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TITLE:

Brain Organoid Generation from Induced Pluripotent Stem Cells in Home-Made Mini Bioreactors

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SUMMARY:

Here we describe a protocol for generating brain organoids from human induced pluripotent stem cells (iPSCs). To obtain brain organoids in large quantities and of high quality, we use home-made mini bioreactors.

ABSTRACT:

The iPSC-derived brain organoid is a promising technology for *in vitro* modeling the pathologies of the nervous system and drug screening. This technology has emerged recently. It is still in its infancy and has some limitations unsolved yet. The current protocols do not allow obtaining organoids to be consistent enough for drug discovery and preclinical studies. The maturation of organoids can take up to a year, pushing the researchers to launch multiple differentiation processes simultaneously. It imposes additional costs for the laboratory in terms of space and equipment. In addition, brain organoids often have a necrotic zone in the center, which suffers

from nutrient and oxygen deficiency. Hence, most current protocols use a circulating system for culture medium to improve nutrition.

Meanwhile, there are no inexpensive dynamic systems or bioreactors for organoid cultivation. This paper describes a protocol for producing brain organoids in compact and inexpensive home-made mini bioreactors. This protocol allows obtaining high quality organoids in large quantities.

INTRODUCTION:

Human iPSC-derived models are widely used in the studies of neurodevelopmental and neurodegenerative disorders[1]. Over the past decade, 3D brain tissue models, so-called brain organoids, essentially complemented traditional 2D neuronal cultures[2]. The organoids recapitulate to some extent the 3D architecture of the embryonic brain and allow more precise modeling. Many protocols are published for the generation of organoids representing different brain regions: cerebral cortex[3,4,5], cerebellum[6], midbrain, forebrain, hypothalamus[7,8,9], and hippocampus[10]. There have been multiple examples of using organoids to study human nervous system diseases[11]. Also, the organoids were implemented in drug discoveries[12] and used in studies of infectious diseases, including SARS-Cov-2[13,14].

The brain organoids can reach up to several millimeters in diameter. So, the inner zone of the organoid may suffer from hypoxia or malnutrition and eventually become necrotic. Therefore, many protocols include special bioreactors[8], shakers, or microfluidic systems[15]. These devices may require large volumes of expensive cell culture media. Also, the cost of such equipment is usually high. Some bioreactors consist of many mechanical parts that make them difficult to sterilize for reuse.

Most protocols suffer from the "batch effect"[16], which generates significant variability among organoids obtained from the identical iPSCs. This variability hinders drug testing or preclinical studies requiring uniformity. The high yield of organoids enough to select organoids of uniform size may partially solve this problem.

The time factor is also a significant problem. Matsui et al. (2018) showed that brain organoids require at least six months to reach maturity[17]. Trujillo et al. (2019) also demonstrated that electrophysiological activity occurred in organoids only after six months of cultivation[18]. Due to the long organoid maturation time, the researchers often launch new differentiation before completing the previous one. Multiple parallel processes of differentiation require additional expenses, equipment, and laboratory space.

We have recently developed a mini bioreactor that mainly solves the problems mentioned above[19]. This home-made bioreactor consists of an ultra-low adhesion or untreated Petri dish with a plastic knob in the center. This plastic knob prevents crowding of organoids and their conglutination in the center of the Petri dish, which is caused by the rotation of the shaker. This paper describes how this inexpensive and simple home-made mini bioreactor allows generating high-quality brain organoids in large quantities.

PROTOCOL:

NOTE**:** Use sterile technique throughout the protocol, excluding steps 1.2 and 1.3. Warm all culture media and solutions to 37 °C before applying to cells or organoids. Cultivate cells in a CO_2 incubator at 37 °C in 5% CO_2 upon 80% humidity. The protocol scheme is shown in **Figure 1.**

1. Transforming Petri dishes into mini bioreactors

1.1. Cut sterile 15 mL centrifuge tubes in rings of 7-8 mm in height; autoclave the rings.

1.2. Break low-adhesion, untreated or microbiological Petri dishes into crumbs. Dissolve about 1 g of plastic crumbs in 10 mL of chloroform overnight to prepare liquid plastic.

CAUTION: Work in a fume hood.

1.3. Check that the resulting liquid plastic is viscous enough for pipetting; its drop retains a spherical form and does not spread on the surface. If it is very liquid, add more plastic crumbs. If it is thick, then add chloroform.

1.4. Make a plastic knob in the center of a sterile ultra-low adhesion 6-cm Petri dish. There are two equally suitable ways, as detailed below.

1.4.1. Put the autoclaved plastic ring on the center and drop the liquid plastic to the inside of the ring.

1.4.2. Without any plastic ring, drop the liquid plastic on the center of the Petri dish.

1.5. Leave the dishes open for 2-3 h in a laminar flow hood until dried complete. Treat the dried dishes with ultraviolet radiation for 15-20 min.

2. Induction of neuronal differentiation of iPSCs

2.1. Cultivate iPSCs in the medium for pluripotent stem cells up to 75-90% confluence in 35 mm Petri dishes precoated with a matrix consisting of extracellular proteins.

2.2. Prepare medium A-SR. See **Table 1** for details.

2.3. Aspirate the cultivation medium and add 2 mL of A-SR medium at Day 0 of differentiation.

2.4. Prepare medium A (see **Table 2**).

2.5. Cultivate cells in medium A for two weeks from Days 2-14, refreshing medium in Petri dishes every other day.

3. The formation of spheroids from neuroepithelial precursor cells at Day 14

3.1. At Day 14, make spheroids from neuroepithelial precursor cells using a special 24-well culture plate containing approximately 1,200 microwells in each well (**Figure 2C**). Follow the procedure given below.

NOTE: At this differentiation stage, a 35 mm Petri dish usually contains 3 – 3.5 x 10^6 neuroepithelial precursor cells. Thus, one 35 mm Petri dish with neuroepithelial precursor cells is sufficient for 3 – 4 wells of 24-well culture plate with microwells.

3.2. Prepare a 24-well culture plate with microwells: To each well, add 1 mL of medium A. Centrifuge briefly at 1,300 x *g* for 5 min in swinging bucket rotor fitted with plate holder. Control under the microscope that there are no bubbles in microwells.

3.3. Prepare the medium B (**Table 3**).

3.4. Remove the medium from the Petri dish with neuroepithelial precursor cells; wash the cells with 2 mL of DMEM/F12. For the cell detachment, treat the cells with 1.5 mL of 0.48 mM EDTA solution prepared in PBS. Control the cell detachment under the microscope.

3.5. Harvest the cells into a 15 mL tube. Add 5 mL of DMEM/F12 in the tube to wash the cells. Centrifuge at 200 x *g* for 5 min. Remove the supernatant and resuspend cells in 2 mL of medium B.

3.6. Check the cell concentration and viability by Trypan Blue staining and a hemocytometer. Calculate the volume of suspension needed to contain 1 x 10^6 viable cells in total.

3.7. Transfer the cell suspension containing 1 x 10^6 cells into each well of a 24-well plate with microwells. Add medium B up to 2 mL into each well, gently pipette cells up and down several times, and centrifuge briefly 100 x *g* for 1 min to capture cells in the microwells.

NOTE: The number of cells per well should not exceed 1 x 10^6. Otherwise, spheroids from neighboring microwells fuse.

3.8. Check under the microscope that cells are evenly distributed in microwells. Repeat pipetting and centrifugation if cells are distributed unevenly.

3.9. Incubate the plate overnight to let cells aggregate in spheroids.

4. Obtaining and cultivation of organoids

4.1. The next morning (Day 15), check the quality of spheroids under the microscope. Ensure that they are transparent and smooth, if healthy (**Figure 2A,B**). Carefully collect the spheroids from each well into a 15 mL tube, leave the spheroids to precipitate by gravity for 2-3 min, and then remove the supernatant.

4.2. Add to the spheroids 2 mL of the matrix thawed during the same time on ice. Mix gently by pipetting and incubate at room temperature for 30 min.

4.3. To wash excess of the matrix, add to the tube 8 mL of medium B. Pipette gently, then centrifuge the tube for 1 min at 100 x *g*.

NOTE: Do not exceed the time and the speed of centrifugation to avoid the irreversible aggregation of spheroids.

4.4. Remove the supernatant. Add to the tube 20 mL of medium B, pipette gently. Split the spheroid suspension between two mini bioreactors. Place the mini bioreactors into a 15 cm Petri dish to prevent the evaporation of water and to avoid contamination.

4.5. Put the Petri dish with mini bioreactors on an orbital shaker. Cultivate the organoids at a rotation rate of 70-75 rpm.

4.6. On Day 16, prepare medium C (**Table 4**).

4.7. Transfer the organoids into 15 mL tube. For 5 min, let them fall to the bottom, aspirate the supernatant, add 5 mL of medium C. Return the organoids into the mini bioreactors.

NOTE: Be careful not to lose the spheroids, which are transparent and barely visible.

4.8. Cultivate spheroids in medium C for two weeks, refreshing the medium every two days. At the end of these two weeks, leave about 100 spheroids per mini bioreactor for the following cultivation. Freeze excessive spheroids in a freezing medium in liquid nitrogen.

4.9. On Day 30, prepare medium D (**Table 5**).

4.10. Change the cultivation medium to medium D, which is a maturation medium. Refresh cultivation medium every 2-3 days for three weeks, then use medium D without BDNF and GDNF.

REPRESENTATIVE RESULTS:

The protocol scheme is shown in **Figure 1**. The protocol included five media in which iPSCs differentiated into brain organoids during at least one month. The differentiation was started then iPSCs reached the 75-90% confluence (**Figure 2A,B**). The first signs of differentiation towards neurons were observed on days 10-11 of iPSC cultivation in medium A when cells began to cluster into "rosettes" (**Figure 2C**). At days 14-15, iPSCs differentiated into neuroepithelial progenitors.

99% of cells were positive on neuroepithelial marker SOX1 and did not express pluripotent cell markers TRA-1-81 and OCT4 (**Supplementary Figure S1**). We then harvested the cells using EDTA-containing solution and transferred them in medium B to a special 24-well culture plate with microwells (**Figure 2D**). The cells immediately after transfer to microwells, as shown in **Figure 2E**. Each microwell promoted the aggregation of cells into a single spheroid. The spheroids from all microwells were of the same size and contained about 100 cells (**Figure 2J**). After the formation of spheroids, they were coated with freshly thawed matrix and transferred in medium C into home-made mini bioreactors (**Figure 2H**). The mini bioreactors were rotated on an orbital shaker at the rate of 70-75 rpm. The spheroids differentiated into brain organoids, which all grew up evenly, and morphogenesis proceeded identically in all organoids.

The organoids grew during the first three months, then their growth slowed down and eventually stopped. The maximal size of brain organoids cultured for six months was about 6 mm. Larger organoids had a loose central zone, often with cavities or necrotic areas (**Figure 3**). Immunohistochemical staining of cryosections of organoids at d45 of differentiation revealed large clusters of SOX2-positive cells, indicating immature neurons (**Figure 4B**). The two-month-old organoids expressed neuronal and glial markers, such as tyrosine hydroxylase (TH), PAX6, beta-III-tubulin (TUBB3), MAP2, and GFAP proteins (**Figure 4A, 4C**, and **4D**). The maximal cultivation time for organoids of high quality was about ~7 months.

Thus, the formation of spheroids of the identical size followed by the cultivation under dynamic conditions in mini bioreactors results in standard-sized organoids developed through identical morphogenesis.

FIGURE AND TABLE LEGENDS:

Figure 1: The main stages of the protocol. In the beginning, iPSCs are cultivated in a commercial medium for pluripotent stem cells up to 70-95% confluence. The next stage of the protocol consists of two steps. First, for 1-2 days, the medium for pluripotent stem cells is changed to medium A-SR. Second, cells are cultivated in medium A for two weeks. Upon forming rosettes of neuroepithelial cells, the cells are transferred into the culture plate with microwells with medium B to form spheroids. The day after, the spheroids obtained are transferred into mini bioreactors with the medium C. The further maturation of organoids proceeds in medium D.

Figure 2: The key morphological structures observed under the microscope during protocol execution. (**A**) The iPSCs in the low confluence, insufficient for the start of differentiation. (**B**) The iPSCs in the confluence, suitable to launch differentiation. (**C**). The clusters of neuroepithelial progenitors, so-called rosettes before harvesting. (**D**) The empty microwells on the bottom of the culture plate. (**E**) The cells just after seeding in culture plate with microwells. (**F**) After overnight incubation, the cells aggregate in the spheroid in each microwell. (**J**). The spheroid of good quality. H. The spheroids after matrix coating.

Figure 3: Hematoxylin-eosin staining of organoids. (**A-D**) Normal organoids with the dense central zone. (**E**) The organoid with an epithelium-lined cavity. (**F**) The organoid with the necrotic

central zone.

Figure 4: Immunohistochemical staining of organoids. The organoids had clusters of cells expressing markers of neural progenitors (SOX2, B) and neural cells (TH, PAX6, A; TUBB3, C; GFAP, MAP2, D). DAPI was used to stain nuclear DNA.

Table 1: Composition of medium A-SR.

Table 2: Composition of medium A.

Table 3: Composition of medium B.

Table 4: Composition of medium A.

Table 5: Composition of medium D.

Supplementary Figure S1: At d14 of the differentiation, the cell population purity was assessed using flow cytometry and RT-PCR. (**A**) More than 98% of the cells lost a pluripotency marker TRA-1-81. (**B**) Most of the cells (>99%) exhibited a neuroepithelial marker SOX1. (**C**) The expression of POU5F1 gene coding a key pluripotency factor OCT4 decreased drastically in three different iPSC lines (IPSRG2L, IPSPDL2.15L, IPSPDP1.5L).

DISCUSSION:

The described protocol has two crucial steps allowing the generation of high-quality organoids of uniform size. First, the organoids grow from spheroids which are near identical in cell number and cell maturity. Second, the home-made bioreactors provide each organoid a uniform environment, where organoids do not crowd or stick together.

The cell quality and state of cell maturation are essential to perform the protocol. It is critical to start neuronal differentiation at 75-90% confluence of iPSCs. If cell density is too low, the iPSCs can differentiate into non-neuronal directions. It is important not to exceed two weeks of neuronal induction of iPSCs because neuronal progenitors later become more vulnerable. During differentiation, the cells and the organoids should be regularly supplied with the fresh medium because starvation leads to the sharp decline of organoid quality. Only short-term breaks are permitted in dynamic cultivation.

Some modifications of the protocol are allowed. Any inert biological materials can be applied to make a knob in the central part of the mini bioreactor: fluoroplastic, polyethylene, polypropylene. If a Petri dish for microbiology is used to prepare the liquid plastic, then the resulting mini bioreactors should be checked for neuronal cytotoxicity. The Petri dishes of different diameters can serve as a base for the bioreactor. However, then the adjustment of rotation speed is necessary. Also, the rotation speed needs to be adjusted if the volume of the medium is changed. For example, for 8 mL of the medium in a 6 cm Petri dish, the optimal speed is 70-75 rpm.

The recipe of the medium can be reformulated to obtain more mature brain organoids or organoids specific to different brain regions[20]. Also, the culture plate with microwells is suitable for the formation of complex spheroids. For example, it is possible to mix neuronal progenitors with endothelial progenitors to receive a vascularized brain organoid[21]. Other iPSC derivatives can also be aggregated into spheroids in a culture plate with microwells to obtain other organoids: chondrospheres[22], intestinal organoids[23], etc.

The protocol requires changing medium every 2-3 days during organoid maturation, which can take half of the year. So special care should be taken when using sterile techniques. It is allowed to use the prophylactic dose of antimicrobials for the prevention of mycoplasma infection.

The protocol limitation arises from the limited diffusion of oxygen and nutrients into the center of large organoids. Most current protocols for organoid generation suffer from this problem[24]. In our conditions, growth stops then the organoid reaches 6 mm. The organoids of larger size developed necrotic zone in the center. Probably, this problem can be solved using vascularization[21] or hyperoxygenation[25].

In comparison with other bioreactors, home-made mini bioreactors have apparent advantages in terms of cost and affordability. In addition, they are small. We can keep several dozen home-made bioreactors on one orbital shaker in the incubator. It is impossible to maintain these many bioreactors in the incubator when using stirred bioreactors.

In conclusion, the presented protocol is helpful for biomedical and pharmacological studies where in vitro modeling of the human brain is required. We believe that by varying the differentiation media composition, it is possible to obtain brain organoids of different brain regions and different degrees of maturity. Moreover, the use of mini bioreactors is most likely not limited to neural differentiation and, if the protocol is modified, they can also be used to establish other organoids from pluripotent or adult stem cells.

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DISCLOSURES:
The authors have nothing to disclose.

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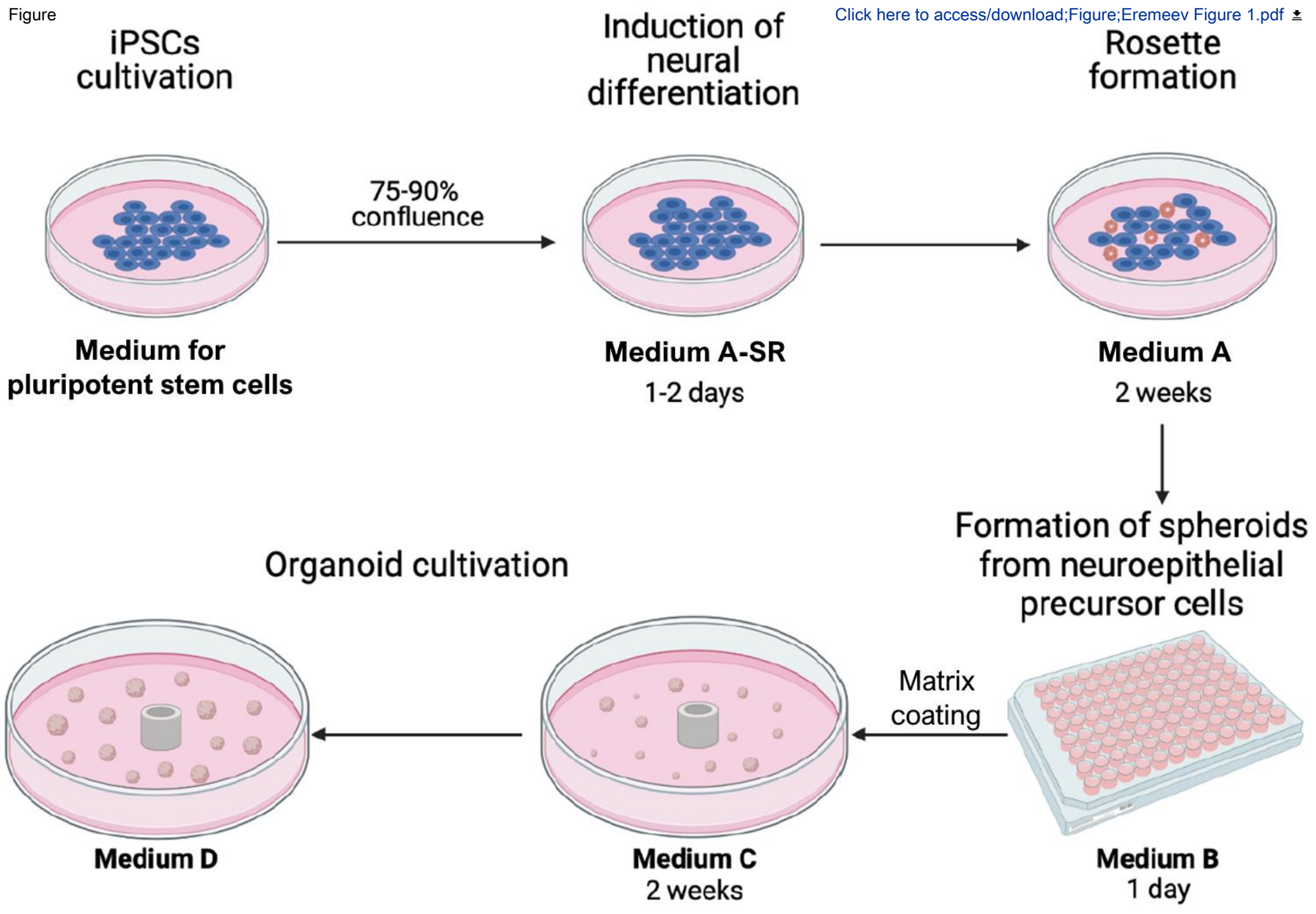
Figure



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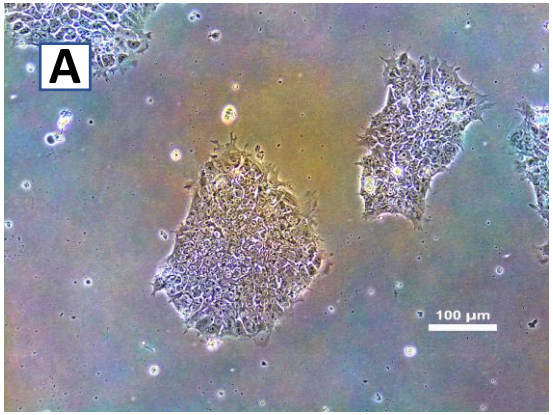

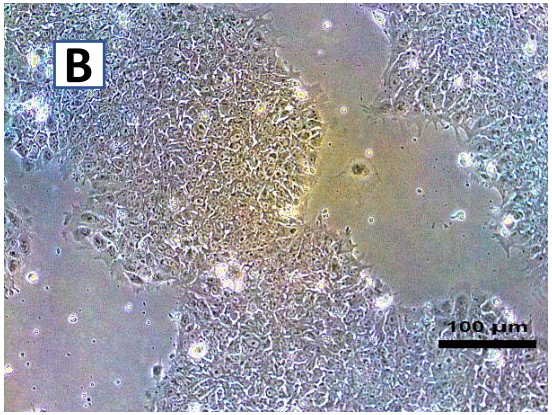

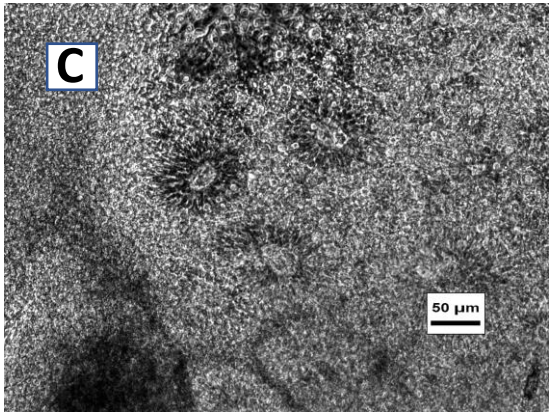

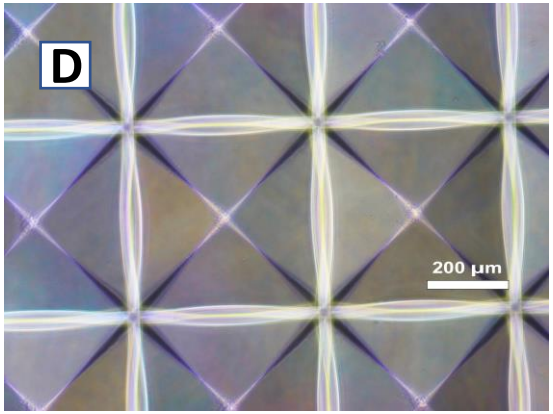

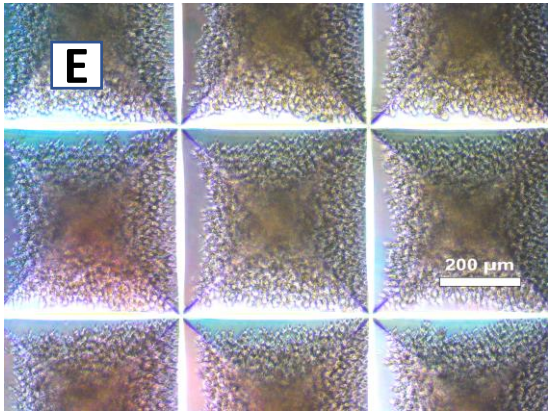

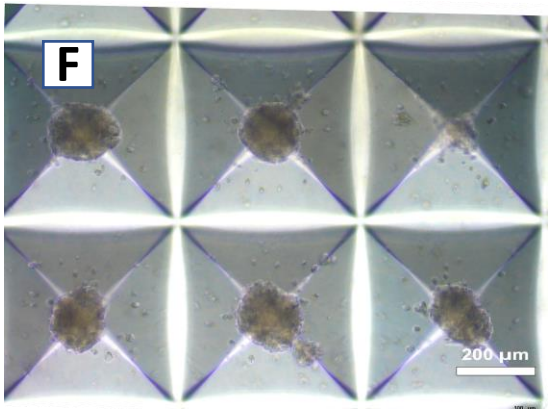

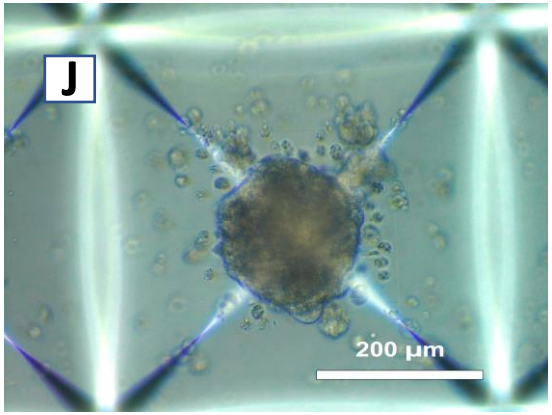

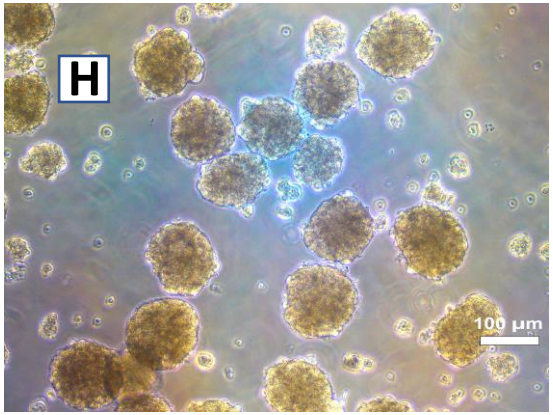

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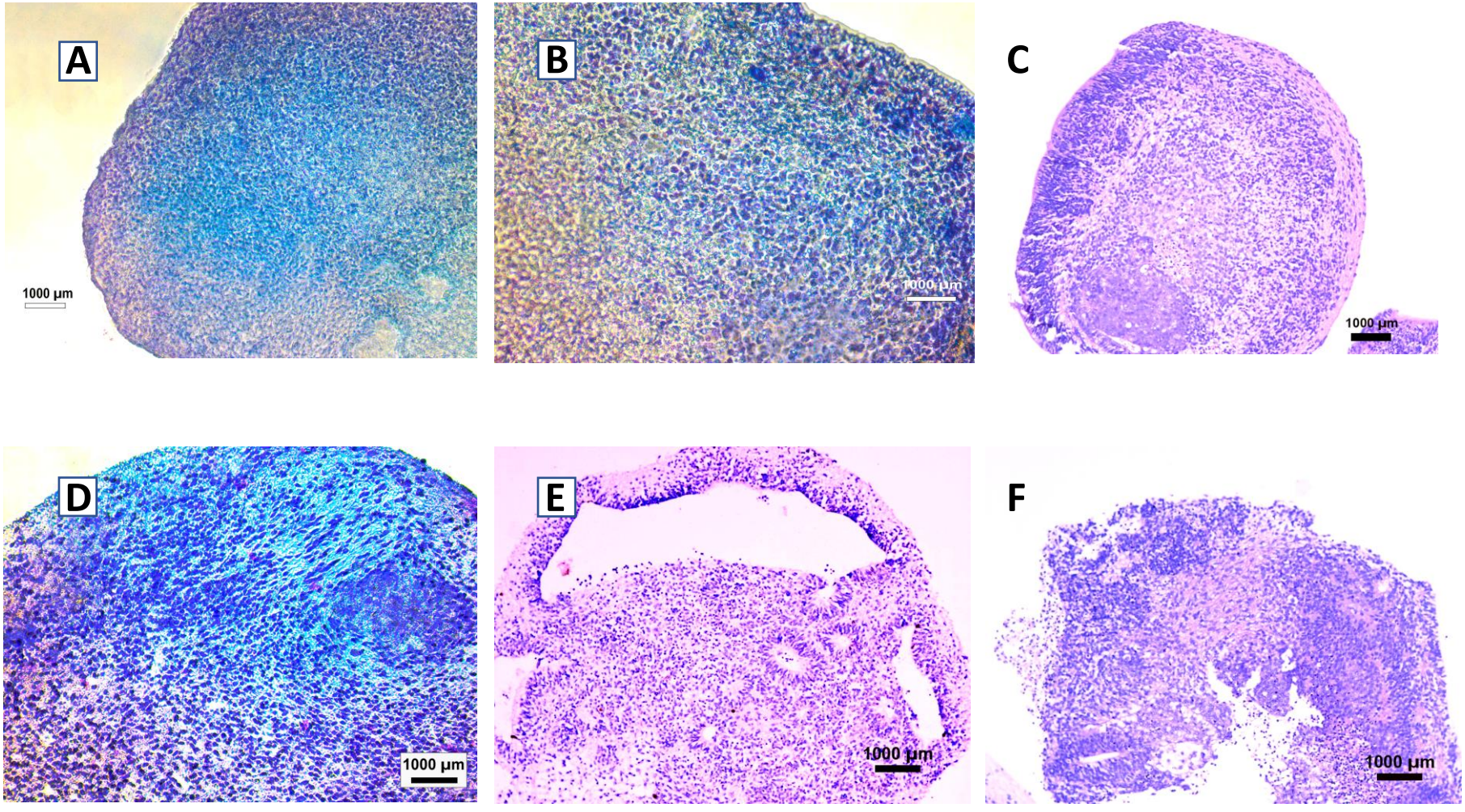

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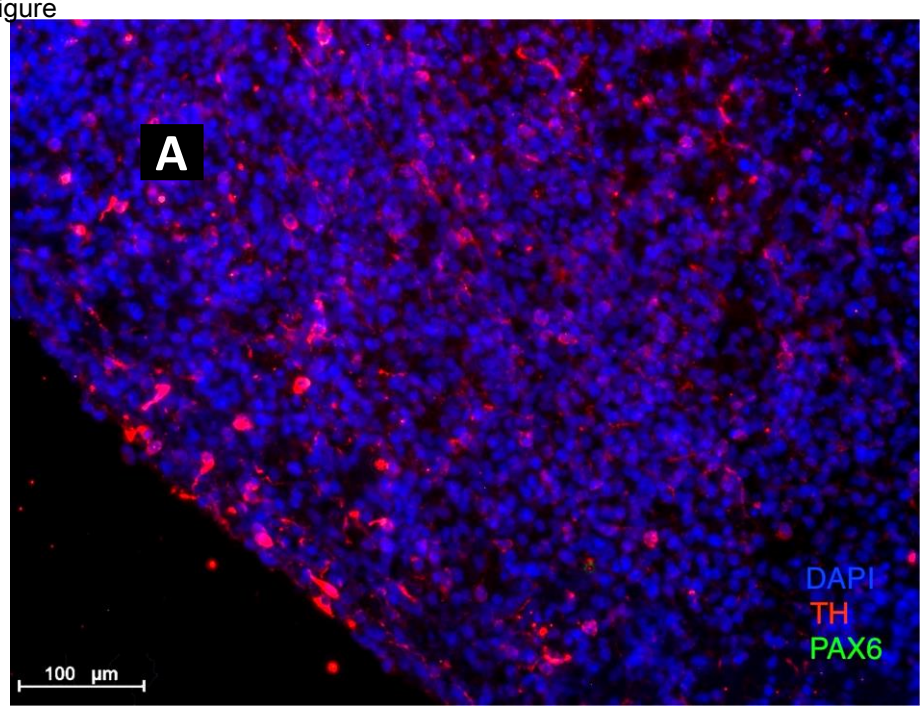

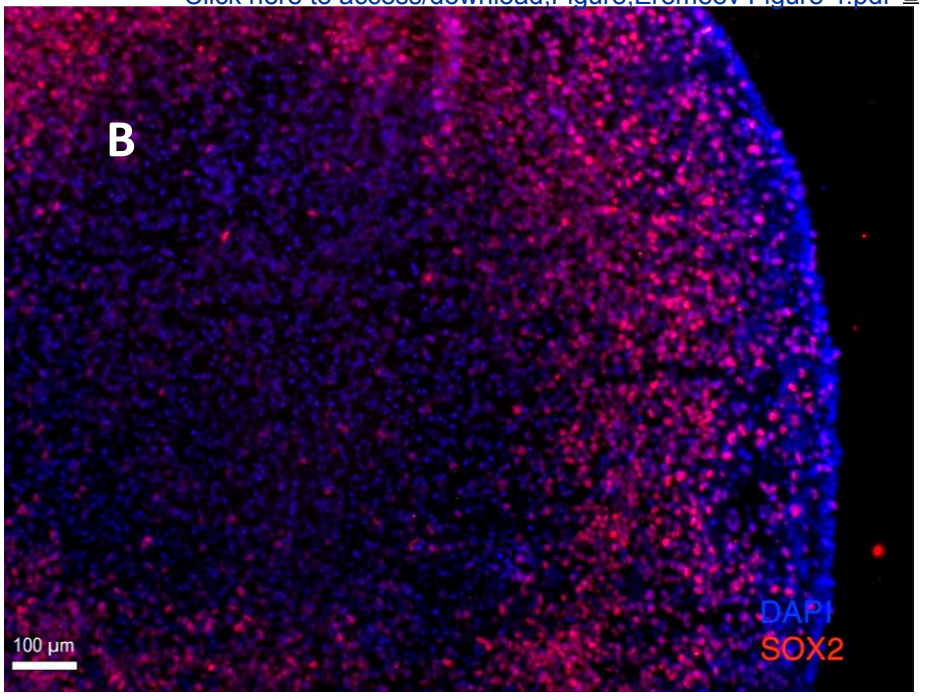

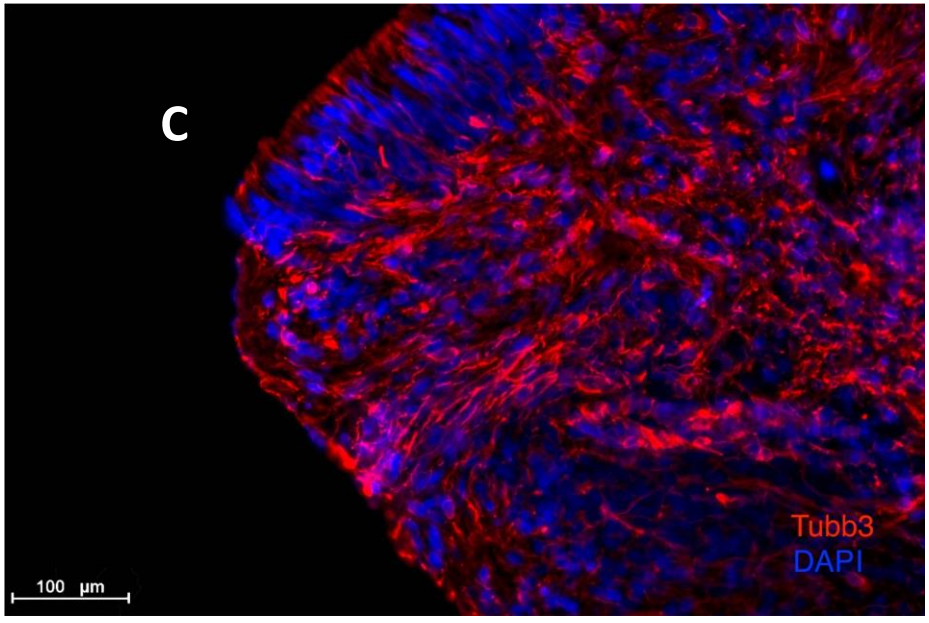

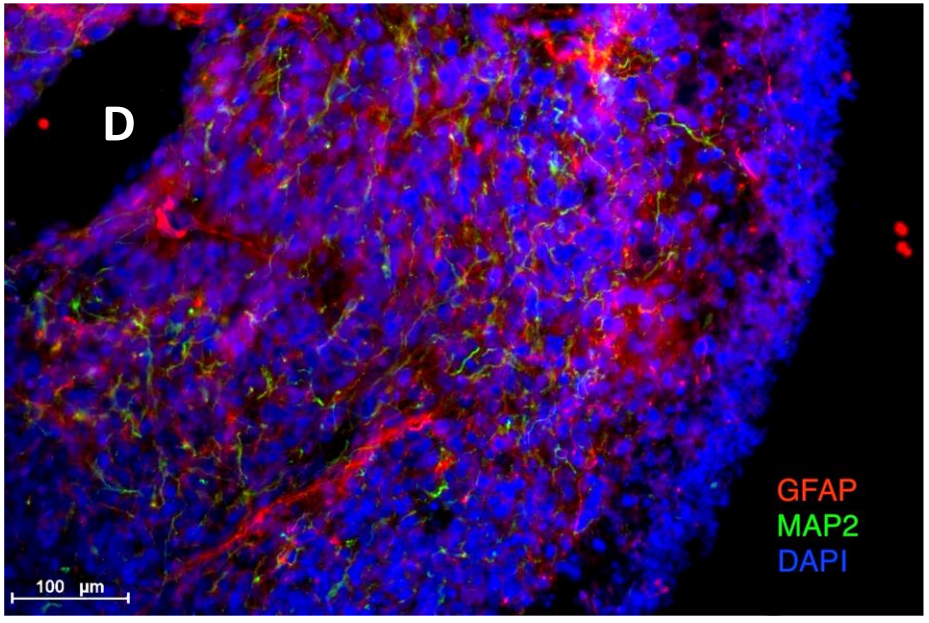

Table 1. Composition of medium A-SR.

Components for medium A-SR	Concentration
DMEM/F12 medium	add up to 100%
Serum Replacement	1%
N2 supplement	1%
Neuronal supplement B	2%
L-alanyl-L-glutamine	2 mM
β-Mercaptoethanol	50 µM
SB431542	10 µM
Dorsomorphin	3 µM
LDN193189	0.1 µM
Penicillin-Streptomycin solution	1x

Table 2. Composition of medium A.

Components for medium A	Concentration
DMEM/F12 medium	add up to 100%
N2 supplement	1%
Neuronal supplement B	2%
L-alanyl-L-glutamine	2 mM
β-Mercaptoethanol	50 µM
SB431542	10 µM
Dorsomorphin	3 µM
LDN193189	0.1 µM
Penicillin-Streptomycin solution	1x

Table 3. Composition of medium B.

Components for medium B	Concentration
DMEM/F12 medium	add up to 100%
N2 supplement	1%
β-Mercaptoethanol	50 μM
SB431542	10 μM
Y-27632	5 μM
Dorsomorphin	5 μM
LDN193189	0.1 μM
Penicillin-Streptomycin solution	1x

Table 4. Composition of medium C.

Components for medium C	Concentration
DMEM/F12 medium	add up to 100%
N2 supplement	1%
Neuronal supplement B	2%
L-alanyl-L-glutamine	2 mM
β-Mercaptoethanol	50 µM
Purmorphamine	3 µM
bFGF	10 ng/mL
Penicillin-Streptomycin solution	1x

Table 5. Composition of medium D.

Components for medium D	Concentration
Basal medium for neuronal cell maintenance	add up to 100%
Neuronal supplement B	2%
L-alanyl-L-glutamine	2 mM
β-Mercaptoethanol	50 µM
BDNF	20 ng/mL
GDNF	20 ng/mL
Penicillin-Streptomycin solution	1x

Dear Vineeta Bajaj,

We are pleased to send a revised version of the manuscript “Brain Organoid Generation from Induced Pluripotent Stem Cells in Home-Made Mini Bioreactors.”

We corrected the manuscript and the video to address editorial comments. In the manuscript, the red color of the font highlights the correction.

All corrections in the manuscript and the video are described below, along with editorial comments highlighted in blue font.

On behalf of all authors,

Alexandra Bogomazova

Editorial comments:

Text manuscript:
1. The editor has formatted the text as per the journal standards. Please retain and use the attached file for revision.
2. Please address all the specific comments marked in the manuscript.

We corrected improper value for volume

Video:
Editing Video and Audio:

- 9:44, 10:00 Please sync the Video and audio in the interview section

We synchronized the video and the audio for this part.

- Capitalize all the keywords in the title main card

We capitalized all letters in the keywords in the title main card.

- The audio does not match the JoVE standard (-12 to -6dB) of the footage

Please Increase the volume during the procedure

We corrected the audio.

Supplemental File (Figures, Permissions, etc.)

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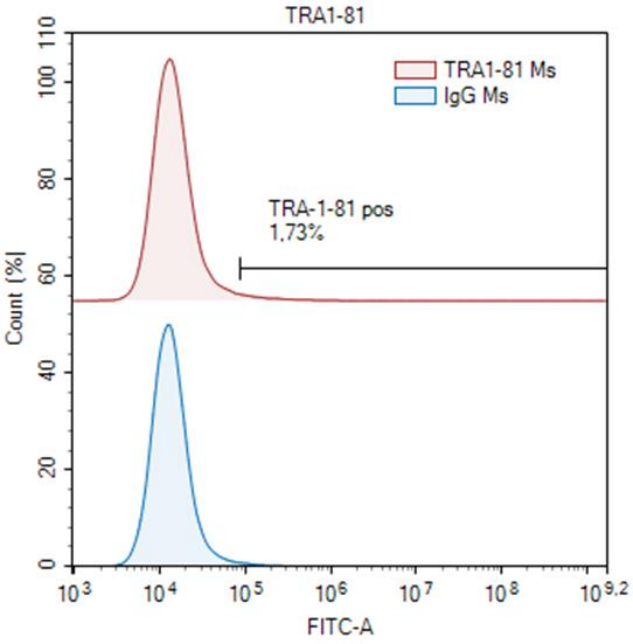
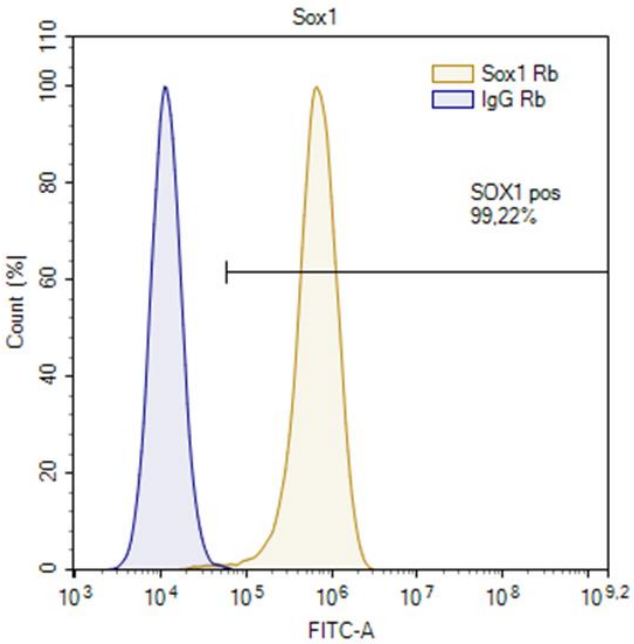
A



B



C