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A Static Self-Directed Method for Generating Brain Organoids from Human Embryonic Stem Cells

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Alisha DSouza, PhD
Senior Review Editor, JoVE

Dear Dr. DSouza:

Thank you for the opportunity to submit our revised manuscript, entitled "**A Static Self-Directed Method for Generating Brain Organoids from Human Embryonic Stem Cells"**. We are very grateful for the opportunity to address the Editor comments and would like to thank you for your constructive review of our manuscript. We have now used track changes to identify all of the manuscript edits and addressed each of the editorial comments individually below. We hope that with these new corrections that you will find our revised organoid protocol of use to the scientific community and suitable for publication in *JoVE*.

Sincerely,

Samuel G. Katz, M.D., Ph.D.

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Response to Editors

Editorial Comments:

1) I have edited some steps and highlighting slightly to meet our style requirements. (line 84)
    a. We agree with these changes.
2) I edited the protocol to replace Matrigel with the word matrix. (line 89)
    a. We agree.
3) I edited the protocol to replace dispase with the word protease. (line 102)
    a. We agree.
4) Mention wavelength. (line 187)
    a. We added 260 and 280 nm.
5) Please remove this (“Images were taken with a Nikon TMS-F microscope”) and add to the table of materials. (lines 319-320)
    a. Done.
6) Please remove this (“Images were taken with an Olympus BX43”) and add to the table of materials. (line 325)
    a. Done.
7) Please obtain reprint permission prior to acceptance. (line 330)
    a. Done, please see uploaded Supplemental file.
8) Define the error bars (line 330)
    a. We added to our description of the error bars (lines 329-330).
9) Add a legend. Table 1 was missing from the submission. Please add it.
    a. We added a legend and uploaded the Table (line 337)

TITLE:

A Static Self-Directed Method for Generating Brain Organoids from Human Embryonic Stem Cells

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KEYWORDS:

Brain organoids, human embryonic stem cells, neurodevelopment, neuronal differentiation, disease modeling, simplified, growth factors

SUMMARY:

This protocol was generated as a means to produce brain organoids in a simplified, low cost manner without exogenous growth factors or basement membrane matrix while still maintaining the diversity of brain cell types and many features of cellular organization.

ABSTRACT:

Human brain organoids differentiated from embryonic stem cells offer the unique opportunity to study complicated interactions of multiple cell types in a three-dimensional system. Here we present a relatively straightforward and inexpensive method that yields brain organoids. In this protocol human pluripotent stem cells are broken into small clusters instead of single cells and grown in basic media without a heterologous basement membrane matrix or exogenous growth factors, allowing the intrinsic developmental cues to shape the organoid's growth. This simple system produces a diversity of brain cell types including glial and microglial cells, stem cells, and neurons of the forebrain, midbrain, and hindbrain. Organoids generated from this protocol also display hallmarks of appropriate temporal and spatial organization demonstrated by brightfield images, histology, immunofluorescence and real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR). Because these organoids contain cell types from various parts of the brain, they can be utilized for studying a multitude of diseases. For example, in a recent paper we demonstrated the use of organoids generated from this protocol for studying the effects of hypoxia on the human brain. This approach can be used to investigate an array of

otherwise difficult to study conditions such as neurodevelopmental handicaps, genetic disorders, and neurologic diseases.

INTRODUCTION:

Due to myriad practical and ethical limitations, there has been a great deal of difficulty in studying the human brain. While studies utilizing rodents have been critical to our understanding of the human brain, the mouse brain has many dissimilarities[1,2]. Interestingly, mice have a neuronal density that is at least 7 times less than the primate brain[3,4]. Although primates are closer to humans than rodents from an evolutionary standpoint, it is not practical for most researchers to work with them. The purpose of this protocol was to recapitulate many important features of the human brain using a simplified and less expensive method without the need for a heterologous basement membrane matrix or exogenous growth factors while maintaining brain cell diversity and cellular organization.

Formative work from the Sasai lab used the serum free culture of embryoid bodies (SFEBq) method to generate two- and three- dimensional neuronal cell types from signalized embryonic stem cells (ESCs)[5,6]. Many human brain organoid methods have followed a relatively similar path from signalized ESCs[7,8]. In contrast, this protocol starts with clusters of detached human ESCs (hESCs), similar to the initial steps of seminal work of the Thomson and Zhang laboratories prior to the plating steps[9,10] as well as the initial step of the brain organoid protocol of the Pasca laboratory before the addition of exogenous growth factors[11]. Basement membrane matrices (e.g., matrigel) have been utilized in many brain organoid protocols and it has been shown to be an effective scaffold[8]. However, most commonly used basement membrane matrices do not come without complications as they co-purify with unknown quantities of growth factors with batch to batch variability during production[12]. In addition, these matrices can complicate imaging, and increase the risk of contamination and cost.

While human brain organoids can be used to answer many questions, there are certain limitations to bear in mind. For one, starting from embryonic stem cells, organoids more closely resemble immature brains than aged brains and as such may not be ideal models for diseases that occur in old age, like Alzheimer's disease. Second, while our protocol found markers of forebrain, midbrain and hindbrain development which are useful to study the effect of a treatment or disease on cells from multiple brain regions in concert, other protocols can be followed to concentrate on specific brain regions[13,14]. Finally, another limitation of organoid models is that of size, while the average length of a human brain approximately 167 mm, brain organoids made with the use of agitation grow up to 4 mm [8] and the organoids formed by this protocol grow to 1–2 mm by 10 weeks. Nonetheless, this protocol provides an important tool to study human brain tissue and the interaction of multiple cell types.

PROTOCOL:

1. Stem cell maintenance

1.1. Maintain H9 hESCs on a layer of growth factor reduced basement membrane matrix (see the **Table of Materials**, henceforth simply referred to as matrix) according to the manufacturer's instructions.

1.1.1. To coat one 6-well plate or one 10 cm dish, combine 100 µL of matrix with 5.9 mL of ice-cold Dulbecco's modified Eagle medium (DMEM)/F12 media. Wrap plates in paraffin film and store overnight at 4 °C. Use them on the next day for passaging cells after the excess matrix/media is aspirated.

1.2. Culture the cells week to week, using mTESR-1 media in a 37 °C, low oxygen incubator (5% O_2, 5% CO_2). Refresh media daily. Weed out differentiating cells from the culture between passages using glass tools.

1.3. Four to six days prior to utilizing the H9 cells to produce organoids, dissociate the cells with a neutral protease (e.g., dispase, henceforth referred to simply as protease), rinse with DMEM/F-12, and plate as 30–60 cell clusters across 4 plates (6-well or 10 cm) at ~20% confluency. Two days prior to harvest, transition them to a regular incubator (21% O_2, 5% CO_2). Plates should reach ~80% confluency when starting organoid formation.

2. Dissociation of the hESCs for organoid culture

2.1. Aliquot the protease stock solution (5 U/mL).

NOTE: We typically freeze down 1 mL aliquots at -20 °C for use over several months.

2.2. Dilute the protease stock solution to the working concentration by adding 1 mL of the stock solution plus 5 mL of DMEM/F12 for each 6-well or 10 cm plate of hESCs.

2.3. Aspirate and remove cell culture media, then cover the hESCs with the protease solution. Place plates in the incubator for 10–15 min or until the edges of the colonies round up and begin to separate from the matrix.

2.4. Tilt the plate, aspirate the protease solution, and gently wash the cells with DMEM/F12 three times. Use 2 mL/well for each wash when using a 6-well plate and 6 mL when using a 10 cm plate. Make sure colonies stay attached to the matrix when performing this step.

2.5. Add back about 1.5 mL of fresh mTESR media to each well (or 5 mL for a 10 cm plate) and flush the cells off the plate using gentle pipetting.

2.6. Using a 10 mL pipette, gently aspirate and dispense hESC within the plate until they reach approximately 1/30th of their original size. Colony clusters should resemble ~250–350 µm sized squares at the completion of these steps.

3. Generation of organoids

3.1. Transfer cells into a single ultra-low attachment T75 flask containing 30 mL of mTESR media without basic fibroblast growth factor (bFGF).

3.2. The next day, tilt the flask(s) such that the live cells pool in the corner (this may take 5–10 min on the first day, but will get quicker as the clusters get larger).

NOTE: If there are a large number of cells that have adhered to the bottom of the flask at this step or any subsequent steps, transfer the cells to a new flask. It is normal to have a high population of dead cells for the first two days. When performing media changes, be sure to remove as much of the cell debris as possible.

3.3. Once the cells settle, aspirate off the media and dead cells leaving about 10 mL of media containing the live cells.

3.4. Add ~20 mL of low bFGF media (DMEM/F12 supplemented with 1x N2, 1x B27, 1x L-glutamine, 1x NEAA, 0.05% bovine serum albumin (BSA), and 0.1 mM monothioglycerol (MTG) supplemented with 30 ng/mL bFGF.

3.5. Check the cells on day 2. If most of the cells look healthy and bright, there is no need to do anything. However, if more than a third of the cells appear dark, replace the media (using the same tilting technique as in step 3.2) with ~20 mL of low bFGF media supplemented with 20 ng/mL bFGF.

3.6. On day 3, replace the media (using the tilting technique in step 3.2) with 20 mL of low bFGF media supplemented with 10 ng/mL bFGF.

3.7. On day 5, replace the medium (using the tilting technique in step 3.2) with 20 mL of neural induction media (NIM: DMEM/F12, 1x N2 supplement, 0.1 mM MEM NEAA, 2 µg/mL heparin).

NOTE: If there are any large clusters of cells or organoids that are much larger than the others, they should be removed from the culture. Size is estimated by appearance under the microscope; for example, using an eyepiece with reticle. The majority of organoids are similarly sized (roughly 100 ± 20 µm). We removed organoids that were approximately 2x smaller or larger than the others.

3.8. Replace half of the medium (~15 mL) (using the tilting technique) with NIM every other day.

3.9. After 3 weeks in culture, add 100x penicillin/streptomycin to the media (NIM: DMEM/F12, 1x N2 supplement, 0.1 mM MEM NEAA, 2 µg/mL heparin) at a final concentration of 1x if desired. Refresh the media every other day.

NOTE: In this fashion, we maintained the organoids for up to 6 months in culture.

4. RNA extraction and preparation

4.1. Gently extract approximately 15 organoids (depending upon size) from the flask using a 10 mL pipette and place into a 1.5 mL tube.

4.1.1. Gently pellet the organoids in the centrifuge (200 x *g* for 1 min), and rinse with 1x Dulbecco's PBS (DPBS) three times.

4.2. Extract RNA using validated system or protocol (e.g., RNeasy kit).

4.3. Measure the optical density value of each sample at 260 and 280 nm.

4.4. Prepare cDNA using a validated system or protocol (e.g., iScript cDNA synthesis kit).

4.5. Perform qRT-PCR using pre-validated primers (**Table 1**) including at least one housekeeping gene.

5. Immunohistochemistry

5.1. Fixation

5.1.1. Prepare a 4% paraformaldehyde (PFA) solution and place it at 4 °C.

5.1.2. Using a sterile razor, cut the tip off of a sterile transfer pipette.

5.1.3. Gently extract organoids using the cut transfer pipette, as they can be easily broken apart, especially when they grow large, and place them into a 6-well plate with additional media or DPBS.

5.1.4. Tilt the plate, aspirate the media, and replace with 1x DPBS. Rinse the cells with 1x DPBS two additional times.

5.1.5. Replace the DPBS with 4% PFA solution and place on a shaker at 4 °C.

NOTE: While we fixed for 2 days (for small organoids) to 7 days (for organoids >3 months), shorter times (e.g., 16–24 h) may also be possible.

5.1.6. Prepare 30%, 20% and 10% sucrose solutions in DPBS.

5.1.7. After fixation in PFA, replace with the 10% sucrose solution and place on a shaker at 4 °C for 24 h.

5.1.8. Replace the 10% sucrose with 20% sucrose and place on a shaker at 4 °C for 24 h.

5.1.9. Replace the 20% sucrose with 30% sucrose and place on a shaker at 4 °C for 24 h.

5.2. Frozen sections

5.2.1. Prepare a flat layer of dry ice and place a labeled plastic mold on top of it.

5.2.2. Pour a thin layer of optimal cutting temperature medium (OCT) into the mold and let it start to harden (within a few seconds).

5.2.3. Place a few organoids on the top of the OCT in the mold using a transfer pipette with the tip cut off and pay close attention to the location of the organoids.

5.2.4. Slowly add in OCT until the mold is full and the organoids are covered. Let it harden completely for an additional 10 min.

NOTE: While freezing over 10 min helps ensure ideal relative placement of multiple organoids for sectioning, it is possible to use an ethanol-dry ice mix or liquid nitrogen to freeze more quickly.

5.2.5. Mark the relative location of the organoids with a marker to make it easier to find them when cutting.

5.2.6. Place the molds in a bag or box and store at -80 °C until ready to cut the sections.

5.2.7. Using a cryostat, slice 10 µm sections and place the tissue onto labeled, positively charged slides.

5.3. Staining

5.3.1. Prepare blocking solution (0.3% Triton X-100, 4% normal donkey serum in PBS).

5.3.2. Use a hydrophobic pap pen to draw around the perimeter of the tissue.

5.3.3. Rinse the slides with PBS 3 times for 5 min each.

5.3.4. Replace PBS with blocking solution for 1 h at room temperature.

5.3.5. Replace the blocking solution with antibody solution (antibody at appropriate concentration, 0.1% Triton X-100, 4% normal donkey serum in PBS) at 4 °C overnight.

5.3.6. On the following day, wash the slide 3 times with PBS for 10 min each.

5.3.7. Replace PBS with the appropriate secondary antibody (1:1000) diluted in antibody solution for 1 h at room temperature.

5.3.8. Rinse 3 times for 10 min each time with 1x PBS.

5.3.9. Apply the 4',6-diamidino-2-phenylindole (DAPI) stain and rinse three times for 10 min each with 1x PBS.

5.3.10. Affix coverslips to the front of the slides with mounting solution, let dry at room temperature in the dark, and store in the dark at 4 °C.

REPRESENTATIVE RESULTS:

Figure 1 shows representative brightfield images of several time points to demonstrate what the cells/organoids look like throughout the different stages of the protocol. The hESCs were removed from the tissue culture plate, broken into small pieces, and placed in a T75 ultra-low attachment flask where they formed spheres. It is important to note that the cells look bright and similar in size, without dark, dying cells in the centers of these clusters. The cells were gradually weaned off bFGF. On day 5, they were placed into neural induction media and they remained in this media throughout the culture period. Although the organoids get larger and thus darker over time, it is important to take note of the neural rosette-like structures (black arrows) that are present throughout the brain organoid development and expand. The rosettes indicate the initiation of neural differentiation and contain features of the embryonic neural tube, displaying epithelial characteristics and surrounding an apical lumen[15].

Staining of the organoids with hematoxylin and eosin at 5 months in culture indicated that there were not vast amounts of necrosis even in the centers, which was of initial concern given the stagnant culture system (**Figure 2A**). These organoids demonstrated a histologic morphology similar to the human cortex based on light microscopic evaluation by an experienced neuropathologist (**Figure 2B**). By histology, many unique cell morphologies were observed resembling glia (blue arrow head), neurons (red arrowhead), cells with Cajal-Retzius morphology (black arrows), and neuropil (orange arrow head) (**Figure 2B,C**).

To take a more in depth look at gene expression within the cells, qRT-PCR was performed. For the results shown in **Figure 3**, each bar represents 3 separate batches of cells grown independently and harvested at the specified time point. These samples were then run in triplicate with a primer pair to the indicated gene in addition to the housekeeping gene, GAPDH. The glutamate transporter, Vglut1 (**Figure 3A**), was expressed at 2.5 weeks, increased at 5 weeks, and remained consistent through 5 months in culture. A forebrain marker, Foxg1 (**Figure 3B**), was expressed at low levels until 5 weeks in culture. The deep layer marker, Tbr1 (**Figure 3C**), peaked around 5 weeks and decreased subsequently, whereas the upper layer marker, Satb2 (**Figure 3D**), increased over time.

The expression of the ventral markers Eng1 (**Figure 3E**) and Hoxb4 (**Figure 3F**), as well as the oligodendrocyte marker, Olig2 (**Figure 3G**), all increased over time. In contrast, the stem cell

marker, Sox2 (**Figure 3H**), decreased over time. The glial marker, GFAP (**Figure 3I**), peaked at 5 weeks and remained relatively constant subsequently. In addition, immunofluorescence data was consistent with the qRT-PCR data. At 10 weeks there was a robust expression of Foxg1 (**Figure 4A**). Sox2 expression was more confined to areas resembling the subventricular zone (SVZ) (**Figure 4B,C**). Interestingly, there was also some expression of the outer radial glial cell marker, HopX (**Figure 4D**).

FIGURE AND TABLE LEGENDS:

Figure 1. Overview of organoid growth conditions and morphology. (**A**) Schematic of media changes. (**B–J**) Representative images of organoids as they matured. (**B**) H9 hESCs were utilized to form the brain organoids. Organoids on (**C**) day 2 in 20 ng/mL bFGF media and (**D**) day 4 in 10 ng/mL bFGF media. (**E– J**) Organoids in neural induction media (NIM) on days 8 (**E**), 10 (**F**), 35 (**G**, **H**), and 70 (**I**, **J**). Arrows point to neural rosettes. Bar = 200 μm.

Figure 2. Organoids shared histologic similarities to human brain tissue. H&E staining of the organoids at 5 months (**A**) with some layering resembling the human cortex (**B**). At higher magnification many cell morphologies were observed including glia (blue arrow head), neurons (red arrowhead), neuropil (orange arrow head), and cells with Cajal-Retzius morphology (black arrows) (**B, C**).

Figure 3. Expression of neurodevelopmental genes within the brain organoids over time. Quantitative RT-PCR data using SYBR green evaluating the expression of Vglut1 (**A**), Foxg1 (**B**), Tbr1 (**C**), Satb2 (**D**), En1 (**E**), Hoxb4 (**F**), Olig2 (**G**), Sox2 (**H**), and GFAP (**I**). Error bars represent mean ± standard deviation ($n \geq 3$). This figure has been modified from[16]. See **Table 1** for primer information.

Figure 4. **Expression of neurodevelopmental proteins within the brain organoids at 10 weeks.** Immunofluorescence revealed a robust expression of Foxg1 (**A**), localized expression of Sox2 (**B,C**) and the presence of HopX (**D**).

Table 1. Primer sequences used for quantitative RT-PCR in **Figure 3**.

DISCUSSION:

Similar to other organoid models, this is an artificial system that comes with several caveats. Although there was little batch to batch variation in terms of overall expression levels, individual organoids did exhibit differences. For example, the location of Sox-2 positive areas were not identical in every organoid (**Figure 3**). While qPCR is suitable to look for overall changes in batches of cells, additional techniques such as single cell RNAseq will be utilized in future studies to gather more information on a cell-by-cell basis. Another limitation of this system, is that it does not integrate vasculature within the organoids as has been done in some of the more recent studies[17-19]. However, transitioning the hESCs from a low to high oxygen environment may more closely resemble the anaerobic to aerobic transitioning in a developing embryo.

The critical steps within this protocol are the formation of the neurospheres as well as the appropriate maintenance including media changes with the proper culture media to ensure healthy cells and adequate nutrients to the growing organoids without overcrowding. To troubleshoot inadequate cell proliferation or differentiation, we recommend starting with a fresh batch of low passage ESCs and freshly prepared media including supplements. Occasionally there can be batch variation of reagents and materials. Thus, we recommend purchasing multiple bottles of reagents such as N2 and ultra-low attachment flasks which are from the same lot as long as they can be utilized in a reasonable amount of time.

Unlike many other brain organoid protocols, this method does not use a bioreactor; instead the cells stay relatively stagnant aside from media changes. This is similar to previous work with neurospheres, which were eventually broken apart to make 2D neuronal cultures[20]. In this model the cells are kept in a 3D format and allowed to grow for up to 6 months in culture. It was found that when utilizing small clusters instead of aggregating single cells that the organoids looked brighter, which we interpreted as less necrotic. As previously reported, when the brain organoid clusters were evaluated by histology and immunofluorescence at 5 months, there were no obvious areas of necrosis[16]. Although starting from small clusters of cells introduces a little variety in the size of organoids formed, the majority of organoids were of a roughly similar size.

The use of a heterologous basement membrane matrix and a bioreactor have both advantages and disadvantages. Certain cell types, or larger brain organoids might prefer growth under one condition or another. Basement membrane matrices or other hydrogels might be beneficial to selectively add growth factors to particular regions or create specific molds. Although basement membrane matrices have been shown to support three-dimensional organization and differentiation[15], it is worth emphasizing that some of these products have a poorly defined and variable composition that includes quantities of growth factors[12]. In addition to simplifying the workflow while culturing the brain organoids, the absence of a basement membrane matrix might also improve three-dimensional imaging techniques.

The development of this brain organoid model system offers a new approach for many potential applications. For example, toxic insults like hypoxia, hyperglycemia, hypercapnia, and infection among others, may be tested with this system. In addition, neurodevelopmental disorders may be studied with this system by starting with either genetically modified stem cells or patient-specific human induced pluripotent stem cells (hPSCs). The ability to add different cell types during organoid culture also offers the possibility to study tumor-brain interactions. Given the simplicity of the protocol and lack of expensive, specialty materials we hope that this approach may be considered by laboratories both within and outside of the field as one potential method with its own unique benefits to further advance this rapidly progressing and exciting discipline.

ACKNOWLEDGMENTS:

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generous unrestricted gift from Joseph and Lucille Madri.

DISCLOSURES:

S.G.K. is a SAB member for Dansar IT and Gene-in-Cell.

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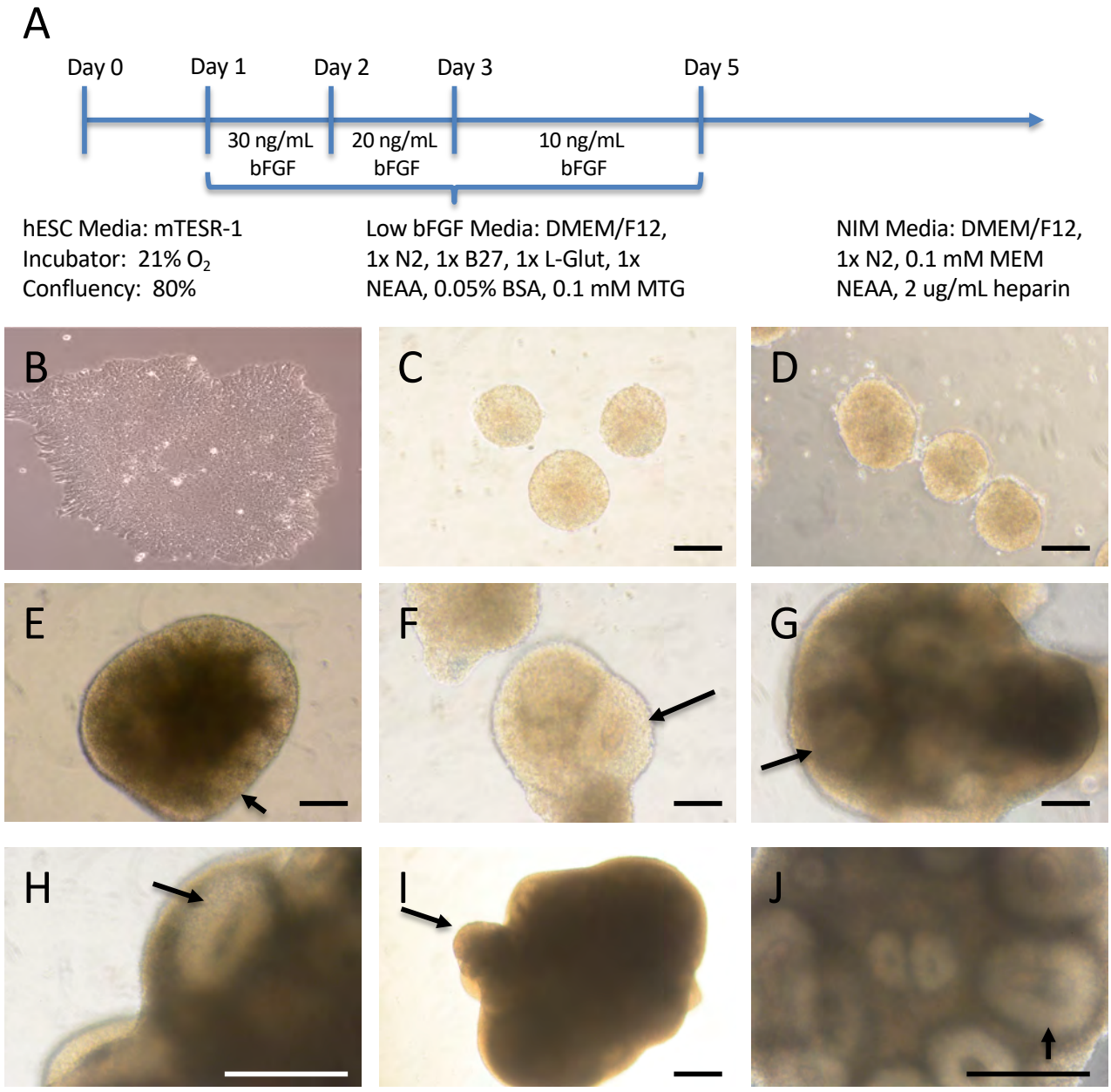

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Figure 1

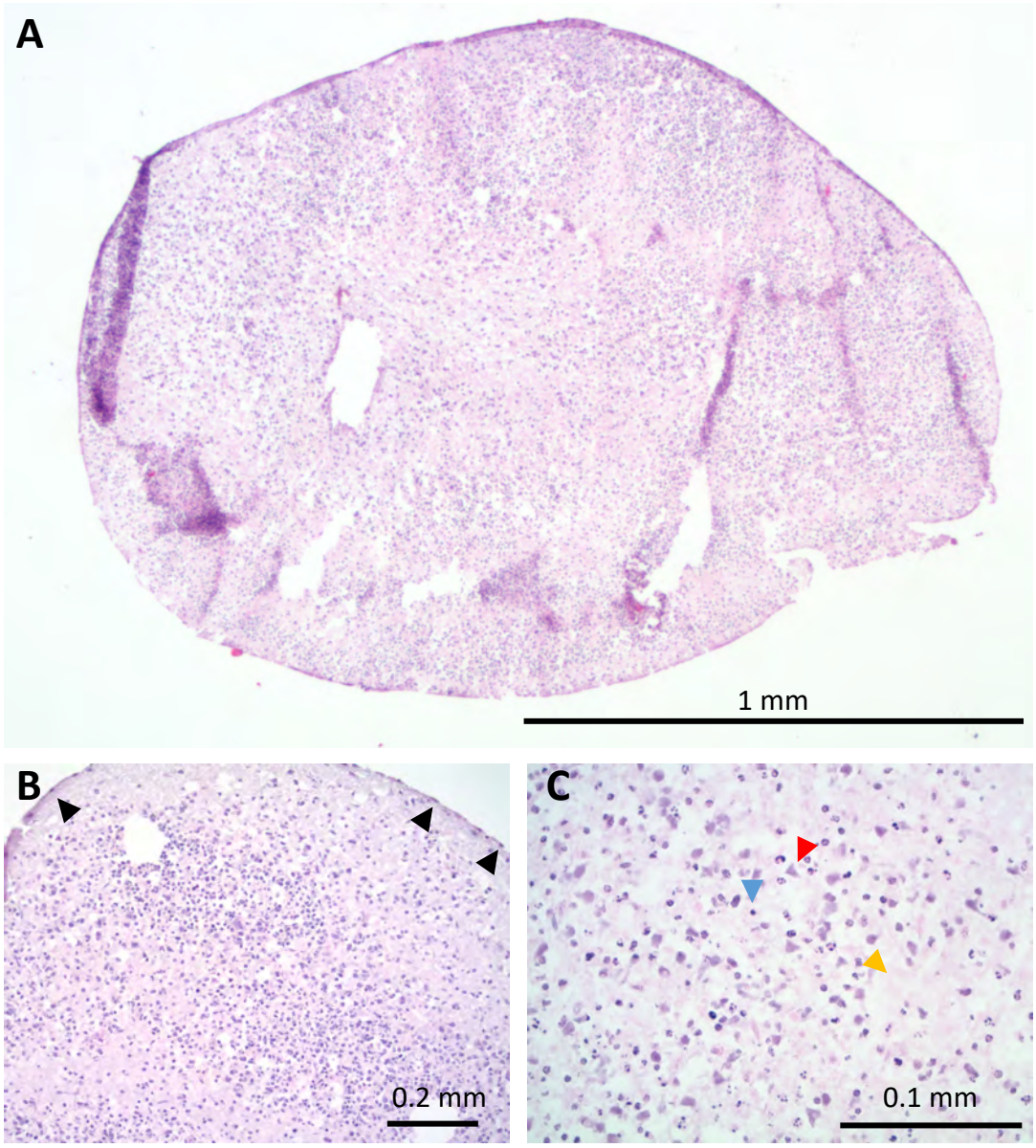

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Figure 2

Figure

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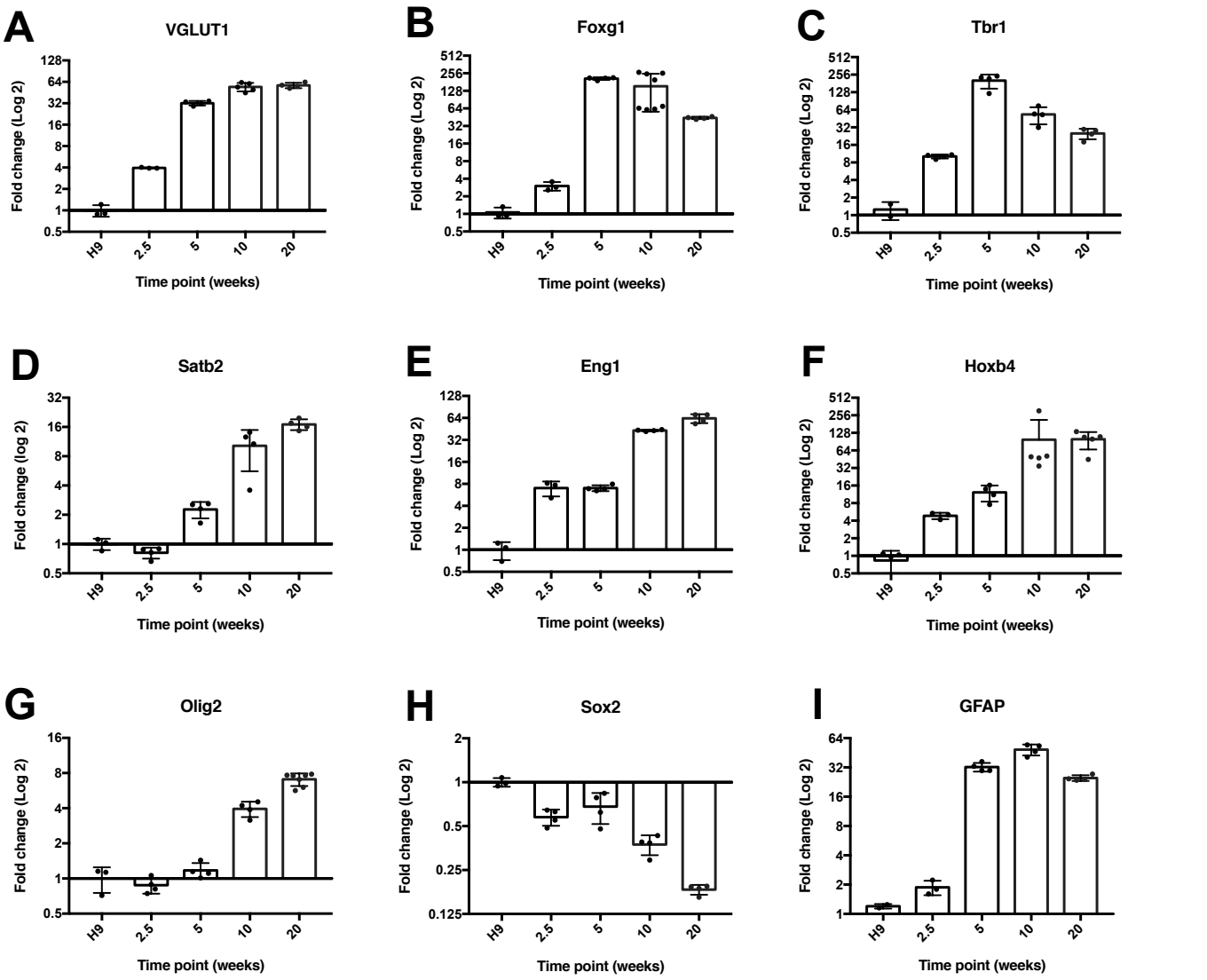

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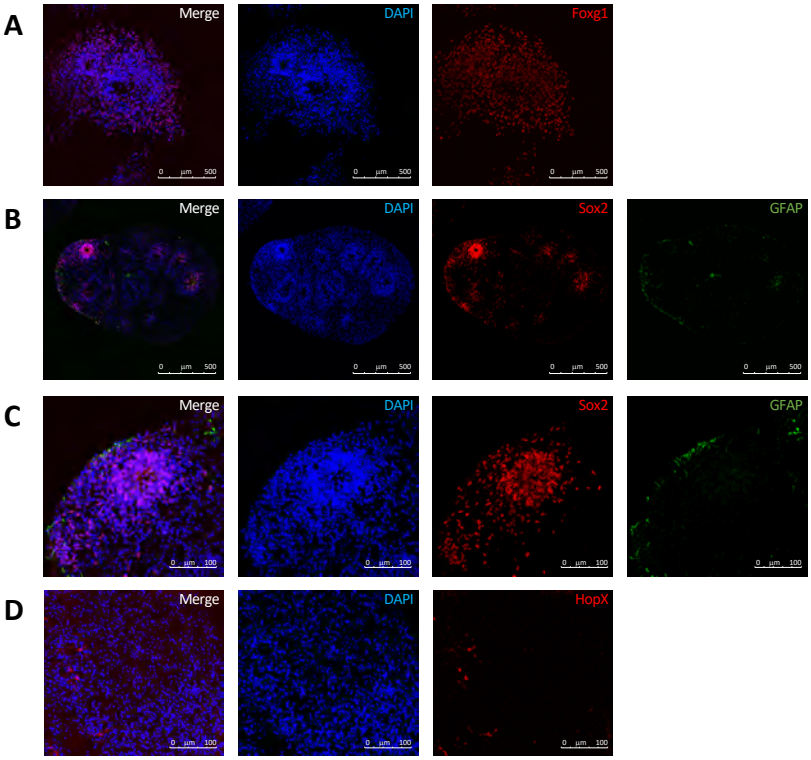
Figure 3

Figure

Click here to access/download;Figure;FIGURE 4.pdf



Boisvert et al.
Figure 4

Table

Gene		Sequence (5’ to 3’)	Amplicon	Exon
En1	F	GGACAATGACGTTGAAACGCAGCA	149	2
	R	AAGGTCGTAAGCGGTTTGGCTAGA		2
Foxg1	F	AGAAGAACGGCAAGTACGAGA	188	1
	R	TGTTGAGGGACAGATTGTGGC		1
GAPDH	F	ACCACAGTCCATGCCATCAC	449	8
	R	CACCACCCTGTTGCTGTAGCC		9
GFAP	F	AGAGATCCGCACGCAGTATG	80	4
	R	TCTGCAAACTTGGAGCGGTA		5-Apr
Hoxb4	F	AAAGCACCCTCTGACTGCCAGATA	80	2
	R	ATGGGCACGAAAGATGAGGGAGA		2
Olig2	F	CCCTGAGGCTTTTCGGAGCG	451	1
	R	GCGGCTGTTGATCTTGAGACGC		2
Satb2	F	TAGCCAAAGAATGCCCTCTC	94	6
	R	AAACTCCTGGCACTTGGTTG		7
Sox2	F	CCCAGCAGACTTCACATGT	150	1
	R	CCTCCCATTTCCCTCGTTTT		1
Tbr1	F	GTCACCGCCTACCAGAACAC	101	4
	R	ACAGCCGGTGTAGATCGTG		6
Vglut1	F	CAGAGTTTTCGGCTTTGCTATTG	183	5-Apr
	R	GCGACTCCGTTCTAAGGGTG		6

Name	Company	Catalog Number	Comments
Alexa Fluor 488 goat anti-mouse	Thermo Fisher Scientific, Waltham, MA, USA	A11029	
Alexa Fluor 546 goat anti-rabbit	Thermo Fisher Scientific, Waltham, MA, USA	A11035	
B27 Supplement	Gibco , Waltham, MA, USA	17504-044	
bFGF	Life Technologies , Carlsbad, CA, USA	PHG0263	
BSA	Sigma–Aldrich, St. Louis, MO, USA	A9647	
BX43 microscope	Olympus, Shinjuku, Tokyo, Japan		
DAPI stain	Thermo Fisher Scientific, Waltham, MA, USA	D1306	
Dispase	STEMCELL Technologies , Vancouver, Canada	07913	
DMEM/F12	Thermo Fisher Scientific, Waltham, MA, USA	11330-032	
DPBS	Gibco , Waltham, MA, USA	10010023	
FluroSave	MilliporeSigma, Burlington, MA	345789	
GFAP antibody	NeuroMab, Davis, CA	N206A/8	
Growth Factor Reduced Matrigel (Matrix)	Corning , Corning, NY, USA	356231	
H9 hESCs	WiCell , Madison, WI, USA	WA09	
Heparin	Sigma–Aldrich, St. Louis, MO, USA	9041-08-1	
iQ SYBR Green Supermix	Bio-Rad, Hercules, CA, USA	1708880	
iScript cDNA Synthesis Kit	Bio-Rad, Hercules, CA, USA	1708891	
L-glutamine	Gibco , Waltham, MA, USA	25030-081	
Monothioglycerol	Sigma–Aldrich, St. Louis, MO, USA	M6145	
mTESR media	STEMCELL Technologies , Vancouver, Canada	85850	
N2 NeuroPlex	Gemini Bio Products, West Sacramento, CA, USA	400-163	
Nanodrop	Thermo Fisher Scientific, Waltham, MA, USA	ND-2000	
NEAA	Gibco , Waltham, MA, USA	11140-050	
Normal Donkey Serum (NDS)	ImmunoResearch Laboratories Inc., West Grove, PA, USA	017-000-121	
OCT	Sakura Finetek, Torrance, CA, USA	25608-930	
PFA	Electron Microscopy Sciences, Hatfield, PA	RT15710	
qPCR machine	Bio-Rad, CFX96, Hercules, CA, USA	1855196	
RNeasy kit	Qiagen, Hilden, Germany	74104	
Sox2	MilliporeSigma, Burlington, MA	AB5603	
TMS-F microscope	Nikon, Melville, NY, USA		
Triton X-100	Sigma–Aldrich, St. Louis, MO, USA	T8787-100ML	
Ultra-low attachment T75 flasks	Corning , Corning, NY, USA	3814	

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Author(s):	Boisvert, EM, Means RE, Michaud M, Thomson JJ, Madri JA, Katz SG

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Date:	June 3, 2019

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July 23, 2019

Alisha DSouza, PhD
Senior Review Editor, JoVE

Dear Dr. DSouza:

Thank you for the opportunity to submit our revised manuscript, entitled "**A Static Self-Directed Method for Generating Brain Organoids from Human Embryonic Stem Cells"**. We are very grateful for the opportunity to address the Editor and Reviewer comments and would like to thank them for their constructive review of our manuscript. We have now answered all of the questions raised in point-by-point format (see below) and made the requested corrections to the manuscript. We hope that with these new descriptions that you will find our revised organoid protocol of use to the scientific community and suitable for publication in *JoVE*.

Sincerely,

Samuel G. Katz, M.D., Ph.D.

Boisvert et al.
Response to Reviewers

Editorial Comments:

• Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammatical errors.

We have carefully proofread the manuscript to remove spelling and grammatical errors.

• **Protocol Detail:**
1) 3.9: What concentration of pen-strep?

We clarify that 100X P/S is used for a final concentration of 1X (lines 168-171).

• **Discussion:** JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please ensure that the discussion covers the following in detail and in paragraph form (3-6 paragraphs): 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol.

We have focused our discussion on the method and specifically cover 1) modifications and troubleshooting (lines 336-343), 2) limitations of the technique (lines 326-334), 3) significance with respect to existing methods (lines 345-363), 4) future applications (lines 365-373), and 5) critical steps (lines 336-343).

• **Figures:**
1) Please add scale bars to all panels in Fig 1, 2, 4.
2) Fig 3: define error bars.

Scale bars have been added to Figures 1, 2 and 4. Error bars have been defined as standard deviation within the figure legend (line 318-319).

• **References:**Please spell out journal names.

We have spelled out full journal names.

• **Commercial Language:** JoVE is unable to publish manuscripts containing commercial sounding language, including trademark or registered trademark symbols (TM/R) and the mention of company brand names before an instrument or reagent. Examples of commercial sounding language in your manuscript are Matrigel, dispase.

We have removed the use of Matrigel from the summary, abstract, introduction, representative results and discussion and now refer to it as a basement membrane matrix (lines 27, 35, 56, 65, 67, 355-362). However, we have left it

within the protocol itself as it describes a specific product that contains about 94% laminin 1, 4% type IV collagen and 2% proteoglycans, in addition to a number of growth factors. It is commonly used as a coating gel of tissue culture plates in the ES cell field and we do not know how the cells would grow using a substitute, which would not have the same composition. We noticed that over 30 *JoVE* publications use the term Matrigel in their abstracts by pubmed. Please let us know if this is a suitable compromise or if you have another suggestion to how to refer to it within the protocol.

Similarly, dispase is the name of an enzyme, which cleaves fibronectin, collagen IV and to a lesser extent collagen I. Many different companies sell dispase including Stem Cell, Sigma-Aldrich, and Thermo-Fisher. We also found dispase in nearly 10 abstracts from *JoVE*. Nonetheless, we defined it as a neutral protease the first time it is mentioned (line 98) and keep it as a lower-case word so it is not confused as a specific product name. Again, if you have another suggestion as how not to refer to the enzyme we used, please let us.

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Figure 3 is the only one that has been modified from a previous publication. The other images, while similar to previous images, are new. We will attain a letter from the editor upon conditional acceptance of the manuscript.

Comments from Peer-Reviewers:

Please note that the reviewers raised some significant concerns regarding your method and your manuscript. Please revise the manuscript to thoroughly address these concerns. Additionally, please describe the changes that have been made or provide explanations if the comment is not addressed in a rebuttal letter. We may send the revised manuscript and the rebuttal letter back to peer review.

Reviewer #1:

Boisvert et al. describe a protocol to make human brain organoids out of H9 hESCs and attempt to characterize the system using histology and microscopy or gene expression

analyses. The study is interesting and the area of brain organoids is still growing, and no doubt we are still in the beginning of using this valuable system to model neuropsychiatric and other diseases. However the manuscript needs significant revisions and there are some comments (below) to address for it to be suitable for publication and to be useful to the field.

I am not sure what is the value of a system that gives an organoid size (1-2 mm by 10 wks) that is at best ½ of what people have achieved already in the literature like 4-5 mm.

We agree that size is one criteria that should be considered when choosing among different protocols for the system that is best for the investigator's question, which is why we originally included this point (lines 77-81). However, size is not the only criteria that should be used to determine usefulness. Differentiation state and diversity of cell types, responsiveness to perturbations, comparisons to animal studies, ease of use, cost, and risk of contamination should should also be considered. As some of the largest organoids have necrotic centers, some applications might benefit from smaller organoids with less necrosis.

Step 1.3 of the protocol, what is the point of transitioning from low to high oxygen incubators? Is there any point even in growing the stem cells in low oxygen? Please explain and clarify in the text.

When the Yale Stem Cell Center (YSCC) Core Facility in opened in 2006, low oxygen incubators were utilized for hESC growth under the reasoning that in the blastocysts these cells reside in an anaerobic environment. Although a few papers have since shown that their hESC grew equally well in 21% and 5% oxygen, the YSCC continued to grow their cells at 5% oxygen since they tended to grow a little faster without any major differences to differentiation. Although we have not tested if this is a critical factor in our protocol, it is possible that the transition of the cells from low to high oxygen may more closely resemble the natural differentiation process within a developing embryo. In fact, oxygen tension has been shown to be important in both in vitro and in vivo rodent studies as well as human clinical studies. We now add this discussion to the text (lines 333-334).

Please make a schematic figure to describe your procedure of generating organoids per your protocol, sort of summarize the method schematically.

As requested, we have added a schematic to Figure 1.

Step 3.4, Line 141, which kind of B27, with or without vitamin A? Per experts in the field, removing retinoic acid is required earlier (to prevent the stem cells going into the wrong fate (see papers on the role of RA on neuroectoderm or neural tube development, which same concept applies to differentiating organoids in vitro)), and adding it later is good for best neuronal maturation later.

As listed in the table of materials, we used B27 supplement from Gibco (Cat. No. 17504-044), which is without Vitamin A. While there is good support in using RA in 2D cultures to attain specialized neurons from embryoid bodies, we were interested in seeing what develops innately in 3D organoids. In addition, we reasoned that unlike in 2D cultures where each cell is equally exposed to the added growth factor, a 3D structure would experience a poorly controlled gradient with higher concentrations on the outside compared to the inside of the organoid.

Do you have an idea about the stepwise development of organoids per your protocol? Like what stage is ectoderm or neural epithelium or mature neurons? That'd be very useful to clarify in the MS.

As we report in the protocol, we have an initial appreciation for when the neural epithelium is formed as we are using very similar steps as previous work to make the EBs, when "neural tube" like structures are first seen (Figure 1, Day 8), and when more mature and ventral genes are expressed (Figure 3). Although the beginning steps are quite similar to protocols which have well characterized the neural epithelium (Xia, X and Zhang, S.C. Differentiation of Neuroepithelia from Human Embryonic Stem Cells *Methods Mol Bio* 2009; 549: 51-58 and Hu, B.Y., and Zhang, S.C. Directed Differentiation of Neural-Stem Cells and Subtype-Specific Neurons from hESCs *Methods Mol Bio* 2010; 636: 123-37) we agree that a more detailed investigation regarding differentiation can be explored with our method; however we feel like it is beyond the scope of this report.

I see you start induction with bFGF and then you gradually withdraw it. But I see that your medium has B27 and N2 even from the beginning. Usually these are required at later stages of organoid development (after you remove the bFGF completely), they have factors to help induce and mature the neurons , but as long as you have bFGF the organoid is still in the EB (embryoid-body)-like, stem cell-rich, stage, and these factors are thus pointless at this stage.

We agree and now clarify in the schematic added to Figure 1 that there is no B27 or N2 in the mTESR1 medium used as the base media up to day 5, while we are

weaning the cells off of bFGF. On day 5, we removed the bFGF completely and switch to neural induction media (NIM), which does contain B27 and N2.

Step 3.7, your neuronal medium lacks important factors for neuronal maturity like neurobasal (works better than DMEM/F12 for neurons) and importantly B27 which is rich in >20 factors that enhance neuronal functions. I am thus afraid that your system may be giving rise to immature neurons, especially in the absence of matrigel here which for sure helps improve neuronal maturation.

The marker gene expression shown in Figure 3 supports the development of mature neurons within this system. In our initial approach, for these concerns we compared a 50/50 Neurobasal base media, but did not observe significant differences. Martigel is a very undefined product with known batch to batch variability in associated growth factors (Hughes CS et al. Proteomics 2010). In the past, as a laboratory focusing on the extracellular matrix, we have purified type IV collagen and laminin from the EHS tumor and reconstituted them in the desired ratios to generate gels of differing properties. We look upon not using Matrigel, a poorly defined product, in the preparation as an advantage.

"Note: If there are any large clusters of cells or organoids that are much larger than the others, they should be removed from the culture." Please describe the expected and acceptable size range at this and other steps of the protocol. Please describe to people new to the field how to measure the size. Please define clearly and specifically the size range and all possible criteria to rule out failed organoids from the rest of the protocol or from the analysis.

We now describe our estimation of size (lines 160-163). We have included images with scale bars for readers to gain insight into the approximate expected size at various time points.

Step 3.9, please state the maintenance medium at this step here, and for how long can you maintain the organoids in it? And how often to change it?

We now specify that we used the NIM media, feeding every other day for up to 6 months (lines 168-171).

Fig 1 and 2 please describe the microscope type used for each figure.

We now describe that the images in figure 1 were taken with a Nikon TMS-F (lines 307-308) and the images in figure 2 were taken with an Olympus BX43 (line 314)

Step 4.5, for the qPCR assay, please state the primer and probe sequences, including their sequences, vendor, and where in the transcript (which exons Or exon-exon junctions they recognize), and amplicon size. Tabulated form would be very clear to the readers. Is this intercalating-dye (SYBR)-, or probe-based assay? Please clarify in the paper.

The qPCR assay was completed using SYBR-green as now mentioned (line 317). Primer information is placed in Table 1.

Step 5.5, I am perplexed by the extensive fixation durations (days to a week)!! Why is that? Wouldn't standard fixing conditions (like 16-24 hr) just do the job? If a mouse brain can be sufficiently fixed in 16-24 hr, then I assume an organoid, which is tremendously smaller, should be doable by the same conditions. Please explain and clarify in the paper.

We agree that 16-24 hours may be sufficient, however we increased the incubation time to ensure full fixation and did not test this variable. We have added the possibility of shorter fixation times to the protocol as we do not believe this is a critical step (lines 204-206).

Step 5.7.1, Instead of dry ice (which freezes an organoid in like 10 min), wouldn't it better to use liquid nitrogen, or ethanol-dry ice mix which can freeze it down in under a minute to aminute? Quick freezing is good for later analysis and histology results.

The longer time to freeze the organoids is helpful to ensure proper orientation of multiple organoids and less waste when cutting on the cryostat. However, we agree that if one can perform these tasks more quickly that other methods of freezing are acceptable. We have added this explanation to the protocol (lines 226-228).

Representative Results of Fig 1, line 259, what are those "rosette like structures"? Please explain and clarify to the readers in the paper.

We have added a brief description of neural rosettes and refer the reader to an excellent review on the topic (lines 271-273).

Line 264-5: I am not sure if there is anything in the Fig2B that shows "an organization similar to the human cortex"? You have not stained for cortical layer markers, like many in the field did- All I can see there is just a cluster of blue cells.

Line 265-8: your red, blue, orange and green arrow-heads!! How can we see those are neurons or glia or whatever kind of cell types you're referring to? Please remove this claim or stain for markers of these cell types and show high-magnification, zoomed-in, images showing clear staining and morphology for any cell type you claim there to be. Otherwise this whole paragraph is just a vast overstatement. Likewise, Lines 267-8, your claims about potential synapse cannot be justified by the images shown in Fig2C. Others in the field have analyzed synapses and they show clear zoomed-in images with one or few cells and clear punctated structures that stain for synaptic markers (you can pubmed "organoids, synapses" and you'll find several papers have done that). Even with confocal miscroscopy and staining for specific synaptic markers, I am not even sure if you can see synapses at 40x mag power which is used in Fig2C (yes, you'll get a

signal or brightness at 40X but to make out individual synapses you'll need 63-100x mag power).

We appreciate that immunofluorescent analysis of organoids is a useful technique to further evaluate the organoids, which we include in Figure 3. However, we also believe that standard histologic analysis is a useful adjunct when performed by an experienced neuropathologist. We have modified our language to avoid overstatements, by qualifying that these are histologic morphologies of cells that in the experience of our pathologist resemble glia, neurons etc. Since we do not follow-up on the histologic appearance of synapse formation by other methods, we have removed this statement from the text (lines 277-279).

Fig 3, please indicate that the time unit for the x-axis label "time point" is days. Please correct the typo 'vglu1" to "VGLUT1"; for all the genes use the correct designation for human genes, all capital and italics.

We changed the labels on Figure 3 to "VGLUT1" and "Time point (weeks)"

Fig 4, Foxg1 staining is not punctated on single cells (like Sox2 for instance), I am afraid this is just background, especially many commercial antibodies for this marker don't work well. Please check or confirm with another marker or another antibody. It is also difficult to see GFAP or Hopx staining, perhaps you could add a high-mag inset to the figure

The Foxg1 antibody that we used was Ab18259, from Abcam. Based on benchsci.com over 22 published papers use this antibody for immunofluorescence including papers on cerebral organoids (Lancaster MA and Knoblich JA, Nat. Protoc. 2014). Additional images (including stains for GFAP and HopX) can be found in our previous publication (Boisvert EB et al. Cell Death Dis 2019).

Line 285, perhaps you mean confined not refined.

We changed to "confined", now on line 298.

Discussion, Line 325, you have not stained for markers of necrosis or apoptosis to tell whether or not there is reduced cell death by either mechanism of the two. "looked brighter" doesn't necessarily mean no dead cells in there, nor is looking bright the test for apoptosis or necrosis. Please remove this part, or if you wanna keep the cell-death claim please provide scientific evidence like histochemical staining or immunoblotting for death markers.

We have added in a reference for immunohistochemical staining for cleaved caspase-3 and phosphor-MLKL that was previously performed (lines 350 – 352).

Lines 328-333, absence of matrigel has, to the best of my knowledge, negative effects on neuronal maturity and synapses and synaptic function.

Within the discussion we have taken a more conservative approach to emphasize that each variable can have distinct benefits and detractions. While Matrigel has been used extensively in the field to support neuronal growth, it's effects and mechanism of action have not been extensively studied. Are the "beneficial" effects due to the ECM or to the growth factor cocktail? As discussed earlier, what has been analyzed is a batch to batch variability in the composition of Matrigel (Hughes CS et al. Proteomics 2010), which may explain the variability in cell and organoid culture systems that use it. We have added a sentence to the discussion to acknowledge the positive aspects of Matrigel (lines 358-361).

Line 336, I am afraid your system is not the best organoid system to study neurodevelopmental disorders, given all the possible effects on neuronal maturity and synaptic development I described above, like no matrigel, which besides its scaffold and basement-membrane kind of purpose, also provides important factors for neuronal maturity I believe. And like no neurobasal medium. If there are other diseases (like ischemia, hypoxia, infection, hypoglycemia ..etc.) that you feel your system can fit better than neuropsychiatric diseases per see, you can highlight those.

All model systems are approximations with no *in vitro* system that mimics *in vivo* perfectly. However, we have demonstrated correlation of this *in vitro* system (Boisvert et al. *Cell Death Dis* 2019) with our previously published *in vitro* and *in vivo* modeling of hypoxic effects (Li Q et al. *Am J Pathol* 2015). Different systems with or without Matrigel each have their advantages and disadvantages. We only mention neurodevelopmental disorders among several other diseases and insults that one could explore in this system (e.g. hypoxia, hyperglycemia, infection, tumorigenesis). In no way do we mean to detract from the other potential approaches that an investigator may try, but to suggest that this is another variant to consider. As such, we have modified our concluding statement to emphasize that this is one method with its own benefits (lines 370-373).

Fig1 legend, please state in the legend what the arrows in the figure point to.

We now specify in the legend that the arrows point to neural rosettes (line 307).

Reviewer #2:

Manuscript Summary:
Boisvert, et al. introduced a method to generate brain organoids from human pluripotent stem cells and validate the neural phenotypes with bright field images, histology, immunofluorescence, and qRT-PCR. The authors are suggested to address the following issues.

Major Concerns:
1. The sizes of the spheroids should be controlled within a normal distribution range rather than rely on removal of unintended aggregates which decreases the output and causes waste of the reagents.

As discussed above, we now describe our estimation of size (lines 160-163). In practice, unintended aggregates occurred <1% of the time and do not amount to a large loss of reagents.

2. The method should be verified on at least two ESC and iPSC lines to demonstrate the potential for wide applications.

Different cell lines are known to have different proclivities for differentiation. While we agree that trying additional cell lines within this system is important, we feel like it is beyond the scope of this paper. However, since H9 is one of the most widely used hES cell line, especially for neuronal differentiation, we believe that this protocol will be useful for many groups.

3. Markers for pluripotency and other germ layers should be tested in the assays as controls to prove the specific differentiation to the neural ectoderm.

Although we show that the pluripotency marker, Sox2, decreases as the organoids mature (here and Boisvert EM et al. *Cell Death Dis* 2019), we agree that this can be better explored in future work. Using single cell RNA-sequencing, Quadrato et al. found that their 6 month old brain organoids contained a cluster of 3,027 cells (out of ~ 66,000 cells; ~5%) that expressed mesodermal markers (Quadrato G et al. *Nature* 2017). Therefore, we suspect that differentiation of other germ layers does occur to a certain extent, which be analyzed by single cell analysis in future work.

Minor Concerns:
1. Scale bars should be added in all the figures.

Scale bars have been added to Figures 1, 2 and 4.

2. Panels in each figure should be labelled alphabetically instead of using colour box.

We have labelled each image in Figure 1 alphabetically.

3. Colour contrast and brightness should be adjusted for consistency in figure 2.

We have adjusted the contrast and brightness of the different panels in figure 2 using white balance.

4. Explanation should be provided for using DMEM/F12 as the basal medium instead of a neural basal medium.

As discussed above, in our initial approach we compared a 50/50 Neurobasal base media, but did not observe significant differences.

We thank both reviewers and the editors for your careful and thoughtful review of our work. We believe that the changes made have improved the manuscript and appreciate your important contributions.

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If you have any questions or concerns, please feel free to contact me directly.

Kind regards,

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Dear Editor,

I would like permission to reuse data for a new article from one of my figures published in Cell Death and Disease. The original data (attached as CDD figure) can be found in figure 3 of Boisvert, E. M., Means, R. E., Michaud, M., Madri, J. A., Katz, S. G. Minocycline mitigates the effect of neonatal hypoxic insult on human brain organoids. Cell Death and Disease. 10 (4), 325, (2019). The new figure (attached) is for a methods paper under review at JoVE: Boisvert E. M., Means, R. E., Michaud, M., Thomson, J. L., Madri, J. A., Katz, S. G. A Static Self-Directed Method for Generating Brain Organoids from Human Embryonic Stem Cells. The figure legend will say: " This figure has been modified from [citation]", with the citation to our original paper. Thank you for your consideration,

--sam

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