditional 2D cultures and *in vivo* mouse models. In 2013, Lancaster *et al.* described a novel 3D model called a cerebral organoid, that recapitulated different areas of the human brain³⁴. Kept in culture, these organoids formed a complex self-organized neuronal tissue composed of a mixed population of neurons, astrocytes and oligodendrocytes. In contrast to neurospheres, the cells were organized in layers that, at early stages, included a ventricular zone composed of progenitors. Neurons within these organoids were functional and had spontaneous electrical activity in networks. Interestingly, brain organoids could be cultured for long periods to obtain morphologically and functionally mature cells, in contrast to neurosphere cultures³⁴⁻³⁷.

Since 2013, different types of brain organoids have been generated based on adaptations of the initial protocol published by Lancaster. Earlier, the protocols involved no external addition of growth factors in the medium, resulting in self-differentiating cells. However, different laboratories now directly drive the stem cells towards specific fates. The key to efficient brain organoid generation is a defined combination of inductive signals and physical factors that drive pluripotent stem cells to form 3D brain organoids. The modulation of these factors gives rise to multiple types of brain organoids that can be used to model neurodevelopmental and neurodegenerative disorders that affect distinct regions within the brain. Protocols now exist for making human cerebral^{34,38,39}, forebrain-like (dorsal and ventral)^{40,41}, cerebellar⁴², cortical-like (dorsal and ventral)^{43,44}, hippocampal and choroid plexus-like tissue⁴⁵, midbrain^{35,36,41}, hypothalamic⁴¹, pallium and subpallium⁴⁶ brain organoids.

iPSC-derived human brain organoids recapitulate brain development and can be used to study normal neurodevelopment. Brain organoid development recapitulates the early to mid-fetal development, and the epigenomic signatures of the human foetal brain^{41,47,48}. So far, cerebral organoids have been used to study pathologies including microcephaly³⁴, Zika virus infection^{49–52}, and autism spectrum disorders^{46,53}.

Recently, human brain organoids have been used to investigate aspects of neurodegenerative disorders. Two groups generated cerebral organoids from iPSCs of Alzheimer's disease (AD) patients carrying familial mutations for presenilin 1 or amyloid precursor protein duplication, and successfully recapitulated the aggregation of beta-amyloid protein and tau pathology (hyperphosphorylation and aggregation), the two neuropathological markers of AD, in a human model. Treatment of the 3D cultures with drugs targeting either amyloid-beta aggregation or tau phosphorylation decreased the pathological markers^{54,55}. These promising results demonstrated that human brain organoids represent a relevant model for drug discovery. The development of different types of brain organoids represents a major advance in the stem cell field. In particular, the development of midbrain organoids represents a new drug discovery tool for PD. Two groups published similar protocols to generate the human midbrain organoids, based on specific inductive signals introduced at specific stages in the 3D cultures to drive the stem cells towards a midbrain fate^{35,36}. They showed that the midbrain organoids are composed of functional midbrain neurons producing neuromelanin granules, a by-product of dopamine synthesis. Of the neuronal population, 30% was myelinated due to the presence of oligodendrocytes. Interestingly, Monzel et al., showed the presence of nodes of Ranvier and spontaneous saltatory transmission^{35,36}. Considering the mix of neuronal populations connected within the midbrain organoids, they represent an interesting model to discover new pathological mechanisms involved in PD.

In this paper, we provide a standardized protocol for a robust derivation of iPSCs lines into 3D midbrain organoids. This protocol is an adaptation of the Nature protocol paper initially published by Lancaster in combination with discoveries from Jo *et al.* and Monzel *et al.*^{34–36} in order to successfully produce high quality midbrain organoids with a successful midbrain fate generation rate of near 100% per batch. We also describe a cryosectioning protocol that we adapted to produce high-quality histological sections from midbrain organoids, overcoming difficulties resulting from the particular texture of cultured tissue as well as their small size, relative to rodent brains. Taken

together, this article extensively explains the methods involved in generating these iPSC-derived organoids and their histological analysis.

Materials

The materials, reagents and equipment listed in this document can be substituted. However, the performance of the protocol may not be the same and may need to be optimized or redeveloped upon significant modifications to the materials and/or methods. It is also important to note that there is a significant lot-to-lot variability for certain reagents. In order to monitor this variability, we recommend a systematic test of new batches.

List of materials, reagents and equipment for 3D midbrain generation

For growing human iPSCs, the quality of reagents is critical. Variability in the quality of any of these materials or in associated manufacturing processes will lead to inconsistent quality, which has been reported to negatively impact human iPSCs cultures. See Table 1 and Table 2.

Background information on media

Neuronal induction medium: A cell-permeable, highly potent and selective inhibitor of Rho-associated, coiled-coil containing protein kinase (ROCK) enhances survival of iPSCs when dissociated to single cells and improves embryonic bodies formation⁵⁶. Midbrain is of ectodermal origin, thus neuroectodermal differentiation towards a floor plate can be induced with the help of dual-SMAD inhibition factors, Noggin and SB431542 and a Wnt pathway activator, CHIR99021 (chemical name: 6- [[2-[[4-(2,4-dichlorophenyl)-5-(5-methyl-1H-imidazol-2-yl)-2-pyrimidinyl]amino [ethyl]amino]-3-pyridinecarbonitrile)⁵⁷. Complementary to these factors, heparin plays a role in enhancing the activity of Wnt signaling⁵⁸. 2-mercaptoethanol regulates oxidative stress to maintain cell growth and avoid cell death due to

Table 1. List of media and biochemicals.

| Reagents | Supplier/ manufacturer | Catalogue number |
|---|------------------------|------------------|
| mTeSRTM1 Basal medium/ mTeSRTM1 5x supplement | STEMCELL Technologies | 05851/05852 |
| Dulbecco's Modified Eagle Medium/ Nutrient Mixture F-12 (DMEM/F12) | Gibco | 10-565-018 |
| Neurobasal | Thermo Fisher | 21103-049 |
| B27 without vitamin A | Invitrogen | 12587010 |
| Glutamax | Gibco | 35050-061 |
| Minimum Essential Medium- Non-Essential Amino Acids (MEM-NEAA) | Invitrogen | 11140050 |
| 2-mercaptoethanol | Gibco | 21985023 |
| Heparin | Millipore Sigma | H3149 |
| SB431542 | Selleck Chemicals | S1067 |
| Noggin | PeproTech | 120-10C |
| CHIR99021 | Selleck Chemicals | S2924 |
| ROCK inhibitor | Tocris Bioscience | 1254 |
| Sonic Hedgehog (SHH) | PeproTech | 100-45 |
| Fibroblast Growth Factor 8 (FGF8) | PeproTech | 100-25 |
| Insulin | Millipore Sigma | 19278 |
| Laminin | Millipore Sigma | L2020 |
| Penicillin-Streptomycin | Millipore Sigma | P0781 |
| Brain-derived Neurotrophic Factor (BDNF) | PeproTech | 450-02 |
| Glial cell-derived Neurotrophic Factor (GDNF) | PeproTech | 450-10 |
| Ascorbic acid | Millipore Sigma | A5960 |
| Dibutyryl- cyclic AMP (db-cAMP) | Millipore Sigma | D0627 |
| Accutase | Gibco | A11105-01 |
| Matrigel® reduced growth factor | BD Biosciences | 356230 |
| N2 | Gibco | 17504-044 |
| Antibiotics-Antimycotic | Gibco | 15240-062 |

| Consumables and equipment | Supplier | Catalogue No. |
|--|---------------------------|---------------|
| Cell culture dishes, 10cm | Thermo Fisher | 08772E |
| 96 well plates U-bottomed wells ultra low attachment | Corning | CLS7007 |
| 5ml serological pipettes, wrapped | Thermo Fisher | 1367811D |
| 15ml Falcon Conical tube | Thermo Fisher | 352097 |
| 50ml Falcon Conical tube | Thermo Fisher | 352098 |
| 6 well plates, ultra-low attachment | Corning Co-star | CLS3471 |
| 10µl ultrafine long tips (Autoclave before use in cell culture) | Harvard Apparatus | DV-P1096-FR |
| 100-250µl non-sterile ultrafine tips, refill package (autoclave before use in cell culture) | VWR | 89368-954 |
| 100-1250µl non-sterile ultrafine tips, refill package (autoclave before use in cell culture) | VWR | 89079-470 |
| Nitrile glove, Small (Box of 100, other sizes available from XS-XL) | Diamed | TECNITE-SPF |
| Pipette Set, Gilson | Mandel Scientific | |
| Pipette Aid, Drummond | VWR | 53498-105 |
| 25°C incubator | Thermo Fisher | S50909 |
| Centrifuge for 96 well plates | Eppendorf | 022625501 |
| Orbital shaker | Scientific Industries Inc | SI-M100 |
| Multichannel pipette | Gilson | |
| 50mL reagent reservoir | Corning | 4870 |
| Box of 1mL cut tips autoclaved | | |
| Manual repeater pipette | Gilson | F164072 |
| Distritips mini ST for manual repeater | Gilson | F164140 |

Table 2. List of consumables and equipment.

toxicity. Retinoic acid, a metabolite of vitamin A, is a potent caudalizing factor that we exclude from our media to promote midbrain differentiation. See Table 3.

Midbrain patterning medium: The patterning can be influenced by sonic hedgehog, SHH and fibroblast growth factor FGF8 as they are responsible for guiding the cells towards the mesencephalic fate⁵⁹. To avoid the dorsal influence on organoids patterning, vitamin B27 without vitamin A is considered appropriate⁶⁰. See Table 4.

Tissue Growth Induction Medium: The presence of insulin and laminin promote the growth of tissue embedded. See Table 5.

Final Differentiation Medium: The presence of brainderived neurotrophic factor (BDNF), is reported to play a potential role in developing cholinergic, dopaminergic, serotonergic and gamma-aminobutyric acid (GABA) ergic neurons, along with promoting the function and survival of other neuronal populations⁶¹ The other growth factor, glial cell-derived neurotrophic factor (GDNF) also affects neuronal differentiation, maturation and neurite growth by enhancing

Table 3. Composition of neuronal induction medium.

| Final Concentration | Recipe (50 ml) |
|-----------------------------------|----------------|
| DMEM-F12 + Anti /Neurobasal (1:1) | 25 mL + 25 mL |
| 1:100 N2 | 0.5 mL |
| 1:50 B27 without vitamin A | 1 mL |
| 1% Glutamax | 0.5 mL |
| 1% MEM-NEAA | 0.5 mL |
| 2-mercaptoethanol | 0.175 µL |
| 1 µg/mL Heparin | 50 µL |
| 10 µM SB431542 | 50 µL |
| 200 ng/mL Noggin | 50 µL |
| 0.8 µM CHIR99021 | 13.5 µL |
| 10 µM ROCK inhibitor | 50 µL |

Note: DMEM-F12+ Anti is an initial mix of 5 mL of Antibiotics-Antimycotics to 495 mL of DMEM-F12
 Table 4. Composition of midbrain patterning medium.

| Final Concentration | Recipe (50 ml) |
|-----------------------------------|----------------|
| DMEM-F12 + Anti /Neurobasal (1:1) | 25 mL + 25 mL |
| 1:100 N2 | 0.5 mL |
| 1:50 B27 without vitamin A | 1 mL |
| 1% Glutamax | 0.5 mL |
| 1% MEM-NEAA | 0.5 mL |
| 2-mercaptoethanol | 0.175 μL |
| 1 µg/mL Heparin | 50 µL |
| 10 µM SB431542 | 50 µL |
| 200 ng/mL Noggin | 50 µL |
| 0.8 µM CHIR99021 | 13.5 µL |
| 100 ng/mL (or 200ng/mL) SHH | 25 µL |
| 100 ng/ mL FGF8 | 50 µL |

Note: DMEM-F12+ Anti is an initial mix of 5 mL of Antibiotics-Antimycotics to 495 mL of DMEM-F12

Table 5. Composition of tissue growth induction medium.

| Final Concentration | Recipe (50 mL) |
|------------------------------|------------------|
| Neurobasal | 50 mL |
| 1:100 N2 | 0.5 mL |
| 1:50 B27 without vitamin A | 1 mL |
| 1% Glutamax | 0.5 mL |
| 1% MEM-NEAA | 0.5 mL |
| 2-mercaptoethanol | 0.175 µL |
| 2,5 µg/mL insulin | 12.5 µL |
| 200 ng/mL laminin | 8.5 µL |
| 100 ng/mL (or 200 ng/mL) SHH | 25 µL (or 50 µL) |
| 100 ng/ mL FGF8 | 50 µL |
| Penni/Strep | 0.05 mL |

myelination. It has also been reported to induce dopaminergic phoneotype⁶². See Table 6.

iPSC lines

For growing midbrain organoids from human iPSCs, the quality of iPSCs is critical. Variability in the iPSCs maintenance will negatively impact midbrain organoids generation (section "Protocol description for iPSC culture and maintenance"). The observations provided in this method were generated from two iPSCs lines from healthy individuals, NCRM-1 and XCl-1 (Table 7).

List of material, reagents and equipment for 3D midbrain histological processing and cryosections See Table 8.

Table 6. Composition of final differentiation medium.

| Final Concentration | Recipe (50 ml) |
|----------------------------|----------------|
| Neurobasal | 50 mL |
| 1:100 N2 | 0.5 mL |
| 1:50 B27 without vitamin A | 1 mL |
| 1% Glutamax | 0.5 mL |
| 1% MEM-NEAA | 0.5 mL |
| 2-mercaptoethanol | 0.175 µL |
| 10 ng/mL BDNF | 25 µL |
| 10 ng/mL GDNF | 25 µL |
| 100 µM ascorbic acid | 25 µL |
| 125 µM db-cAMP | 12.5 µL |
| Penni/Strep | 0.05 mL |

Table 7. Summary of iPSCs used.

| iPSC line name | Supplier |
|-------------------|---|
| NCRM-1 | NIH CRM Lonza Contract https://nimhstemcells.org/crm.html |
| XCI-1 | Dr Xianmin Zeng's laboratory The Buck Institute for Research on Aging https://xcell-app-prod.s3-us-west-1.amazonaws. com/file/spina/attachment/2/69_CNS_iPSC_review_ SCTM.pdf |

Table 8. Material for histological sections.

| Reagents and equipment | Supplier | Catalogue No. |
|--|-------------------|---------------|
| Formaldehyde | Thermo Scientific | 28908 |
| Frosted microscope slides | Fisher Scientific | 12-550-15 |
| Cryomolds | Fisher Scientific | 22-363-553 |
| Microtome blades, low profile | Fisher Scientific | 12-634-1C |
| Optimal Cutting Temperature (OCT) medium | VWR | 75806-668 |
| Cryostat | Thermo Scientific | 14-071-401 |

List of antibodies See Table 9.

Method

Protocol description for iPSC culture and maintenance General principles for culturing and maintaining human *iPSCs.* Human iPSCs are sensitive to many stresses, including shear stress, heat shock, and changes in media formulation

| - | | |
|---|---------------------------|---------------|
| Antibodies | Supplier | Catalogue No. |
| Tyrosine Hydroxylase (TH), rabbit, polyclonal | Pel Freez | P40101-150 |
| β-Tubilin III (TUJ1), mouse, monoclonal | Millipore | MAB5564 |
| MAP2, chicken, polyclonal | EnCor Biotechnology | CPCA-MAP2 |
| Donkey anti-chicken Alexa 488 | Jackson ImmunoResearch | 703-545-155 |
| Donkey anti-mouse Dylight 550 | Abcam | ab96876 |
| Donkey anti-rabbit Dylight 650 | Abcam | ab96894 |

Table 9. Summary of antibodies used.

and must be manipulated with extreme care at all steps of the protocol.

Technical and safety considerations for manipulating IPSCs

- The iPSC cells should not have been passaged more than 10 times.
- The cells need to be a minimum of passage 2 after thawing for generation of midbrain organoids.
- Do not work with colonies that present with differentiated areas (Figure 1a). We recommend removing the differentiated areas during daily maintenance and to never exceed 5% of differentiated areas prior to organoid generation for optimal organoid formation.
- Sterile technique must be used at all times when working with cells or in preparing reagents and materials.
- Human iPSCs lines are to be handled within a Class II biosafety laminar flow hood to protect the worker from possible biohazardous agents.

Preparing Matrigel® hESC-qualified matrix coated culture dishes, thawing frozen human iPSCs cryovials, and daily maintenance of human iPSCs

- Start by thawing an aliquot of 150-200 μl Matrigel® hESC-qualified Matrix at 4°C, 15–20 minutes prior to use (depends on the dilution factor of each lot per manufacturer instructions).
- Prepare DMEM/F-12 solution by removing 5 mL of DMEM/F-12 from a 500 mL bottle and adding 5 mL of anti-anti. Place the bottle at 4°C until cold.
- Once the Matrigel® is thawed and DMEM/F-12 is cold, prepare a coating solution diluted as per manufacturer instructions and mix well.
- Immediately use the diluted Matrigel® solution to evenly coat the tissue culture dishes (2 ml/ 60 mm dish and 5 ml/ 100mm dish) by swirling the tissue culture dish in each direction, multiple times. Incubate at 37°C for a minimum of one hour before use.

- Meanwhile, warm up mTeSRTM 1 and DMEM/F-12 medium at room temperature (RT) for a minimum of one hour. Do not use a water bath during this process because the media components would be degraded.
- Once the tissue culture dish is coated and mTeSR[™] 1 and DMEM/F-12 medium are warmed, get the frozen cryovial of iPSCs from the liquid nitrogen storage container, and quickly thaw the vial by warming in a 37°C water bath. Continuously shake the cryovial until only a small frozen pellet remains.
- Using a 5 mL glass pipette, transfer the contents of the cryovial to a 5 mL solution of DMEM-F12 (with antianti), in dropwise manner and gently pipette up and down 1–2 times.
- Centrifuge the cells at 1200 rpm for 3 minutes at RT. After centrifugation, aspirate the medium, leaving the cell pellet intact. Using a 5 mL glass pipette, gently resuspend (1–2 times) the cell pellet in 3 mL of mTeSR[™] 1 medium containing Y27632 (1:1000 dilution).
- Take the coated tissue culture dish from 37°C and aspirate out the coating medium. Transfer the cells in mTeSRTM 1 medium to the coated dish and place in a 37°C, 5% CO₂ incubator. Do not disturb the dish for 24h, to allow the cells to attach.
- Change the medium every day with mTeSRTM 1 without Y27632, until the cell confluency reaches 70% and cells are close in contact.
- Daily, visually identify regions of differentiation under the microscope and remove them by aspiration under the sterile hood.

Passaging of human iPSCs

- When the cells reach 60–70% confluency, they are ready to be split.
- Aspirate out the medium from the dish and wash the cells with 3 mL DMEM/F-12 (with anti-anti).

a.



b.





d.

e.



FD day 1 FD day 5 FD day 5 FD day 5

Figure 1. Midbrain organoid generation, timeline and steps of tissue formation. a) Quality of iPSCs suitable for midbrain organoids formation. **b**) Timeline for midbrain organoid generation. PBMCs (peripheral blood mononuclear cells) from individuals were collected and reprogrammed into iPSCs. Uniform embryoids bodies are formed from iPSC colonies and then patterned into neuronal midbrain fate with inductive signals. EBs are then embedded in Matrigel scaffold to promote growth of tissue. The tissue formed was cultured on an orbital shaker for several months. c) EB with smooth edge 48 hours after formation. d) Extrusion of buds on EB after midbrain patterning. e) Typical EB 24 hour after embedding in Matrigel®. f) Day 1, 5 and 15 after transferring the tissue into final differentiation media. Scale bar= 500 µm.

- Add gentle cell dissociation medium (5 ml for 100 mm dish and 1 mL for 60 mm dish) at RT until the cells at the edge of the colony begin to detach from each other. (Note: Do not leave cells for longer as the cell viability will be affected)
- Aspirate out the gentle cell dissociation medium from the dish and wash the dish with DMEM/F-12.
- Add 5mL DMEM/F-12 to the dish and gently detach the colonies by scrapping with a cell scrapper. Using a glass pipette, transfer the detached cell aggregates in a Falcon tube.
- Centrifuge the Falcon tube with cells at 1200 rpm for 3 minutes.
- After the centrifugation is done, check the pellet and aspirate out the supernatant (do not let the pellet dry so leave 100μ L-200 μ l DMEM after aspiration).
- Using a glass pipette, resuspend the pellet gently to break the cell aggregates in mTeSRTM 1 medium with 10 μ M Y27632.
- For seeding cells, add 10% of the original cell suspension to a new coated dish when passaging between similar size dishes. For a small to large dish passage-take 20% of the original cell suspension and from a large to small dish, take 5%.
- Incubate the dishes at $37^{\circ}C$ until the colonies reach 70% confluency.

Generating midbrain organoids

Eight days are necessary to generate midbrain organoids from iPSC colonies. Then, the organoids are grown with constant agitation for maturation. The timeline is described in Figure 1b.

Seeding of iPSCs – Day 0

- Start with one 10 cm dish of iPSCs at 70% confluency, cultured in mTeSR[™] 1 to obtain 96 midbrain organoids from a 96-well ultra-low attachment plate.
- Dissociate cells using Accutase by removing medium from cells, wash cells once with DMEM-F12 +Anti-Anti and add 5 ml Accutase at RT. Incubate cells for 3 minutes at 37°C, then stop reaction with DMEM-F12+Anti-Anti. Transfer the medium with the cells into a 15 mL Falcon tube and centrifuge for 3 min, at 1200 rpm in a regular Falcon centrifuge. Remove the supernatant.
- Using a 1mL tip combined with a 200 μl tip resuspend cells in 5 ml of neuronal induction medium (Table 3) by pipetting gently up and down 3 times. Count cells. *Note: The 200* μl *tip is at the end of the 1 ml tip.*
- Plate 10,000 cells/well in an ultra-low attachment 96 well U-bottomed plate with a multichannel pipette and add neuronal induction medium (Table 3) to a total of 200 μ /well with a multichannel pipette.
- Centrifuge the plate for 10 min at 1200 rpm at 37°C.
- Incubate cells for 48 hrs at 37°C in a regular cell incubator.

Change medium – Day 2

Observation: EBs should have smooth and round edges (Figure 1c)

- Change medium to neuronal induction medium WITHOUT ROCK inhibitor using a multichannel pipette.
- Incubate cells for 48 hrs at 37°C in a regular cell incubator.

Change medium – Day 4

Observation: EBs should reach a diameter of $>300 \mu m$ (typically 400-600 μm) with smooth and round edges

- Change medium to midbrain patterning medium (Table 4) using a multichannel pipette.
- Incubate cells for 48 hrs at 37°C in regular cell incubator.

Change medium and embedding in Matrigel® – Day 7

Observation: Neuroectoderm buds should have started to extrude before the embedding (Figure 1d)

- Transfer aliquots of Matrigel® reduced growth factors to ice and let it reach 4°C.
- Remove the medium from each well with a multichannel pipette and add 30 µl of reduced growth factor Matrigel® with the manual repeater-pipette and sterile distritips.
- Incubate the plate for 30 min at 37°C in a regular cell incubator.
- Add 200 μ l of tissue growth induction medium (Table 5) and incubate for 24 hours at 37°C in a regular cell incubator.
- Autoclave a box of 1mL cut tips if not already done.

Transfer the organoids to final differentiation medium – Day 8 Observations: Neuroepithelium should be more developed (Figure 1e)

- Add 3 mL of final differentiation medium per well of an ULTRA LOW ATTACHMENT 6 well plate.
- Transfer the midbrain organoids with a cut 1000 µl pipette tip into the 6 well plate (5 organoids/well).
- Put the 6 well plates on the orbital shaker (set at 70 rpm) in the 37°C regular cell incubator and change the medium every 2–3 days using final differentiation medium (Table 6). When the midbrain organoids reach 1 mm in diameter, increase the final differentiation media volume to 5 mL to provide enough nutrients (Figure 1f).

Observations: From day 1 in final differentiation media to day 50 the organoids should grow from 600 μ m to approximately 4 mm (Figure 2). The presence of dopaminergic neurons is tested by immunofluorescence staining with tyrosine hydroxylase antibody (Figure 3a–c). In parallel, the functionality of dopaminergic neurons is tested at 35 days by treating the midbrain organoids with 100 μ m of dopamine. The appearance of black/ brown dots after 10 days correspond to neuromelanin granules, a by-product of dopamine synthesis (Figure 4).



Figure 2. Difference in size expected from day 1 to day 50 in final differentiation media. The hMOs will grow from 600µm to 4mm. Scale bar= 4mm.



Figure 3. Typical cytoarchitecture of hMOs at day 30. a) Cryosection of hMO. The immunofluorescence staining for tyrosine hydroxylase reveals dopaminergic neurons (red) and nuclei (blue). We observe multiple ventricule-like structures or "rosettes". **b**) A typical rosette in hMOs is composed of neural progenitor cells (squares) and differentiated cells (triangles). On the outside layer of the rosette, we observe the dopaminergic neurons stained with anti-tyrosine hydroxylase TH (red), and the neurons stained for MAP2 (green) and TUJ1 (yellow) (stars). The cells negative for MAP2, TH and TUJ1 are progenitors (squares). Scale bar=200µm. **c**) Higher magnification of differentiated neurons. We observe the presence of dopaminergic neurons revealed by TH staining. Scale bar = 70µm.

Histological processing of organoids by cryosectioning *Tissue fixation and cryoprotection*

• Remove medium from plates and fix organoids with submersion in fresh 4% formaldehyde solution for 1h at RT or O/N at 4°C in fume hood.

HAZARD WARNING: Use care handling formaldehyde solutions. Follow instructions according to the product MSDS.

- Wash organoids with PBS 3 times for 5 min to remove formaldehyde.
- Incubate organoids in 20% sucrose solution at 4°C until the organoids sink. This is usually achieved O/N or after 3 days.

а.

СТІ

NOTE: Do not extend the incubation for longer than 3 days, as this impacts the quality of sectioning. In rare occasions, the tissue does not sink after a long time because it includes low-density components such as Matrigel®. However, this does not impact subsequent procedures.

Block embedding

• Transfer organoids from the sucrose solution to a cryomold using a pipette with a cut tip. We typically embed up to 9 organoids per block. If embedding different types of organoids (different ages, cell lines, etc.) in the same block, care must be taken not to mix the organoids.



100 µm dopamine

Figure 4. Dopaminergic neurons release by-products of dopamine synthesis. a) At day 35 in final differentiation media, midbrain organoids are treated with 100 µm dopamine for 10 days. Under dopamine treatment, brown/black areas appeared. **b**) Fontana Masson staining confirmed the presence of neuromelanin granules with dopamine treatment. Black dots are extracted with colorimetric selection from GIMP software and quantified by ImageJ (ref. (63)). **c**) Statistical analysis of neuromelanin granules number in GraphPad Prism, unpaired t-test, p*** <0.001, 4n.

- After all organoids are placed in the mold, remove all the sucrose solution with a paper tissue, taking care that the organoids do not stick to the paper.
- Slowly pour optimal cutting temperature (OCT) mounting medium directly on top of the organoids to ensure they stay on the bottom of the cryomold. If embedding different types of organoids, be careful to maintain their organization.
- Use a needle to place organoids in the desired positions, while taking care not to move organoids upwards. Space the organoids about 1 mm from each other.
- Freeze organoids by placing the mold in a -80°C freezer or in the gaseous phase of a liquid N₂ container. When moving the mold, take care not to tilt excessively, which may displace the organoids.
- Once completely frozen, blocks may be stored long term inside a closed container to prevent drying in a -80°C freezer.

Cryosectioning

• Set cryostat temperature and place all blocks to be cut in the same session inside the cryostat chamber to equilibrate the temperature.

NOTE: The relationship between the cryostat temperature and the actual temperature at the block surface after mounting varies according to the cryostat model. Using a thermal probe, we found that a surface temperature of -9° C allows easy production of high-quality samples. However, the precise setting necessary to achieve this temperature must be determined for each machine.

- Prior to, or shortly after, removal of the block from mold, cut one corner of the block to keep track of the block orientation in the subsequent steps.
- Trim the block edges using a razor blade, maintaining a margin of 1-2 mm of OCT around the area containing organoids.

WARNING: Use care when handling the razor blade inside of the cryostat. Avoid manually cutting blocks that are not equilibrated with the cryostat temperature, as these become harder and need more strength to be cut, which may lead to injuries.

- Pour an amount of OCT on the sample holder (chuck) sufficient to cover the entire bottom surface of the block.
- Press the block on top of the OCT layer on the sample holder using a heat extractor to orient the block as horizon-tally as possible. Wait until OCT freezes completely.
- Place the mounted block in the microtome head and cut sections in the desired thickness. We routinely produce sections with a thickness ranging between 10 and 20 µm.
- If necessary, flatten sections using a pair of brushes and pick sections using a slide kept at room temperature (direct mount method).

• Let slides air dry for 30–60 min and proceed with histological staining. The slides may also be kept in boxes at -80°C for long term storage.

Observations: Cryosections on 30-day aged midbrain organoids reveal the presence of rosettes in the tissue (Figure 3a). The progenitors are localized in the center of rosettes while the more mature neurons localized on the external layer. The differentiated cells are positive for neuronal markers such as MAP2 and TUJ1. TH is more specific for dopaminergic neurons (Figure 3bc).

Immunostainings, images acquisition and statistical analysis

Immunostaining and Fontana Masson staining. Cryosections were rehydrated in PBS for 15 min and surrounded with a hydrophobic barrier using a barrier pen. The sections were then blocked for one hour at room temperature in a humidified chamber, with blocking solution (5% of normal donkey serum, 0.05% BSA, and 0.2% Triton X-100 in PBS. They are then incubated overnight at 4°C with primary antibodies diluted in blocking solution (See Table 9). The following day, cryosections were washed three times in PBS, fifteen minutes each, and then incubated in secondary antibodies diluted in blocking solution (See Table 9) for one hour at room temperature. Then we washed the sections three times in PBS for fifteen minutes each. Hoerscht (diluted 1/5000 in PBS) was incubated 10 min on sections, followed by a wash in PBS for 10 min. Finally, we mounted the section with an aqua-mounting media and visualized the staining under a confocal microscope (Figure 3).

Fontana Masson stainings (Figure 4b) were performed with an Abcam staining kit (#ab150669) following provider's instructions on regular paraffin sections of hMos.

Imaging. iPSCs colonies (Figure 1a) were imaged with an inverted microscope Motic AE2000 and the Moticam BTO camera associated, while the EBs images (Figure 1c-f) were taken with a transmitted light microscope EVOS XL Core. The hMos (Figure 2 and Figure 4a) were imaged with a ZEISS Stemi 508 stereomicroscope combined with a ZEISS Axiocam ERc 5s camera. The fluorescence images (Figure 3) were acquired with a Leica TCS SP8 confocal. Fontana Masson stainings (Figure 4b) were acquired with a clinical microscope Olympus BX46 and an Olympus DP27 digital color camera associated.

Raw fluorescent images were opened in ImageJ software (version 2.0.0-rc-69/1.52i) with a red, blue, green or yellow color associated to each channel, before all images were merged to create a merged image. Black dots from Fontana Masson stainings pictures were extracted with colorimetric selection from GIMP software (version 2.8.22) and quantified by ImageJ (version 2.0.0-rc-69/1.52i) following the method described in 63.

Statistical analysis. The statistical analysis was performed in GraphPad Prism v7.0. For the quantification of neuromelanin

granules, we performed a normality test followed by a parametric unpaired t-test, $p^{***} < 0.001$.

Results and observations

We observed that good quality of iPSCs is determinant to successfully generate high-quality of hMOs. iPSCs colonies are maintained daily and passaged in order to present no differentiated area (Figure 1a). If the colonies still presented less than 5% of differentiated areas after the precautions described, we removed the differentiated areas prior to the generation of hMOs to ensure an optimal quality of hMOs. The process to generate hMOs from iPSCs, at 70% confluency, takes eight days (Figure 1b). Then, embryoid bodies formed from the iPSCs, were differentiated toward midbrain fate in stationary culture and embedded in Matrigel® to promote the formation of the tissue. Indeed, we observed the progressive appearance of the tissue within the EB (Figure 1c-f). Once the EBs presented multiple bud extrusion (Figure 1e), they were transferred to shaking culture to promote the growth of the tissue (Figure 1b and 1f). After 50 days of shaking culture in final differentiation media, the hMOs grew up to approximately four millimeters (Figure 2).

Immunostainings on cryosections of hMOs thirty days-aged, showed a typical cytoarchitecture (Figure 3a) with multiple rosettes. The center of the rosettes was composed of neural progenitors cells, negative for MAP2 and TUJ1 (Figure 3b squares) while the outside layer is composed of more matured cells, including neurons MAP2 positive (Figure 3b arrows and stars). As expected for hMos, we detected the presence of tyrosine hydroxylase (TH) cells (Figure 3c). Finally, to test the functionality of the dopaminergic neurons, we treated hMos at day 35 with dopamine. We observed the appearance of brown/black areas, suspected to be neuromelanin granules accumulations, also known as by-products of dopamine synthesis (Figure 4a)³⁶. Silver stains were showed to bind to neuromelanin granules in the substantia nigra⁶⁴. Thus, we confirmed the presence of neuromelanin granules in hMOs by Fontana Masson staining (Figure 4b) and observed a significant increase of neuromelanin granules after dopamine treatment (Figure 4b-c). Raw images and data are available as Underlying data⁶⁵.

Concluding remarks

In our group, we have successfully generated hMBOs from patientderived iPSCs. However, there are various challenges that are associated with the generation of organoids. (i) iPSC lines are very sensitive and require delicate culture techniques to avoid differentiation. This can be achieved by choosing a range of iPSC passage number suitable for generating good EBs. (ii) Batch-tobatch reproducibility is difficult to achieve. It can be controlled by optimizing chemical and physical parameters of media and incubation. (iii) Optimal concentrations of components in the media need to be carefully chosen. There are various chemical factors that contribute to the generation of organoids, and therefore require careful standardization. (iv) Generation of uniform EBs, is the key factor in the generation of organoids. Uniform, smooth and continuous edges of EBs are essential to develop uniform organoids. (v) The speed of the shaker is crucial in the final differentiation of organoids and maintaining 3D organization. (vi) Cryosectioning organoids is a challenging analysis step.

Since the organoids form a structure distinct from that of natural tissue, some protocol adaptations were necessary to consistently generate high quality sections. Furthermore, due to tissue organization and small sizes, sections have to be optimized for each stage of organoid maturation. Other methods such as clearing techniques can be useful to overcome this challenge. So far, we have overcome the challenges and generated numerous high-quality midbrain organoids. It is important to note that the quality of the iPSCs remains the most crucial step in the formation of organoid tissue.

Data availability

Underlying data

Open Science Framework: Generation of human midbrain organoids from induced pluripotent stem cells. https://doi.org/10.17605/OSF.IO/GF39H⁶⁵

This project contains the following underlying data:

- Figure 1a iPSC colonies.jpg (Quality of iPSCs suitable for midbrain organoids formation.)
- Figure 1c EB with smooth edge 48 hours after formation.jpg (EB with smooth edge 48 hours after formation in neuronal induction media.)
- Figure 1d Extrusion of buds on EB after midbrain patterning.jpg (Extrusion of buds on EB after midbrain patterning.)
- Figure 1e Typical EB 24 hours after embedding in matrigel.jpg (Typical EB 24 hours after embedding in Matrigel®.)
- Figure 1f Day 15 after transferring the tissue into final differentiation media.jpg (Typical hMo at day 15 after transferring the tissue into final differentiation media.)
- Figure 1f Day 1 after transferring the tissue into final differentiation media.jpg (Typical hMo at day 1 after transferring the tissue into final differentiation media.)
- Figure 1f Day 5 after transferring the tissue into final differentiation media.jpg (Typical hMo at day 5 after transferring the tissue into final differentiation media.)
- Figure 2 Day 1 in final differentiation media.jpg (At day 1 in final differentiation media, hMOs are approximately 600µm.)
- Figure 2 Day 50 in final differentiation media.jpg (At day 50 in final differentiation media, hMOs are approximately 4mm.)
- Figure 3a Cryosection of hMO.tif (Cryosection of hMO at day 30. The immunofluorescence staining reveals dopaminergic neurons and multiple ventricule-like structures or "rosettes".)
- Figure 3b map2.tif (MAP2 staining reveals the presence of neurons on the outside layer of rosettes.)
- Figure 3b nuclei.tif (Nuclei staining of the section.)
- Figure 3b th.tif (TH staining reveals the presence of dopaminergic neurons on the outside layer of rosettes.)
- Figure 3b tuj1.tif (TUJ1 staining reveals the presence of neurons on the outside layer of rosettes.)

- Figure 3c map2.tif (MAP2 staining reveals the presence of neurons.)
- Figure 3c nuclei.tif (Nuclei staining of the section.)
- Figure 3c th.tif (TH staining reveals the presence of dopaminergic neurons.)
- Figure 3c tuj1.tif (TUJ1 staining reveals the presence of neurons.)
- Figure 4a 45 days hMO with dopamine treatment.JPG (Under dopamine treatment, brown/black areas appeared in hMos.)
- Figure 4a 45 days hMO without dopamine treatment.JPG (hMos, not treated with dopamine, are used as controls.)
- Figure 4b colorimetric extraction on 45 days hMO with dopamine treatment.jpeg (Colorimetric selection from GIMP software on hMos treated with dopamine.)
- Figure 4b colorimetric extraction on 45 days hMO without dopamine treatment.jpeg (Colorimetric selection from GIMP software on control hMos not treated with dopamine.)
- Figure 4b fontana masson on 45 days hMO with dopamine treatment.TIF (Fontana Masson staining on hMo treated with dopamine.)

• Figure 4b fontana masson on 45 days hMO without dopamine treatment.TIF (Fontana Masson staining on hMo not treated with dopamine.)

Grant information

NVM is supported by Jeanne Timmins Costello, Fonds de Recherche du Québéc-Santé and Parkinson Canada fellowships. EAF is supported by a Foundation Grant from the Canadian Institutes of Health Research, the Consortium Québécois sur la Découverte du Médicament, Fonds de Recherche du Québéc-Santé Quebec Parkinson Network and the Michael J. Fox Foundation. TMD is supported by a Parkinson's Canada New Investigator Award, and through funding of the brain organoid program at the MNI by the Van Berkom-Saucier Foundation.

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Acknowledgments

We acknowledge Dr. Xianmin Zeng (Buck Institute, CA, USA) for providing the XCl-1 iPSC line to our laboratory. We also acknowledge Dr. Chen Carol X.-Q. for constituting the bank of iPSCs.

References

- Deng H, Dodson MW, Huang H, et al.: The Parkinson's disease genes pink1 and parkin promote mitochondrial fission and/or inhibit fusion in Drosophila. Proc Natl Acad Sci U S A. 2008; 105(38): 14503–14508.
 PubMed Abstract | Publisher Full Text | Free Full Text
- Langston JW, Ballard PA Jr: Parkinson's disease in a chemist working with 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine. N Engl J Med. 1983; 309(5): 310. PubMed Abstract | Publisher Full Text
- Zhu J, Chu CT: Mitochondrial dysfunction in Parkinson's disease. J Alzheimers Dis. 2010; 20 Suppl 2: S325–334.
 PubMed Abstract | Publisher Full Text
- Kurz A, Double KL, Lastres-Becker I, et al.: A53T-alpha-synuclein overexpression impairs dopamine signaling and striatal synaptic plasticity in old mice. PLoS One. 2010; 5(7): e11464.
 PubMed Abstract | Publisher Full Text | Free Full Text
- Good CH, Hoffman AF, Hoffer BJ, et al.: Impaired nigrostriatal function precedes
 Bodding and the field in the second second
- behavioral deficits in a genetic mitochondrial model of Parkinson's disease. FASEB J. 2011; 25(4): 1333–1344. PubMed Abstract | Publisher Full Text | Free Full Text
- Anglade P, Vyas S, Javoy-Agid F, et al.: Apoptosis and autophagy in nigral neurons of patients with Parkinson's disease. *Histol Histopathol.* 1997; 12(1): 25–31.
 PubMed Abstract
- Hara T, Nakamura K, Matsui M, et al.: Suppression of basal autophagy in neural cells causes neurodegenerative disease in mice. Nature. 2006; 441(7095): 885–889.
 PubMed Abstract | Publisher Full Text
- Pitcairn C, Wani WY, Mazzulli JR: Dysregulation of the autophagic-lysosomal pathway in Gaucher and Parkinson's disease. Neurobiol Dis. 2019; 122: 72–82.
 PubMed Abstract | Publisher Full Text | Free Full Text
- Plotegher N, Duchen MR: Crosstalk between lysosomes and mitochondria in Parkinson's disease. Front Cell Dev Biol. 2017; 5: 110.
 PubMed Abstract | Publisher Full Text | Free Full Text
- Takahashi K, Yamanaka S: Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell.* 2006; 126(4): 663–676. PubMed Abstract | Publisher Full Text
- Welin S: Ethical issues in human embryonic stem cell research. Acta Obstet Gynecol Scand. 2002; 81(5): 377–382.
 PubMed Abstract | Publisher Full Text
- 12. Soldner F, Hockemeyer D, Beard C, et al.: Parkinson's disease patient-derived

induced pluripotent stem cells free of viral reprogramming factors. *Cell*. 2009; 136(5): 964–977. PubMed Abstract | Publisher Full Text | Free Full Text

- Cooper O, Hargus G, Deleidi M, et al.: Differentiation of human ES and Parkinson's disease IPS cells into ventral midbrain dopaminergic neurons requires a high activity form of SHH, FGF8a and specific regionalization by retinoic acid. Mol Cell Neurosci. 2010; 45(3): 258–266.
 PubMed Abstract | Publisher Full Text | Free Full Text
- Byers B, Cord B, Nguyen HN, et al.: SNCA triplication Parkinson's patient's iPSC-derived DA neurons accumulate *a*-synuclein and are susceptible to oxidative stress. PLoS One. 2011; 6(11): e26159.
 PubMed Abstract | Publisher Full Text | Free Full Text
- Devine MJ, Ryten M, Vodicka P, et al.: Parkinson's disease induced pluripotent stem cells with triplication of the α-synuclein locus. Nat Commun. 2011; 2: 440.
 PubMed Abstract | Publisher Full Text | Free Full Text
- Mazzulli JR, Xu YH, Sun Y, et al.: Gaucher disease glucocerebrosidase and *a*-synuclein form a bidirectional pathogenic loop in synucleinopathies. *Cell.* 2011; 146(1): 37–52. PubMed Abstract | Publisher Full Text | Free Full Text
- Seibler P, Graziotto J, Jeong H, et al.: Mitochondrial Parkin recruitment is impaired in neurons derived from mutant PINK1 induced pluripotent stem cells. J Neurosci. 2011; 31(16): 5970–5976.
 PubMed Abstract | Publisher Full Text | Free Full Text
- Cooper O, Seo H, Andrabi S, et al.: Pharmacological rescue of mitochondrial deficits in iPSC-derived neural cells from patients with familial Parkinson's disease. Sci Transl Med. 2012; 4(141): 141ra190.
 PubMed Abstract | Publisher Full Text | Free Full Text
- Imaizumi Y, Okada Y, Akamatsu W, et al.: Mitochondrial dysfunction associated with increased oxidative stress and a-synuclein accumulation in PARK2 iPSCderived neurons and postmortem brain tissue. *Mol Brain*. 2012; 5: 35.
 PubMed Abstract | Publisher Full Text | Free Full Text
- Jiang H, Ren Y, Yuen EY, et al.: Parkin controls dopamine utilization in human midbrain dopaminergic neurons derived from induced pluripotent stem cells. Nat Commun. 2012; 3: 668.
 PubMed Abstract | Publisher Full Text | Free Full Text
- Sánchez-Danés A, Richaud-Patin Y, Carballo-Carbajal I, et al.: Disease-specific phenotypes in dopamine neurons from human iPS-based models of genetic and sporadic Parkinson's disease. EMBO Mol Med. 2012; 4(5): 380–395. PubMed Abstract | Publisher Full Text | Free Full Text

- Chung SY, Kishinevsky S, Mazzulli JR, et al.: Parkin and PINK1 Patient iPSC-Derived Midbrain Dopamine Neurons Exhibit Mitochondrial Dysfunction and a-Synuclein Accumulation. Stem Cell Reports. 2016; 7(4): 664–677. PubMed Abstract | Publisher Full Text | Free Full Text
- Miller JD, Ganat YM, Kishinevsky S, et al.: Human iPSC-based modeling of lateonset disease via progerin-induced aging. Cell Stem Cell. 2013; 13(6): 691–705. PubMed Abstract | Publisher Full Text | Free Full Text
- 24. Chang KH, Lee-Chen GJ, Wu YR, *et al.*: Impairment of proteasome and antioxidative pathways in the induced pluripotent stem cell model for sporadic Parkinson's disease. *Parkinsonism Relat Disord.* 2016; 24: 81–88. PubMed Abstract | Publisher Full Text
- Fernández-Santiago R, Carballo-Carbajal I, Castellano G, et al.: Aberrant epigenome in iPSC-derived dopaminergic neurons from Parkinson's disease patients. *EMBO Mol Med*. 2015; 7(12): 1529–1546.
 PubMed Abstract | Publisher Full Text | Free Full Text
- Holmqvist S, Lehtonen Š, Chumarina M, et al.: Creation of a library of induced pluripotent stem cells from Parkinsonian patients. NPJ Parkinsons Dis. 2016; 2: 16009.
 PubMed Abstract | Publisher Full Text | Free Full Text
- López de Maturana R, Lang V, Zubiarrain A, et al.: Mutations in LRRK2 impair NF-кВ pathway in iPSC-derived neurons. J Neuroinflammation. 2016; 13(1): 295. PubMed Abstract | Publisher Full Text | Free Full Text
- Ma D, Ng EY, Zeng L, *et al.*: Development of a human induced pluripotent stem cell (IPSC) line from a Parkinson's disease patient carrying the N551K variant in LRK2 gene. Stem Cell Res. 2017; 18: 51–53.
 PubMed Abstract | Publisher Full Text
- Ma D, Ng SH, Zeng L, *et al.*: Generation of a human induced pluripotent stem cell (IPSC) line carrying the Parkinson's disease linked LRRK2 variant S1647T. *Stem Cell Res.* 2017; 18: 54–56.
 PubMed Abstract | Publisher Full Text
- Momcilovic O, Sivapatham R, Oron TR, et al.: Derivation, Characterization, and Neural Differentiation of Integration-Free Induced Pluripotent Stem Cell Lines from Parkinson's Disease Patients Carrying SNCA, LRRK2, PARK2, and GBA Mutations. PLoS One. 2016; 11(5): e0154890.
 PubMed Abstract | Publisher Full Text | Free Full Text
- Oliveira LM, Falomir-Lockhart LJ, Botelho MG, *et al.*: Elevated α-synuclein caused by SNCA gene triplication impairs neuronal differentiation and maturation in Parkinson's patient-derived induced pluripotent stem cells. *Cell Death Dis.* 2015; 6: e1994.

PubMed Abstract | Publisher Full Text | Free Full Text

- Orenstein SJ, Kuo SH, Tasset I, et al.: Interplay of LRRK2 with chaperonemediated autophagy. Nat Neurosci. 2013; 16(4): 394–406.
 PubMed Abstract | Publisher Full Text | Free Full Text
- Ren Y, Jiang H, Hu Z, et al.: Parkin mutations reduce the complexity of neuronal processes in iPSC-derived human neurons. Stem Cells. 2015; 33(1): 68–78.

PubMed Abstract | Publisher Full Text | Free Full Text

- Lancaster MA, Renner M, Martin CA, et al.: Cerebral organoids model human brain development and microcephaly. *Nature*. 2013; 501(7467): 373–379. PubMed Abstract | Publisher Full Text | Free Full Text
- Monzel AS, Smits LM, Hemmer K, et al.: Derivation of Human Midbrain-Specific Organoids from Neuroepithelial Stem Cells. Stem Cell Reports. 2017; 8(5): 1144–1154.

PubMed Abstract | Publisher Full Text | Free Full Text

- Jo J, Xiao Y, Sun AX, et al.: Midbrain-like Organoids from Human Pluripotent Stem Cells Contain Functional Dopaminergic and Neuromelanin-Producing Neurons. Cell Stem Cell. 2016; 19(2): 248–257.
 PubMed Abstract | Publisher Full Text | Free Full Text
- Sloan SA, Darmanis S, Huber N, et al.: Human Astrocyte Maturation Captured in 3D Cerebral Cortical Spheroids Derived from Pluripotent Stem Cells. Neuron. 2017; 95(4): 779–790 e776.
 PubMed Abstract | Publisher Full Text | Free Full Text
- Lindborg BA, Brekke JH, Vegoe AL, et al.: Rapid Induction of Cerebral Organoids From Human Induced Pluripotent Stem Cells Using a Chemically Defined Hydrogel and Defined Cell Culture Medium. Stem Cells Transl Med. 2016; 5(7): 970–979.

PubMed Abstract | Publisher Full Text | Free Full Text

- Quadrato G, Nguyen T, Macosko EZ, et al.: Cell diversity and network dynamics in photosensitive human brain organoids. Nature. 2017; 545(7652): 48–53. PubMed Abstract | Publisher Full Text | Free Full Text
- Kadoshima T, Sakaguchi H, Nakano T, et al.: Self-organization of axial polarity, inside-out layer pattern, and species-specific progenitor dynamics in human ES cell-derived neocortex. Proc Natl Acad Sci U S A. 2013; 110(50): 20284–20289.

PubMed Abstract | Publisher Full Text | Free Full Text

- Qian X, Nguyen HN, Song MM, et al.: Brain-Region-Specific Organoids Using Mini-bioreactors for Modeling ZIKV Exposure. Cell. 2016; 165(5): 1238–1254. PubMed Abstract | Publisher Full Text | Free Full Text
- Muguruma K, Nishiyama A, Kawakami H, et al.: Self-organization of polarized cerebellar tissue in 3D culture of human pluripotent stem cells. Cell Rep. 2015; 10(4): 537–550.
 PubMed Abstract | Publisher Full Text

43. Bagley JA, Reumann D, Bian S, et al.: Fused cerebral organoids model

interactions between brain regions. Nat Methods. 2017; 14(7): 743–751. PubMed Abstract | Publisher Full Text | Free Full Text

- Paşca AM, Sloan SA, Clarke LE, *et al.*: Functional cortical neurons and astrocytes from human pluripotent stem cells in 3D culture. *Nat Methods.* 2015; 12(7): 671–678.
 PubMed Abstract | Publisher Full Text | Free Full Text
- Sakaguchi H, Kadoshima T, Soen M, et al.: Generation of functional hippocampal neurons from self-organizing human embryonic stem cellderived dorsomedial telencephalic tissue. Nat Commun. 2015; 6: 8896. PubMed Abstract | Publisher Full Text | Free Full Text
- Birey F, Andersen J, Makinson CD, et al.: Assembly of functionally integrated human forebrain spheroids. Nature. 2017; 545(7652): 54–59.
 PubMed Abstract | Publisher Full Text | Free Full Text
- Camp JG, Badsha F, Florio M, *et al.*: Human cerebral organoids recapitulate gene expression programs of fetal neocortex development. *Proc Natl Acad Sci* U S A. 2015; 112(51): 15672–15677.
 PubMed Abstract | Publisher Full Text | Free Full Text
- Luo C, Lancaster MA, Castanon R, et al.: Cerebral Organoids Recapitulate Epigenomic Signatures of the Human Fetal Brain. Cell Rep. 2016; 17(12): 3369–3384.
 PubMed Abstract | Publisher Full Text | Free Full Text
- Cugola FR, Fernandes IR, Russo FB, et al.: The Brazilian Zika virus strain causes birth defects in experimental models. Nature. 2016; 534(7606): 267–271.
 PubMed Abstract | Publisher Full Text | Free Full Text
- Dang J, Tiwari SK, Lichinchi G, et al.: Zika Virus Depletes Neural Progenitors in Human Cerebral Organoids through Activation of the Innate Immune Receptor TLR3. Cell Stem Cell. 2016; 19(2): 258–265.
 PubMed Abstract | Publisher Full Text | Free Full Text
- Garcez PP, Loiola EC, Madeiro da Costa R, et al.: Zika virus impairs growth in human neurospheres and brain organoids. Science. 2016; 352(6287): 816–818. PubMed Abstract | Publisher Full Text
- Wells MF, Salick MR, Wiskow O, et al.: Genetic Ablation of AXL Does Not Protect Human Neural Progenitor Cells and Cerebral Organoids from Zika Virus Infection. Cell Stem Cell. 2016; 19(6): 703–708. PubMed Abstract | Publisher Full Text
- Mariani J, Coppola G, Zhang P, et al.: FOXG1-Dependent Dysregulation of GABA/Glutamate Neuron Differentiation in Autism Spectrum Disorders. Cell. 2015; 162(2): 375–390.
 PubMed Abstract | Publisher Full Text | Free Full Text
- Raja WK, Mungenast AE, Lin YT, et al.: Self-Organizing 3D Human Neural Tissue Derived from Induced Pluripotent Stem Cells Recapitulate Alzheimer's Disease Phenotypes. PLoS One. 2016; 11(9): e0161969.
 PubMed Abstract | Publisher Full Text | Free Full Text
- Choi SH, Kim YH, Hebisch M, et al.: A three-dimensional human neural cell culture model of Alzheimer's disease. Nature. 2014; 515(7526): 274–278. PubMed Abstract | Publisher Full Text | Free Full Text
- Baek SK, Cho YS, Kim IS, et al.: A Rho-Associated Coiled-Coil Containing Kinase Inhibitor, Y-27632, Improves Vlability of Dissociated Single Cells, Efficiency of Colony Formation, and Cryopreservation in Porcine Pluripotent Stem Cells. Cell Reprogram. 2019; 21(1): 37–50. PubMed Abstract | Publisher Full Text
- Vemuri MC, Rao MS: Generating different neural cell types from iPSCs for screening and cell therapy for CNS disorders. Neural Stem Cells in Health and Disease. 2016; 521–542.
 Publisher Full Text
- Sasaki N, Okishio K, Ui-Tei K, *et al.*: Heparan sulfate regulates self-renewal and pluripotency of embryonic stem cells. *J Biol Chem.* 2008; 283(6): 3594–3606.
 PubMed Abstract | Publisher Full Text
- Kim TE, Lee HS, Lee YB, et al.: Sonic hedgehog and FGF8 collaborate to induce dopaminergic phenotypes in the Nurr1-overexpressing neural stem cell. Biochem Biophys Res Commun. 2003; 305(4): 1040–1048. PubMed Abstract | Publisher Full Text
- Watanabe M, Buth JE, Vishlaghi N, et al.: Self-Organized Cerebral Organoids with Human-Specific Features Predict Effective Drugs to Combat Zika Virus Infection. Cell Rep. 2017; 21(2): 517–532.
 PubMed Abstract | Publisher Full Text | Free Full Text
- Studer L, Spenger C, Seiler RW, et al.: Effects of brain-derived neurotrophic factor on neuronal structure of dopaminergic neurons in dissociated cultures of human fetal mesencephalon. Exp Brain Res. 1996; 108(2): 328–336.
 PubMed Abstract | Publisher Full Text
- Cortés D, Carballo-Molina OA, Castellanos-Montiel MJ, et al.: The Non-Survival Effects of Glial Cell Line-Derived Neurotrophic Factor on Neural Cells. Front Mol Neurosci. 2017; 10: 258. PubMed Abstract | Publisher Full Text | Free Full Text
- Billings PC, Sanzari JK, Kennedy AR, et al.: Comparative analysis of colorimetric staining in skin using open-source software. Exp Dermatol. 2015; 24(2): 157–159.

PubMed Abstract | Publisher Full Text | Free Full Text

- Zecca L, Tampellini D, Gerlach M, et al.: Substantia nigra neuromelanin: structure, synthesis, and molecular behaviour. Mol Pathol. 2001; 54(6): 414–418.
 PubMed Abstract | Free Full Text
- Mohamed NV: Generation of human midbrain organoids from induced pluripotent stem cells. 2019. http://www.doi.org/10.17605/OSF.IO/GF39H

Open Peer Review

Current Peer Review Status: ? ? 🗸 🗸

Version 1

Reviewer Report 07 May 2019

https://doi.org/10.21956/mniopenres.13879.r26158

© **2019 Schuele B.** This is an open access peer review report distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

?

Birgitt Schuele

Department of Pathology, Stanford University School of Medicine, Stanford, CA, USA

In this review Mohamed et al. describe a protocol and cryosectioning method for midbrain organoids from human IPSCs. The basis of this protocol is a combination of three previously published organoid methods by Lancaster 2013, Jo 2016, and Monzel 2017.

It is important to advance protocols for robust differentiation into 3D cultures. Overall, it is a comprehensive and detailed protocol. The manuscript is lacking is a section on troubleshooting. Since the protocol is geared towards disease research, it would be critical to also include disease cell lines in the protocol (e.g. genetic forms of Parkinson's disease) and describe to what extent differences in the differentiation potential are evident and how to overcome them. No data is shown for cytoarchitecture for 50 days organoids. Is the center becoming necrotic in these larger organoids? Please describe and include.

Minor points: Page 3, last paragraph: define "generation rate of near 100%".

Page 5, second paragraph: replace "<u>vitamin B27</u> without vitamin A" with "<u>B27 supplement</u> without vitamin A".

Page 6, tables 5 and 6: change "Penni/Strep" to "Pen/Strep".

Page 7, second paragraph: iPSC lines, especially from a repository can vary widely in passage number between early 10's to 50's and 60's, e.g. after editing. It seems arbitrary to write "10 passages". Please clarify.

Page 7, last paragraph: change "anti-anti" to "antibiotic-antimycotic", also correct spelling in Table 1 and footnote of Table 3 accordingly.

Page 8, timeline b.: panel below days: first two boxes should be deleted, also please what is the difference between "tissue growth" and "organoid growth". The timeline ends with several

months, but organoids are only described macroscopically until day 50 and microscopically until day 35.

Page 8, legend: It is noted that PBMCs were reprogrammed, however, table 7 describes the commercial source of the iPSCs. Please clarify.

Page 9, third point: spelling "scraper" not "scrapper".

Page 13, second paragraph: Re-write sentence starting with "Silver stains..." Sentence structure and context is not clear.

Is the rationale for developing the new method (or application) clearly explained? $\ensuremath{\mathsf{Yes}}$

Is the description of the method technically sound?

Yes

Are sufficient details provided to allow replication of the method development and its use by others?

Yes

If any results are presented, are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions about the method and its performance adequately supported by the findings presented in the article?

Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: stem cell modeling, Parkinson's research

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 06 Jan 2021

nguyen-vi mohamed, Montreal Neurological Institute and Hospital, Montreal, Canada

 It is important to advance protocols for robust differentiation into 3D cultures. Overall, it is a comprehensive and detailed protocol. The manuscript is lacking is a section on troubleshooting. Since the protocol is geared towards disease research, it would be critical to also include disease cell lines in the protocol (e.g. genetic forms of Parkinson's disease) and describe to what extent differences in the differentiation potential are evident and how to overcome them.No data is shown for cytoarchitecture for 50 days organoids. Is the center becoming necrotic in these larger organoids? Please describe and include.

Answer: We thank the reviewer for these comments. We agreed that the ultimate purpose of generating midbrain organoids is to conduct studies in Parkinson's disease (PD) hMOs. Nonetheless, the precise analysis of differences between healthy hMOs or isogenic corrected hMOs compared to PD hMOs is beyond the scope of this manuscript which aims to generate hMOs from iPSC lines. Since we agreed that it's interesting to show that such comparison would be possible in future disease research manuscript, we included new cytoarchitecture data on PD hMOs derived from an iPSC line from a patient carrying a triplication for synuclein (SNCA_Tri) (**Figure 3c and Figure S1b.c**). In **Figure S1b**, SNCA_Tri hMOs reached approximatively 4mm in diameter. In **Figure 3c** you can observe cryosections of 30 day-old SNCA_Tri hMOs, while in **Figure S1c**, you can observe a 100 day-old section from SNCA_Tri hMOs, both stained for TH (red) and MAP2 (green). We complemented the results section accordingly. We also would like to point out that troubleshooting sections are the "NOTE" points. Since another reviewer made a complementary comment for deeper troubleshooting details, we either added a "NOTE" or added more details to the pre-existing one.

Minor points:

• Page 3, last paragraph: define "generation rate of near 100%".

Answer: As recommended by another reviewer, we softened the language around this statement as following: "This protocol is an adaptation of the Nature protocol paper initially published by Lancaster in combination with discoveries from Jo et al. and Monzel et al.34–36 in order to successfully produce high quality midbrain organoids ».

 Page 5, second paragraph: replace "<u>vitamin B27</u> without vitamin A" with "<u>B27</u> <u>supplement</u> without vitamin A".

Answer: Thanks to the reviewer's comment, we have now replaced it with "B27 supplement without vitamin A"

• Page 6, tables 5 and 6: change "Penni/Strep" to "Pen/Strep".

Answer: Thanks to the reviewer's comment, we have now made the adjustments.

 Page 7, second paragraph: iPSC lines, especially from a repository can vary widely in passage number between early 10's to 50's and 60's, e.g. after editing. It seems arbitrary to write "10 passages". Please clarify.

Answer: We thank the reviewer for their comment and clarified it as follows: "The iPSC colonies should not have been passaged more than 10 times after thawing."

• Page 7, last paragraph: change "anti-anti" to "antibiotic-antimycotic", also correct spelling in Table 1 and footnote of Table 3 accordingly.

Answer: We thank the reviewer for their comment and corrected it through the entire manuscript.

 Page 8, timeline b.: panel below days: first two boxes should be deleted, also please what is the difference between "tissue growth" and "organoid growth". The timeline ends with several months, but organoids are only described macroscopically until day 50 and microscopically until day 35.

Answer: We thank the reviewer for their comment and deleted the two first boxes. We agreed that tissue growth and organoid growth are confusing, thus we changed the terminology for

tissue induction and organoid growth throughout the entire manuscript. To clarify, the tissue induction step corresponds to 24h where the EBs sit within the media containing midbrain patterning factors in addition to laminin and insulin which promote the formation of the tissue after the embedding step. After transferring the tissue into final differentiation media, the organoid can be cultured for several weeks or months. Finally, the timeline end point depends on the user experiment. For clarification, we modified the text as following: "EBs then sit 24 hours in tissue induction media post-embedding in Matrigel® scaffold at day 7 to promote growth of tissue. The tissue formed was cultured on an orbital shaker for several weeks or months until their use in experiments."

• Page 8, legend: It is noted that PBMCs were reprogrammed, however, table 7 describes the commercial source of the iPSCs. Please clarify.

Answer: We thank the reviewer for their comment. In this manuscript, we use commercial lines but, in our group, we do reprogramme iPSCs from PBMCs. The schematic was a general representation of the process from patient to hMOs. The method described here started from Day 0 from iPSC lines. We clarified the schematic legend as following: "PBMCs (peripheral blood mononuclear cells) from individuals were collected and reprogrammed into iPSCs. Commercial lines can be alternatively used that were reprogrammed from skin or PBMCs or other somatic sources. Uniform embryoid bodies are formed from iPSC colonies and then patterned into neuronal midbrain fate with inductive signals."

• Page 9, third point: spelling "scraper" not "scrapper".

Answer: We thank the reviewer for his comment and corrected it.

• Page 13, second paragraph: Re-write sentence starting with "Silver stains..." Sentence structure and context is not clear.

Answer: We thank the reviewer for his comment and corrected it as follow: "Silver stains were shown to label neuromelanin granules in the substantia nigra (67)."

Competing Interests: No competing interests were disclosed.

Reviewer Report 23 April 2019

https://doi.org/10.21956/mniopenres.13879.r26159

© **2019 Hester M.** This is an open access peer review report distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.



Mark E. Hester

Center for Perinatal Research, The Research Institute at Nationwide Children's Hospital, Columbus, OH, USA

Mohamed et. al. describes an optimized protocol to generate human midbrain organoids derived from human iPSCs. In this article, the authors utilize multiple hiPSC lines and optimize chemical and physical parameters of media formulations and incubation times to achieve a standardized protocol. This manuscript is very well written and contains many critical details necessary for successful organoid generation and culture. There are a number of minor details that I highlight below that when incorporated would increase the quality and rigor of the current manuscript.

- 1. In the background section, paragraph 2: "Since their discovery, this technology has opened up many new research avenues, including for PD." Please restructure this sentence to the following: "Since their discovery, this technology has opened up many new avenues of investigation, including research for PD."
- 2. In the background section, column 2, paragraph 4: "for a robust derivation of iPSCs lines into 3D midbrain..." "iPSCs" should be singular and read "iPSC lines into 3D..."
- In the materials section, first paragraph, first sentence: "The materials, reagents and equipment listed in this document can be substituted" should be restructured and written: "The materials, reagents and equipment listed in this document can be substituted <u>for</u> <u>comparable items</u>."
- 4. Mention of lot to lot variability is important and those items should designated with an asterisk in Table 1 and defined in the text.
- 5. In the materials section describing NIM, "the help of" can be deleted.
- 6. On page 9, after describing the orbital shaking speed of 70rpm, there should be another sentence following stating the rpm is dependent upon the type of shaker utilized that is based on the throw (shaking diameter) of the shaker.
- 7. Since two hiPSC lines were tested using this protocol, some details should be given describing whether both lines achieved similar efficiencies or not. In addition, details on how many independent experiments were performed to achieve the optimized parameters should be mentioned.

Is the rationale for developing the new method (or application) clearly explained? $\ensuremath{\mathsf{Yes}}$

Is the description of the method technically sound?

Yes

Are sufficient details provided to allow replication of the method development and its use by others?

Partly

If any results are presented, are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions about the method and its performance adequately supported by the findings presented in the article?

Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Molecular and cellular neuroscience

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Author Response 06 Jan 2021

nguyen-vi mohamed, Montreal Neurological Institute and Hospital, Montreal, Canada

Mohamed et. al. describes an optimized protocol to generate human midbrain organoids derived from human iPSCs. In this article, the authors utilize multiple hiPSC lines and optimize chemical and physical parameters of media formulations and incubation times to achieve a standardized protocol. This manuscript is very well written and contains many critical details necessary for successful organoid generation and culture. There are a number of minor details that I highlight below that when incorporated would increase the quality and rigor of the current manuscript.

We thank Dr Hester for his approval and insightful comments. Please find attached a revised version of the manuscript and below our replies to the points raised.

 In the background section, paragraph 2: "Since their discovery, this technology has opened up many new research avenues, including for PD." Please restructure this sentence to the following: "Since their discovery, this technology has opened up many new avenues of investigation, including research for PD."

Answer: Thanks to the reviewer's comments, we have now adjusted the sentence as recommended.

 In the background section, column 2, paragraph 4: "for a robust derivation of iPSCs lines into 3D midbrain..." "iPSCs" should be singular and read "iPSC lines into 3D..."

Answer: Thanks to the reviewer's comments, we have now adjusted the sentence as recommended. We also corrected the typo thought the manuscript "The observations provided in this method were generated with at least 6 independent batches derived from two iPSC lines from healthy individuals (NCRM-1 and XCI-1) or an iPSC line from a patient with PD (EDI001A named as SNCA_Tri in Figures) (Table 7)." / "Human iPSC lines are to be handled within a Class II biosafety laminar flow hood to protect the worker from possible biohazardous agents."

 In the materials section, first paragraph, first sentence: "The materials, reagents and equipment listed in this document can be substituted" should be restructured and written: "The materials, reagents and equipment listed in this document can be substituted <u>for comparable items</u>."

Answer: Thanks to the reviewer's comments, we have now adjusted the sentence as recommended.

• Mention of lot-to-lot variability is important and those items should designated with an asterisk in Table 1 and defined in the text.

Answer: Thanks to the reviewer's comments, we have now added asterisks to **Table 1** and a note to explain. "**Note:** Media and biochemicals with an asterisk are more susceptible to batch-to-batch variability. The main reason explaining this variability is the production source, either animal or human. It is therefore important to keep track of lot numbers and to test new lots received prior hMOs generation. Regarding Accutase solution, we noticed variability in enzyme efficiency from lot to lot. To compensate for a weaker enzyme activity, incubate for longer time with the enzyme on colonies until they detach properly. "

• In the materials section describing NIM, "the help of" can be deleted.

Answer: Thanks to the reviewer's comments, we have now deleted "the help of".

 On page 9, after describing the orbital shaking speed of 70rpm, there should be another sentence following stating the rpm is dependent upon the type of shaker utilized that is based on the throw (shaking diameter) of the shaker.

Answer: Thanks to the reviewer's comments, we have now adjusted the sentence as follow: "Put the 6 well plates on the orbital shaker (set at 70 rpm for the orbital shaker listed- note: speed setting would change based on shaker diameter) in the 37°C regular cell incubator and change the medium every 2-3 days using final differentiation medium **(Table 6)**."

 Since two hiPSC lines were tested using this protocol, some details should be given describing whether both lines achieved similar efficiencies or not. In addition, details on how many independent experiments were performed to achieve the optimized parameters should be mentioned.

Answer: Thanks to the reviewer's comments, we have now added this information in the manuscript as follows: "The observations provided in this method were generated with at least 6 independent batches derived from two iPSC lines from healthy individuals (NCRM-1 and XCl-1) or an iPSC line from a patient with PD (EDI001A named as SNCA_Tri in Figures) **(Table 7)**". Additionally, we added a representative picture of XCl-1 derived hMOs, 50-day aged respectively, to show similar efficiency achievement (Figure 2b). We updated the legends accordingly.

Competing Interests: No competing interests were disclosed.

Reviewer Report 18 April 2019

https://doi.org/10.21956/mniopenres.13879.r26157

© **2019 Corti O et al.** This is an open access peer review report distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

? Olga Corti

Brain and Spine Institute (ICM), Paris, France **Philippe Ravassard** Brain and Spine Institute (ICM), Paris, France

The manuscript by Nguyen-Vi Mohamed and colleagues describes a standardized procedure for the generation and characterization of midbrain organoids from human iPSC lines, developed by adapting and integrating key reference protocols in the field. The production of such organoids is of great importance for studying human brain development and modeling neurodevelopmental and neurodegenerative diseases, in particular Parkinson's disease. The manuscript is well written and the experimental procedures are described in great detail, nicely pinpointing key steps that require special attention and experimental tricks to help perform the experiments under ideal and controlled conditions. Following the provided procedure, any personnel skilled in stem cell culture should be able to easily generate human midbrain organoids in an appropriate environment. The protocol may be improved to ensure a more complete characterization of the quality of the midbrain organoids, so as to fully support the conclusions drawn and increase the impact of this paper.

Major points:

- The authors built on previous work and the full characterization of the dopaminergic neurons present in the organoids is probably beyond the scope of this manuscript. Nevertheless, it would be useful to provide staining for other basic markers to confirm the efficacy and specificity of the differentiation procedure and exclude the presence of other neuronal types that also express the TH marker. The authors may consider checking for: (i) other enzymes of the dopamine biosynthesis pathway, such as GTP cyclohydrolase 1 or DOPA decarboxylase, and the dopamine transporter; (ii) as well as dopamine betahydroxylase, as a marker for noradrenergic/adrenergic neurons.
- 2. Testing for a few midbrain markers would be useful to fully support the generation of midbrain organoids. The standard protocol used to pattern ventral midbrain structures is based on the combination of SHH and Fgf8 signals and it is important to keep in mind that this combination patterns both ventral midbrain and ventral hindbrain in the embryo. For example, both midbrain dopaminergic and hindbrain serotonergic neurons are dependent on these induction signals. It would therefore be of interest to evaluate whether other, more rostral or more caudal brain areas, are also represented in these organoids. A broader characterization of the generated cell types could be achieved by QPCR marker expression analysis and a few additional immunostainings.
- 3. A discussion of the significance of the achievements with respect to previous publications would be useful. In how far do the adaptations to previous protocols proposed here improve the reproducibility or robustness of the approach? The authors mention that the quality of the iPSCs is critical and that"variability in the iPSCs maintenance will negatively impact midbrain organoid generation". What do they mean by quality and variability, and what kind of negative impact is expected?

Additional points:

- 1. The authors explain how to thaw the iPSCs. They should also indicate how the cells are supposed to be frozen, specifically at what density and how many cells per vial
- 2. In Figure 1a, it would be useful to illustrate an example of colony presenting with differentiated areas by comparison with a suitable colony
- 3. There is no reference to the percentage of dopaminergic neurons across the organoids. The size of the organoid is quite important and only a single section is presented in Figure 3. It would be nice to provide a representation with serial sections immunostained for TH, using bright field microscopy and a colorimetric stain (HRP/DAB), to illustrate the global distribution of the TH-positive cells within the organoid.
- 4. In Figure 3 the authors point with white squares at areas surrounding a "ventricle-like" structure, indicating that they are composed of neural progenitor cells. Although it is most likely that these cells are progenitors, it would be nice to include at least an immunostaining for nestin to confirm it
- 5. The quality of Figure 3c should probably be improved. TUJ1 staining usually labels both soma and cellular processes. It is quite surprising not to see stained processes in this enlarged photograph. Projections of Z-stacks confocal images would be useful to capture more precisely the cell morphology. The absence of neuronal processes would rather indicate a very immature stage of differentiation, which would be inconsistent with the presence of neuromelanin in some of the TH-positive cells
- 6. In how far does treatment with dopamine test "functionality". The authors may consider rewording of their statement. In Figure 4, it would be useful to integrate a high magnification picture illustrating the presence of neuromelanin granules within a single/a few TH-positive neuron/s. In how far does the graph in panel c illustrate "number of neuromelanin granules"?

Is the rationale for developing the new method (or application) clearly explained? $\ensuremath{\mathsf{Yes}}$

Is the description of the method technically sound?

Yes

Are sufficient details provided to allow replication of the method development and its use by others?

Yes

If any results are presented, are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions about the method and its performance adequately supported by the findings presented in the article?

Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Parkinson's disease, cellular neuroscience

We confirm that we have read this submission and believe that we have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however we have significant reservations, as outlined above.

Author Response 06 Jan 2021

nguyen-vi mohamed, Montreal Neurological Institute and Hospital, Montreal, Canada

The manuscript by Nguyen-Vi Mohamed and colleagues describes a standardized procedure for the generation and characterization of midbrain organoids from human iPSC lines, developed by adapting and integrating key reference protocols in the field. The production of such organoids is of great importance for studying human brain development and modeling neurodevelopmental and neurodegenerative diseases, in particular Parkinson's disease. The manuscript is well written and the experimental procedures are described in great detail, nicely pinpointing key steps that require special attention and experimental tricks to help perform the experiments under ideal and controlled conditions. Following the provided procedure, any personnel skilled in stem cell culture should be able to easily generate human midbrain organoids in an appropriate environment. The protocol may be improved to ensure a more complete characterization of the quality of the midbrain organoids, so as to fully support the conclusions drawn and increase the impact of this paper.

Major points:

1. The authors built on previous work and the full characterization of the dopaminergic

neurons present in the organoids is probably beyond the scope of this manuscript. Nevertheless, it would be useful to provide staining for other basic markers to confirm the efficacy and specificity of the differentiation procedure and exclude the presence of other neuronal types that also express the TH marker. The authors may consider checking for: (i) other enzymes of the dopamine biosynthesis pathway, such as GTP cyclohydrolase 1 or DOPA decarboxylase, and the dopamine transporter; (ii) as well as dopamine beta-hydroxylase, as a marker for noradrenergic/adrenergic neurons.

2. Testing for a few midbrain markers would be useful to fully support the generation of midbrain organoids. The standard protocol used to pattern ventral midbrain structures is based on the combination of SHH and Fgf8 signals and it is important to keep in mind that this combination patterns both ventral midbrain and ventral hindbrain in the embryo. For example, both midbrain dopaminergic and hindbrain serotonergic neurons are dependent on these induction signals. It would therefore be of interest to evaluate whether other, more rostral or more caudal brain areas, are also represented in these organoids. A broader characterization of the generated cell types could be achieved by QPCR marker expression analysis and a few additional immunostainings.

Answer: We thank the reviewer for raising these two points. We added a complementary qRT-PCR experiment in **Figure 2c**showing the presence of several complementary midbrain markers and dopaminergic markers (EN1, Nurr1, LMX1B, Calb1, TH, MAOB, LMX1A, DDC, COMT) in hMOs when compared to iPSC lines. We didn't detect the presence of dopamine beta-hydroxylase (DBH), as a marker for noradrenergic/adrenergic neurons in hMOs. Interestingly, we detected serotonergic neurons marker (GBX2) as predicted by the reviewer. Recently this population was also reported by other group in hMOs generated from a different protocol (65). This new characterization panel was added to the "**Results and Observations**" section. Additionally, we performed a broader characterization of cell types by single cell RNA sequencing (**Figure 3d-f**) and revealed the presence of others cell types that includes astrocytes, oligodendrocyte precursors, radial glial, neuronal progenitors as reported by other groups (35,65).

1. A discussion of the significance of the achievements with respect to previous publications would be useful. In how far do the adaptations to previous protocols proposed here improve the reproducibility or robustness of the approach? The authors mention that the quality of the iPSCs is critical and that"variability in the iPSCs maintenance will negatively impact midbrain organoid generation". What do they mean by quality and variability, and what kind of negative impact is expected?

Answer: We thank the reviewers for this comment and have extended the discussion in our concluding remarks as follows: "In our group, we have successfully generated hMOs from patientderived iPSCs with similar dopaminergic neuron yield than previously published (36). However, there are various challenges that are associated with the generation of organoids. (i) It is important to note that the quality of the iPSCs remains the most crucial step in the formation of organoid tissue. Differentiated iPSCs would either avoid proper formation of EBs or lead to the formation of non-homogenous EBs that would contribute to variable material for experimentations. iPSC lines are very sensitive and require delicate culture techniques to avoid differentiation. This can be achieved by choosing a range of iPSC passage number suitable for generating good EBs as well as spending extensive effort to remove any cell with differentiation sign. (ii) Batch-to-batch reproducibility is difficult to achieve. It can be controlled by optimizing chemical and physical parameters of media and incubation. (iii) Optimal concentrations of

components in the media need to be carefully chosen. There are various chemical factors that contribute to the generation of organoids, and therefore require careful standardization. (iv) Generation of uniform EBs, is the key factor in the generation of organoids. Uniform, smooth and continuous edges of EBs are essential to develop uniform organoids. The primary step of assessment is the neural induction that results in formation of embryonic bodies. The shape (spherical with smooth edges) of EBs at this stage is the defining factor of organoid formation. The EBs generated by our protocol have consistent shape. Although, to facilitate tissue induction and further develop a three-dimensional structure, Matrigel® is used as a scaffold. Since this scaffold is present only in the early days of organoids, the shape until then is consistent due to this physical parameter in place whereas, once the organoids outgrow the scaffold and are capable of differentiating independently in the medium, the shape might vary slightly from one organoid to another (so far, we have observed slight variations in shape but not to a great extent). As the organoids are generated in a controlled environment, their shape and size are fairly consistent. (v) The speed of the shaker is crucial in the final differentiation of organoids and maintaining 3D organization. (vi) The first protocols for cerebral organoids generation required use of paraffin, one-by-one Matrigel® embedding, and one-by-one transfer into final plates (34). This procedure was time consuming and could led to tissue damaged or contamination by the multiple transfers steps involved. By using our midbrain generation protocol, we enabled a scaled-up production, without touching directly the tissue at any step. This allowed us to generate easily big batches of 500 hMOs derived from multiples iPSC lines for comparison studies. (vii) Cryosectioning organoids is a challenging analysis step. Since the organoids form a structure distinct from that of brain tissue, some protocol adaptations were necessary to consistently generate high quality sections. Furthermore, due to tissue organization and small sizes, sections have to be optimized for each stage of organoid maturation. Other methods such as clearing techniques can be useful to overcome this challenge. So far, we have overcome the challenges and generated numerous high-quality midbrain organoids." Additional points:

1. The authors explain how to thaw the iPSCs. They should also indicate how the cells

are supposed to be frozen, specifically at what density and how many cells per vial **Answer:** We wish to thank the reviewer for pointing this out. We added this information in the new section "Freezing of human iPSC lines".

1. In Figure 1a, it would be useful to illustrate an example of colony presenting with differentiated areas by comparison with a suitable colony.

Answer: We thank the reviewer for this comment, and we have added a picture of differentiated colony in **Figure S1a**.

 There is no reference to the percentage of dopaminergic neurons across the organoids. The size of the organoid is quite important and only a single section is presented in Figure 3. It would be nice to provide a representation with serial sections immunostained for TH, using bright field microscopy and a colorimetric stain (HRP/DAB), to illustrate the global distribution of the TH-positive cells within the organoid.

Answer: We thank the reviewer for this comment. To address it, we ran single cell RNA sequencing to evaluate the global distribution of TH within hMOs. The results and analysis appear now in **Figure 3** and in the "**Results and Observations**" section. The expression of the dopaminergic marker TH in cluster "Neurons -1, Neurons – 2 and NPCs (neuronal precursors)" is 15%, 34% and 11.5% respectively.

1. In Figure 3 the authors point with white squares at areas surrounding a "ventriclelike" structure, indicating that they are composed of neural progenitor cells. Although it is most likely that these cells are progenitors, it would be nice to include at least an immunostaining for nestin to confirm it

Answer: We thank the reviewer for this comment and include FoxA2 staining as a dopaminergic progenitor marker. As you can observe in **Figure 3b**, group of cells in "ventricle-like" structure are also FoxA2, revealing their progenitors state. Additionally, we added this information in "**Results and Observations**" section.

1. The quality of Figure 3c should probably be improved. TUJ1 staining usually labels both soma and cellular processes. It is quite surprising not to see stained processes in this enlarged photograph. Projections of Z-stacks confocal images would be useful to capture more precisely the cell morphology. The absence of neuronal processes would rather indicate a very immature stage of differentiation, which would be inconsistent with the presence of neuromelanin in some of the TH-positive cells

Answer: We apologize for the quality of pictures and replaced them with higher resolution ones in **Figure 3b-c**. We removed TUJ1 staining pictures due to the high antibody background detected. The reviewer can now appreciate better the shape of the cells and the processes for TH and MAP2 stainings.

1. In how far does treatment with dopamine test "functionality". The authors may consider rewording of their statement. In Figure 4, it would be useful to integrate a high magnification picture illustrating the presence of neuromelanin granules within a single/a few TH-positive neuron/s. In how far does the graph in panel c illustrate "number of neuromelanin granules"?

Answer: We thank the reviewer for this comment and reworded our statement. As the reviewer requested, we integrated a higher magnification of TH positive neuron, revealed by regular IHC with DAB chromogen and counterstained with Romanowsky-Giemsa to detect neuromelanin granule in dark green (arrow, Figure 4d) (64). The neuromelanin changes under dopamine treatment were analyzed using GIMP software to specifically extract the pixels associated with neuromelanin staining from of Fontana–Masson-stained sections and quantitating the number of extracted pixels using Image J (63). A histogram of the image was created, which separates the total number of pixels in the image into 255 color categories spanning the visible spectrum. The peak corresponding to the brown-black color (neuromelanin) was determined by cutting and summing the appropriate counts from each channel of the melanin peak. In order to clarify the graph, we provided more quantification details in methods as follow: "Black dots from Fontana Masson stainings pictures were extracted with colorimetric selection from GIMP software (version 2.8.22) and quantified by ImageJ (version 2.0.0-rc-69/1.52i) following the method described in (63). Briefly, using GIMP software the pixels associated with neuromelanin staining were colored extracted, and quantitating the number of extracted pixels using Image J. A histogram of the image was created, which separates the total number of pixels in the image into 255 color categories spanning the visible spectrum. The peak corresponding to the brown-black color i.e. neuromelanin was determined by cutting and summing the appropriate counts from each channel of the melanin peak." Additionally, since we agreed that the neuromelanin numbers were not a direct quantification of the neuromelanin but rather a black pixels quantification, we modified the title axis for "Relative number of neuromelanin granules" in Figure 4c.

Competing Interests: No competing interests were disclosed.

Reviewer Report 11 April 2019

https://doi.org/10.21956/mniopenres.13879.r26160

© **2019 Ryan S.** This is an open access peer review report distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

了 🛛 Scott D. Ryan 问

Department of Molecular and Cellular Biology, University of Guelph (U of G), Guelph, ON, Canada

In this manuscript Nguyen et al. present a protocol for derivation of human midbrain organoids from induced pluripotent stem cells. This paper integrates recent advances in the field stemming from work pioneered by Lancaster and advanced by both Jo et al. and Monzel et al. The current manuscript further addresses technical barriers that were commonly reported in the previous protocols, including adaptations for cryosectioning that maintain tissue integrity for immunohistological processing. Overall, I find the paper to be a useful and technically sound. Some minor changes (outlined below) would help improve clarity of the protocol, prior to final indexing.

- 1. The authors state on pg 3. that this protocol generates near 100% midbrain per batch. This is difficult to assess given the staining in figure 3. The authors should either soften the language surrounding this claim or provide staining for non-neuronal cell types and quantification of cell types present in addition to characterization of A9 verses A10 neurons.
- 2. The inventory list cites Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12) from Gibco (Cat# 10-565-018) that is a medium supplemented with glutamax. They also report that 1% glutamax (cat 35050-061) is added to the medium. Is this an error or are they growing cells in 2% glutamax final?
- 3. Is there an advantage to using the U-bottoms shaped 96-well plate over V-bottom shaped, or should the plates simply not be flat?
- 4. On Pg 9 Why are 5 organoids being cultured per well of 6-well. Did the authors observe any issues with their organoids merging together when 5 organoids were transferred into one well of a 6-well plate? Was this the max number that could be grown together without organoids merging?
- 5. On Pg 11, The authors state why some organoids don't sink in sucrose post fixation as Matrigel may impact on the density of the organoid. Would it be prudent to use an organoid harvesting media to remove excess materiel in this case? Do the authors have evidence organoids that floated are identical post processing to those harvested by traditional organoid harvesting procedures (i.e. Matrigel is removed)?
- 6. In figure 4, the authors treat their organoids with dopamine to enhance maturation. Is this step necessary for maturation (i.e. neuromelanin production)? Do the organoids release dopamine exogenously or are the only capable or metabolizing exogenous dopamine. Does this step introduce any challenges with modeling aspects of PD where DA-quinone production is considered a toxic insult?
- 7. Can the authors comment on the consistency in the shape of their organoids as opposed to size alone. Does the shape correlate with the quality of the differentiation or variability in differentiation?

Is the rationale for developing the new method (or application) clearly explained? Yes

Is the description of the method technically sound?

Partly

Are sufficient details provided to allow replication of the method development and its use by others?

Yes

If any results are presented, are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions about the method and its performance adequately supported by the findings presented in the article?

Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Neurodegeneration, hiPSC, hESC, Parkinson's Disease

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 06 Jan 2021

nguyen-vi mohamed, Montreal Neurological Institute and Hospital, Montreal, Canada

In this manuscript Nguyen et al. present a protocol for derivation of human midbrain organoids from induced pluripotent stem cells. This paper integrates recent advances in the field stemming from work pioneered by Lancaster and advanced by both Jo et al. and Monzel et al. The current manuscript further addresses technical barriers that were commonly reported in the previous protocols, including adaptations for cryosectioning that maintain tissue integrity for immunohistological processing. Overall, I find the paper to be a useful and technically sound. Some minor changes (outlined below) would help improve clarity of the protocol, prior to final indexing.

We are happy to hear Dr Ryan sees the usefulness in the manuscript. We thank the reviewer for his insightful comments. Please find attached a revised version of the manuscript and below our replies to each of the points raised.

1- The authors state on pg 3. that this protocol generates near 100% midbrain per batch. This is difficult to assess given the staining in figure 3. The authors should either soften the language surrounding this claim or provide staining for non-neuronal cell types and quantification of cell types present in addition to characterization of A9 verses A10

neurons.

Answer: We thank the reviewer for raising this point complementary to another reviewer's comment. We added a complementary qRT-PCR experiment in **Figure 2c** showing the presence of several complementary midbrain markers and dopaminergic markers (EN1, Nurr1, LMX1B, Calb1, TH, MAOB, LMX1A, DDC, COMT) in hMOs when compared to iPSC lines. We didn't detect the presence of dopamine beta-hydroxylase (DBH), as a marker for noradrenergic/adrenergic neurons in hMOs. Interestingly, we detected serotonergic neurons marker (GBX2). Recently this population was also reported by other group in hMOs generated from a different protocol (65). This new characterization panel was added to the "Results and Observations" section. Additionally, we performed a broader characterization of cell types by single cell RNA sequencing (*Figure 3d-f*) and revealed the presence of others cell types that includes astrocytes, oligodendrocyte precursors, radial glial, neuronal progenitors as reported by other groups (35,65). Thanks to the reviewer's comments, we also removed the vague statement " 100% midbrain per batch" and have now adjusted the sentence as follow: "This protocol is an adaptation of the Nature protocol paper initially published by Lancaster in combination with discoveries from Jo et al. and Monzel et al.34–36 in order to successfully produce high quality midbrain organoids ».

2- (DMEM/F12) from Gibco (Cat# 10-565-018) that is a medium supplemented with glutamax. They also report that 1% glutamax (cat 35050-061) is added to the medium. Is this an error or are they growing cells in 2% glutamax final?

Answer: We wish to thank the reviewer for pointing this out. We induce the formation of neurons and pattern them toward a midbrain fate in media containing GlutaMAXTM-I at a final concentration of 2% (**Tables 3 and 4**), while the tissue growth media and final differentiation media contains 1% final GlutaMAXTM-I (Tables 5 and 6). To clarify the media formulations, the reference to «DMEM/F-12» was corrected to « DMEM/F-12 + GlutaMAXTM-I » in the revised manuscript.

3- Is there an advantage to using the U-bottoms shaped 96-well plate over V-bottom shaped, or should the plates simply not be flat?

Answer: This is a great point raised by the reviewer, and while we didn't include all the findings in the manuscript, we did test 96-well plates U-bottom and V-bottom, ultra-low attachment, in parallel (data not showed). While the formation of EBs was similar in both types of plates, we do have a preference for the 96-well plates U-bottom for the embedding step based on observations during the embedding steps. With the U-bottom well shape, it permitted a homogenous Matrigel® scaffold around the EBs compared to the V-shape one. Thus, we recommended the use of 96 wells plates U-bottom over V-bottom for optimal embedding. We thank the reviewer for this question and have now added a plate format recommendation in the new version of the manuscript **in Seeding of iPSCs – Day 0 section**.

4- On Pg 9 - Why are 5 organoids being cultured per well of 6-well. Did the authors observe any issues with their organoids merging together when 5 organoids were transferred into one well of a 6-well plate? Was this the max number that could be grown together without organoids merging?

Answer: We thank the reviewer for these questions. We noticed that with proper embedding of EBs in 96 well U-bottom plates, this avoided aberrant fusion of organoids when they are transferred to final differentiation media. It took around 20 days in final differentiation media to see a disruption of the Matrigel® scaffold. This time enables the organoids growth while the Matrigel® scaffold prevents their fusion. We transferred 5 organoids per well in order to decrease the number of media change per week. By putting 5 organoids per well, we limited the number of media changes to three times a week once the hMOs attain the maximum size of 4mm. We added this information into the new version in **section "Transfer the organoids to final differentiation medium – Day 8"**.

5- On Pg 11, The authors state why some organoids don't sink in sucrose post fixation as Matrigel may impact on the density of the organoid. Would it be prudent to use an organoid harvesting media to remove excess materiel in this case? Do the authors have evidence organoids that floated are identical post processing to those harvested by traditional organoid harvesting procedures (i.e. Matrigel is removed)?

Answer: We thank the reviewer for raising this point. During the past few months, we have systematically removed the Matrigel® surrounding organoids before the sucrose immersion step. Since then, we've never observed sinking issue. We added this observation to the note section.

6- In figure 4, the authors treat their organoids with dopamine to enhance maturation. Is this step necessary for maturation (i.e. neuromelanin production)? Do the organoids release dopamine exogenously or are the only capable or metabolizing exogenous dopamine. Does this step introduce any challenges with modeling aspects of PD where DA-quinone production is considered a toxic insult?

Answer: In **Figure 4**, we treat the young organoids with dopamine to trigger dopaminergic metabolism and observe the formation of neuromelanin granules, as a confirmation for the presence of dopaminergic neurons and their functionality. This step is not necessary for maturation of hMOs. We routinely perform this assessment, on several hMOs from every batch produced, as a tool to quickly confirm the midbrain identity of organoids produced. When we age the hMOs to within 5-6 months, we observe in the absence of any external dopamine treatment, a spontaneous formation of neuromelanin granules meaning the hMOs release dopamine exogenously. We thank the reviewer for his comment, and we clarified this point in the result section to avoid future confusion: "This experiment was not necessary for maturation of hMOs but allowed to confirm the presence of dopaminergic neurons, as well as the ability of dopamine synthesis."

7- Can the authors comment on the consistency in the shape of their organoids as opposed to size alone. Does the shape correlate with the quality of the differentiation or variability in differentiation?

Answer: The primary step for assessment is the neural induction that results in formation of embryoid bodies. The shape (spherical with smooth edges) of EBs at this stage is the defining factor of organoid formation. The EBs generated by our protocol have consistent shape. Although, to facilitate tissue induction and further develop a 3-dimensional structure, Matrigel®

is used as a scaffold. Since this scaffold is present only in the early days of organoids, the shape until then is consistent due to this physical parameter in place whereas, once the organoids outgrow the scaffold and are capable of differentiating independently in the medium, the shape can vary slightly from one organoid to another (so far, we have observed slight variations in shape but not to a great extent). As the organoids are generated in a controlled environment, their shape and size are fairly consistent. We thank the reviewer for his comment, and we added this point in the conclusion remarks.

Competing Interests: No competing interests were disclosed.