

Reprint Compendium

Bio-protocol Selections 2021

Stem Cell

Free access to more than 4000 high-quality protocols

- Contributed by 10,000+ scientists
- Each validated in at least one primary publication
- >91% reproducibility (2018 survey of Bio-protocol users)
- ~1000 videos of key procedural steps

Foreword

We are pleased to launch the second Bio-protocol series of reprint collections, comprising some of the most used protocols published in 2019 and 2020 in several research areas. This collection focuses on stem cell.

Established in 2011 by a group of Stanford scientists, Bio-protocol's mission is to improve research reproducibility and usability through the publication of high quality step-by-step peer-reviewed life science protocols. Bio-protocol invites contributions from authors who have published methods in brief, as part of other research articles, and who might want to provide more detail to facilitate use by others. A survey carried out in 2018 showed that, of more than 2300 users who had followed a protocol published in Bio-protocol, 91% (2166 users) were able to successfully reproduce the method they tried.

In this reprint collection, we have selected 20 stem cell research protocols published in Bio-protocol in 2019 and 2020 that we consider the most highly used. We base this classification on metrics such as the number of times a protocol was viewed or downloaded by unique users, and the number of citations it has received in other publications.

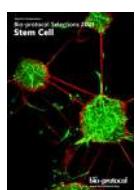
Hopefully, you will find this collection intriguing and visit www.bio-protocol.org to check out the entire archive of protocols. Please feel free to email us (eb@bio-protocol.org) with feedback, and please consider contributing a protocol to Bio-protocol in the future.

The Bio-protocol Editorial Team

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Quantification of Mouse Hematopoietic Progenitors' Formation Using Time-lapse Microscopy and Image Analysis

Isabelle Bergiers^{1,*}, Christian Tischer², Özge Vargel Böyükbaş^{1,\$} and Christophe Lanclin^{1,*}

¹European Molecular Biology Laboratory, EMBL Rome, Epigenetics and Neurobiology Unit Monterotondo, Italy; ²European Molecular Biology Laboratory, EMBL Heidelberg, Advanced Light Microscopy Facility, Heidelberg, Germany; ^{\$}Current address: Department of Stem Cell and Regenerative Biology, Harvard University, Cambridge, MA, USA

*For correspondence: isabelle.bergiers@embl.it; christophe.lanclin@embl.it

[Abstract] *In vitro* differentiation of mouse embryonic stem cells (mESCs) towards blood cells constitutes a well-established system to study the endothelial-to-hematopoietic transition (EHT) at the onset of blood development. Assessing the emergence of small non-adherent round blood cells in the culture without disturbing it is essential to evaluate the progression of EHT and also to test conditions potentially enhancing or repressing this process. Here, we describe how to quantify the formation of mouse hematopoietic progenitors during EHT in normal conditions or following over-expression of eight essential transcription factors using time-lapse microscopy and image analysis.

Keywords: Hematopoietic progenitors, Embryonic stem cells, Differentiation, Inducible cell line, Hemangioblast culture, Microscopy, Time-lapse imaging, Round cell counting

[Background] The first hematopoietic stem and progenitor cells (HSPCs) emerge from endothelial cells in the large arteries of the mouse embryo (de Bruijn *et al.*, 2000; Zovein *et al.*, 2008; Chen *et al.*, 2009;). This evolutionarily conserved event is called endothelial to hematopoietic transition (EHT). As a result of EHT, endothelial cells lose their specific markers, start to express hematopoietic genes, gain round morphology and eventually detach from the endothelial layer (Boisset *et al.*, 2010; Kissa and Herbomel, 2010; Rybtsov *et al.*, 2014). These specific series of events are used to detect EHT activity and monitor the progress of hematopoietic progenitor formation. The widely used method is to quantify endothelial and hematopoietic marker expressing cells. This can be done by gene expression analysis at the single-cell level (Bergiers *et al.*, 2018). Also, protein expression change can be used to assess EHT progression by flow cytometry analysis of endothelial markers such as VE-Cadherin, CD31 and hematopoietic markers such as CD41, CD45, CD43. For spatial information, the same markers can be used for immunofluorescent staining on the fixed tissue. However, all of these techniques require harvesting of the examined cells and therefore represent a single time-point. Besides, the switch between endothelial and hematopoietic gene expressions occurs gradually and in a heterogeneous way so that a marker analysis of a specific time-point is not enough to follow the transition. To complement the gene/protein expression based detection methods, there is a need of a protocol to monitor morphological changes and quantify the round hematopoietic cell progenitors arising through EHT.

To study the hematopoietic cell formation dynamics *in vitro*, researchers developed embryonic stem

cell differentiation protocol (Keller *et al.*, 1993; Kennedy *et al.*, 1997; Sroczynska *et al.*, 2009). The protocol follows the developmental stages of hematopoiesis and recapitulates EHT *in vitro* (Choi *et al.*, 1998; Nishikawa *et al.*, 1998; Palis *et al.*, 1999). Briefly, mouse embryonic stem cells are cultured to form embryoid bodies containing the *in vitro* equivalent of hemangioblast, or blast colony forming cells (BL-CFCs), which is then isolated by cell sorting. Those cells grow and give rise to smooth muscle, endothelial and hematopoietic cells (Keller *et al.*, 1993; Faloon *et al.*, 2000). Time-lapse imaging capturing the BL-CFC culture allows us to visualize cells transitioning into hematopoietic progenitors in real-time. Unlike the endothelial and smooth muscle cells, the cells undergoing EHT become round and bud-off from the endothelial cell layer (Lancrin *et al.*, 2009). Here, by combining *in vitro* time-lapse imaging of an adherent BL-CFC culture with automatic image analysis we introduce a simple and efficient method to quantify those round cells during a culture period, which gives a direct measure of the number of cells undergoing EHT. This protocol enables us to easily test novel parameters affecting EHT rate such as over-expression of certain transcription factors (Bergiers *et al.*, 2018) or testing pathway inhibiting small molecules in the culture media (Vargel *et al.*, 2016). Below, we describe the details of the time-lapse microscopy of BL-CFC culture and image analysis to assess the number of cells underwent EHT.

Materials and Reagents

1. Haemacytometre cover slips (Roth, catalog number: L189.1)
2. BD Falcon Conical Tubes, Polypropylene, 15 ml, high-clarity, dome-seal screw cap (BD Biosciences, catalog number: 352096)
3. BD Falcon Conical Tubes, Polypropylene, 50 ml, high-clarity, flat-top screw cap (BD Biosciences, catalog number: 352070)
4. Costar® 6-well cell culture multiple well plate, flat bottom, with lid (Corning, catalog number: 3506)
5. Millex-GP Syringe Filter Unit, 0.22 µm, polyethersulfone, 33 mm, gamma sterilized (Merck Millipore, catalog number: SLGP033RS)
6. Multi®-safety microcentrifuge tubes, SafeSeal® Tubes (Carl Roth, catalog number: 7080.1)
7. Stericup GP 0.2 µm/150 ml (Merck-Millipore, catalog number: SCGPU01RE)
8. Stericup GP 0.2 µm/500 ml (Merck-Millipore, catalog number: SCGPU05RE)
9. TipOne® 10 µl Graduated Filter Tip (Sterile), Refill (Starlab, catalog number: S1121-2710)
10. TipOne® 1,000 µl Graduated Filter Tip (Sterile), Refill (Starlab, catalog number: S1122-1730)
11. TipOne® 20 µl Graduated Filter Tip (Sterile), Refill (Starlab, catalog number: S1120-1710)
12. TipOne® 200 µl Graduated Filter Tip (Sterile), Refill (Starlab, catalog number: S1120-8710)
13. i8TFs mESC line (Bergiers *et al.*, 2018)
14. D4T endothelial cells (Choi *et al.*, 1998)
15. Ascorbic acid (Sigma, catalog number: A4544)
16. Bovine Serum Albumin (BSA) (Sigma, catalog number: A9418)

17. Doxycycline (Sigma, catalog number: D9891)
18. Fetal bovine serum (FBS) (PAA, catalog number: A15-102)
19. Gelatin (BDH, catalog number: 440454B)
20. IL-6 (R and D, catalog number: 406 ML)
21. IMDM (Lonza, catalog number: BE12-726F)
22. L-glutamine (Gibco, catalog number: 25030-024)
23. Monothioglycerol (MTG) (Sigma, catalog number: M6145)
24. Oxoid™ Phosphate Buffered Saline (PBS) Tablets (Thermo Scientific, catalog number: BR0014G)
25. Transferrin (Roche, catalog number: 10652202001)
26. VEGF (R and D, catalog number: 293-VE)
27. Distilled Water (Thermo Scientific, Gibco™, catalog number: 15230188)
28. 0.1% gelatin solution (see Recipes)
29. 10 mg/ml doxycycline stock solution (see Recipes)
30. 5 mg/ml ascorbic acid stock solution (see Recipes)
31. D4T endothelial cell supernatant (see Recipes)
32. PBS + 0.1% BSA solution (see Recipes)
33. 10 µg/ml VEGF stock solution (see Recipes)
34. 10 µg/ml IL6 stock solution (see Recipes)
35. Conditioned IMDM (see Recipes)
36. IMDM + 20% FBS (see Recipes)
37. MTG dilution (see Recipes)
38. BL-CFC culture medium (see Recipes)

Equipment

1. Falcon® 10 ml Serological Pipet, Polystyrene, 0.1 Increments, Individually Packed, Sterile (Corning, catalog number: 357551)
2. Falcon® 2 ml Aspirating Pipet, Polystyrene, without Graduations, Individually Wrapped, Sterile (Corning, catalog number: 357558)
3. Falcon® 5 ml Serological Pipet, Polystyrene, 0.1 Increments, Individually Packed, Sterile (Corning, catalog number: 357543)
4. 37 °C, 5% CO₂ cell culture incubator (Thermo Scientific, model: Series II Water Jacket)
5. Biosafety cabinet (Tissue culture hood) (Thermo Scientific, model: MSC Advantage)
6. Brightfield inverted Microscope (Leica, model: DMIL LED Inverted)
7. Centrifuge (Eppendorf, model: 5810 R)
8. Computer (Dell Precision T3500)
9. IncuCyte HD (Essen Biosciences)
10. Neubauer counting chamber improved (Roth, catalog number: T729.1)

11. Pipetman P10, 1 to 10 μ l (Gilson, catalog number: F144802)
12. Pipetman Starter Kit (P20, P200, P1000) (Gilson, catalog number: F167300)
13. Pipette controller, PIPETBOY acu 2 (VWR, catalog number: 612-0928)
14. Standard -20 °C freezer (LIEBHERR, model: LCv 4010 MediLine)
15. Standard -80 °C freezer (Heraeus, model: HFU586 Top Freeze)
16. Standard fridge (LIEBHERR, model: LKUexv 1610 MediLine)
17. Vacuum pump BVC control (Vacuubrand, catalog number: 20727200)

Software

1. CellProfiler (Kamentsky *et al.*, 2011; www.cellprofiler.org)
2. Fiji (Schindelin *et al.*, 2012; <http://fiji.sc/Fiji>)
3. IncuCyte software (Essen Biosciences, 2011A Rev2)

Procedure

Prior to the start of the BL-CFC culture, ESCs are differentiated according to previously described protocols (Sroczynska *et al.*, 2009). Flk1 $^{+}$ cells are isolated using magnetic sorting following the manufacturer's instructions (Miltenyi Biotech). The procedure described below has been optimized for adherent BL-CFC culture allowing the use of time-lapse microscopy. The quantities and volumes listed were further adapted for the comparison of two culture conditions: with or without doxycycline.
Note: Before starting, prepare working solutions as described in "Recipes". Careful, BL-CFC culture medium should be prepared fresh.

Day 0

1. Put 1 ml/well of 0.1% gelatin solution (see Recipes) into eight wells of Costar $^{\circledR}$ 6-well cell culture plates (one plate of four wells for each line) and leave at room temperature (RT) for at least 20 min.
2. For each line, introduce 0.425 million Flk1 $^{+}$ cells obtained after embryoid body differentiation of ESCs and counted using a Neubauer counting chamber (Sroczynska *et al.*, 2009) into a 15-ml Falcon tube containing 5 ml of IMDM + 20% FBS.
3. Centrifuge at 290 $\times g$ for 5 min.
4. For each line, remove the supernatant using an aspirating pipet connected to the vacuum pump BVC control, gently tap the bottom of the tube to loosen the cell pellet and resuspend into 8.5 ml of BL-CFC culture medium.

Note: Always use individual pipets and tips for each cell line to avoid cross-contamination.

5. Aspirate the gelatin from the pre-coated Costar $^{\circledR}$ 6-well cell culture plate wells using an aspirating pipet.
6. Resuspend and evenly distribute 2 ml/well of cell suspension into four gelatin-coated wells for

each cell lines.

Note: This means that 0.1 million Flk1⁺ cells are added per well.

7. Agitate the plates parallel to the bench doing vertical and horizontal small jolting movements.

Note: Careful not to do circular movements which would result in the formation of a vortex and, consequently, in all the cells going to the center of the well.

8. Place the plate into a 37 °C, 5% CO₂ cell culture incubator for 24 h.

9. Keep the BL-CFC culture medium in the fridge for the doxycycline dilution on the next day.

Note: The differentiation rate can be affected by cell confluence. Make sure the cells are equally spread across the surface of the wells to insure that the emergence of round blood cells will occur at the same rate everywhere in the wells.

Day 1

1. Prepare 0.2 µg/µl doxycycline dilution by adding 4 µl of 10 mg/ml doxycycline stock solution (see Recipes) to 196 µl of BL-CFC culture medium kept in the fridge from the day before. Mix well.
2. Take out the culture plates from the incubator and check under the microscope that the wells are all similar to each other.
3. Add 10 µl of 0.2 µg/µl doxycycline dilution to each +dox condition well (two wells for each line at a final doxycycline concentration of 1 µg/ml). Add the doxycycline directly in the center of the wells and mix directly after by agitating the culture plates carefully.
4. Add 10 µl of the BL-CFC culture medium to each -dox condition well as control (two wells for each line).
5. Put back the two plates inside the incubator and place them carefully into the IncuCyte HD microscope.

Note: Don't write on top of the wells and avoid splashes of the medium on the lid to prevent malfunctioning of the IncuCyte HD as it takes pictures from the top.

6. While the lid condensation is going away, set up the IncuCyte HD device using the IncuCyte software on the computer:
 - a. Connect to the device and log in.
 - b. Click on "Schedule Upcoming Scans" in the Task List panel on the left (see Figure 1 A).
 - c. In the Drawer Setup window, select an empty vessel on the screen, right-click on the location where you inserted your first plate and click on "New" (see Figure 1).
 - d. Keep this vessel selected and select the following settings in the Scan Setup tab, on the left side of the drawer map (see Figure 1 B):

Tray Type: Microplates

Vessel Type: 6-well Corning

Scan Type: Phase Contrast

Scan Pattern: Select the appropriate scan pattern or create one clicking on "Edit Scan Patterns" on the bottom left of the window (see Figure 1 C). Select 16 images/well for each

6-well.

Note: The IncuCyte can take up to 121 images per well in a 6-well plate format. However, by choosing this option, we would have increased the time of imaging considerably, therefore leading to time points spaced by at least two hours instead of the 15 min needed to capture the morphological changes occurring during the BL-CFC culture.

- e. With the vessel selected, go to the Properties tab (see Figure 1 D) and fill the following fields (see Figure 2 A):

Label: Name of your experiment/vessel type

Cell type: Exact name of the cell line/cell type used

Passage: Number of passages

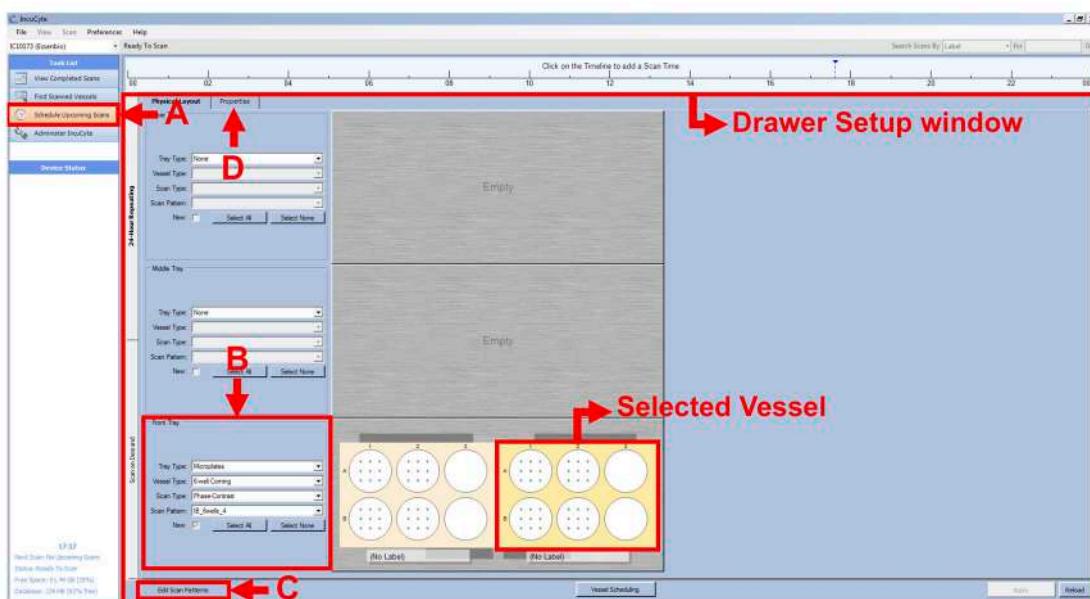


Figure 1. IncuCyte setup

- f. Click on “Plate Map” (see Figure 2 B) and in the new window set up the plate map of your experiment (see Figure 3):
 - i. Add a new compound and name it “+dox” (see Figure 3 A).
 - ii. Select the +dox compound, the wells that you treated with doxycycline and click on “Add +dox” (see Figure 4 A).
 - iii. Select the concentration and click on OK.
 - iv. Click on OK.

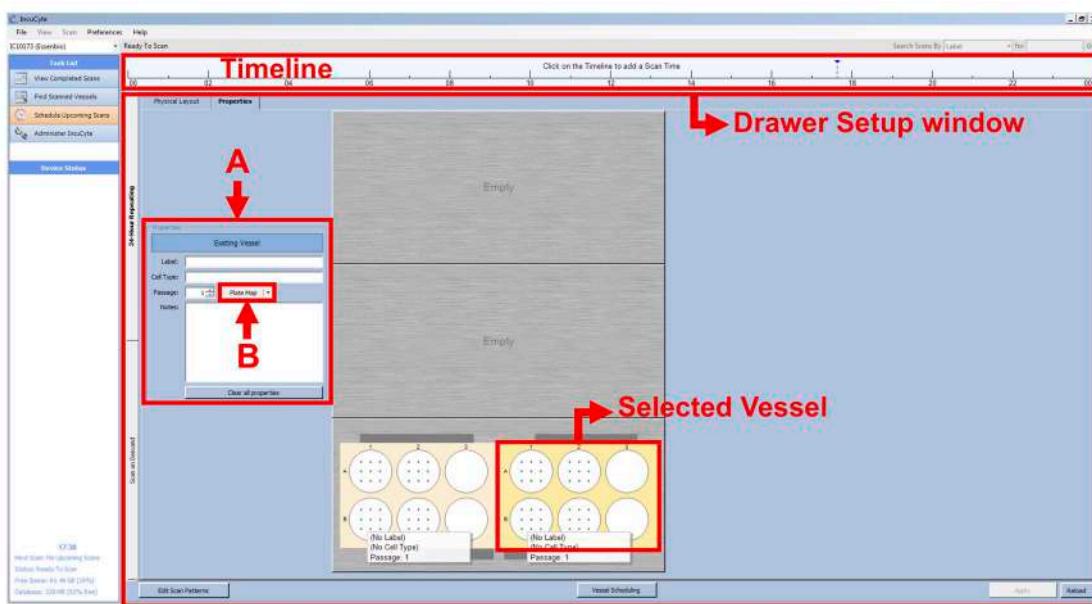


Figure 2. IncuCyte Properties setup

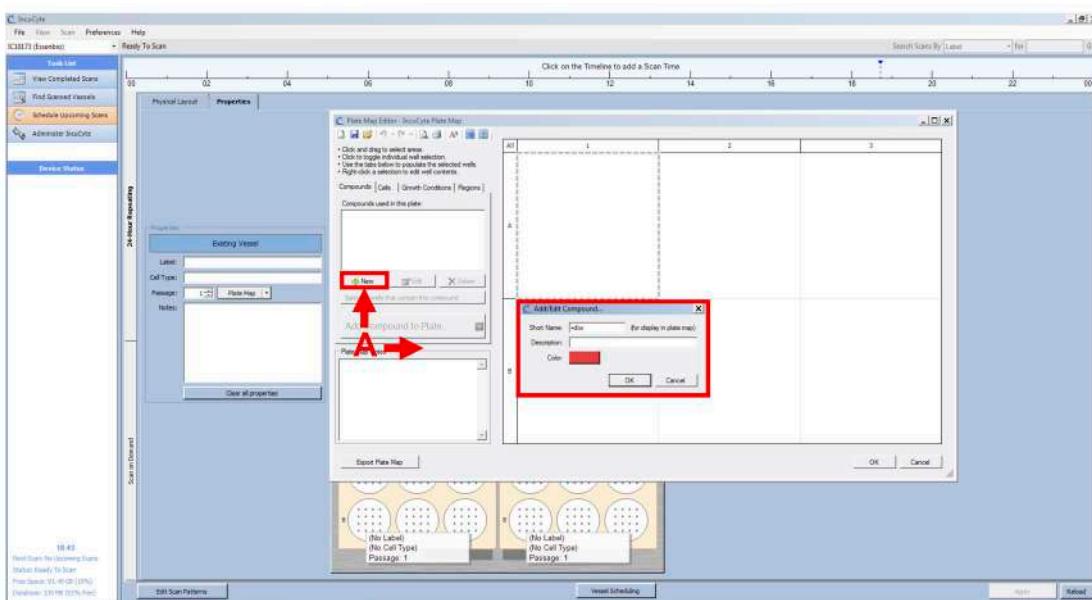


Figure 3. IncuCyte Plate Map initial setup

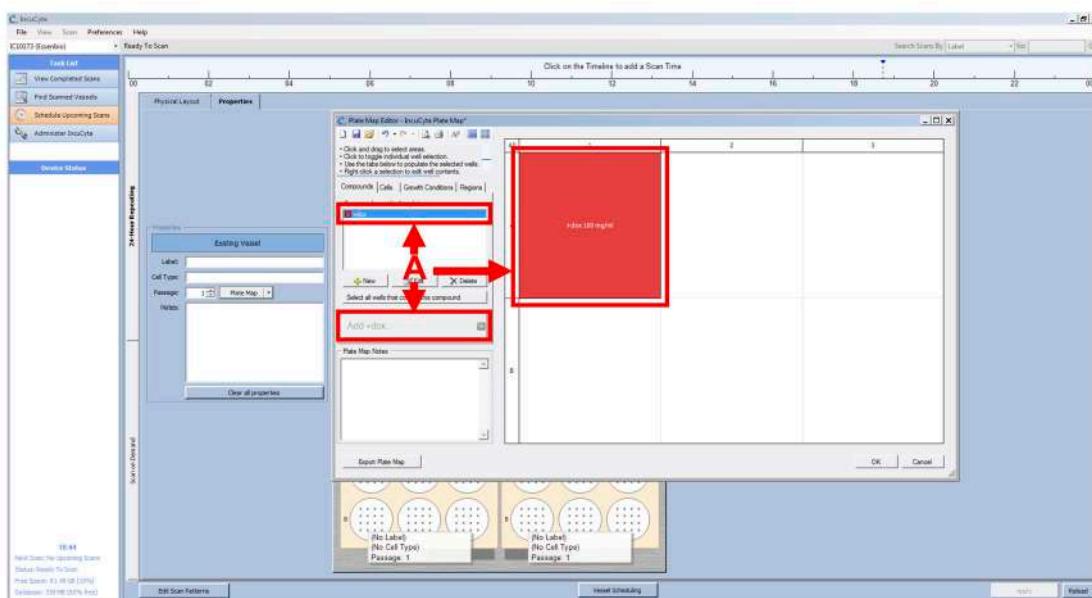


Figure 4. IncuCyte Plate Map setup

- g. Repeat the setup of the vessel (from Day1 Steps 6c-6f) for your second plate.
- h. Right-click on the upper timeline (see Figure 2) and click on “Set intervals”.
- i. Set intervals every 15 min starting from time 0, for a total of 24 h.

Note: Even with a total of 24 h, the Incucyte device will scan your vessels every 15 min until you manually remove your vessels from the software.

- j. Check the grey bars to make sure they are not overlapping (see Figure 5 A), check that IncuCyte device is not scanning (see Figure 5 B) and click on “Apply” (see Figure 5 C).

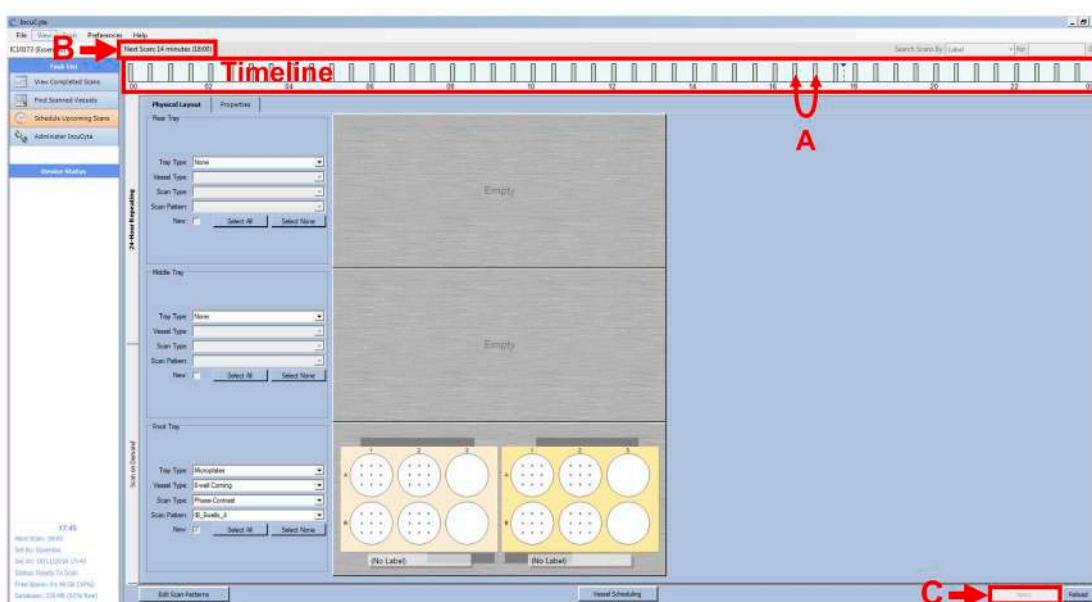


Figure 5. Set Intervals

Note: When the plate is put in the incubator, the difference of temperature between the room and the incubator generates water condensation on the lid (as an example, see Figure 6 A). Wait at least 30 min before initiating the imaging so that the microscope can focus properly on the cells.

- Come back after 15 min to check that everything is working and switch off the computer if you wish.

Day 3

- After 48-h treatment, click on “Schedule Upcoming Scans” in the Task List panel of the IncuCyte software.
- Right-click on the upper timeline and select “Delete Intervals”.
- Click on “Apply” to stop the scanning by IncuCyte.
- Take out your plates and proceed with flow cytometry analysis according to standard protocols.

Data analysis

- Export your images from the IncuCyte software:
 - Click on “Find Scanned Vessels” in the Task List panel of the IncuCyte software (see Figure 6 B).
 - Select one of your cell line vessels (see Figure 6 C) and click on “View Vessel” button in the lower right-hand corner of the screen (see Figure 6 D).

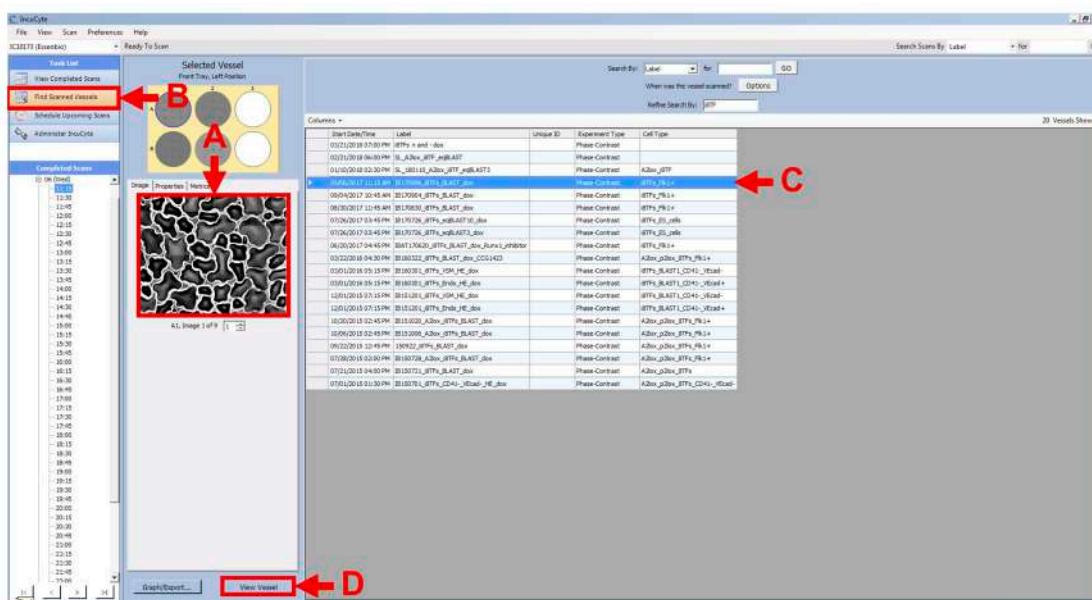


Figure 6. View Vessels

- c. Check that the scans are fine (no dust, condensation, etc.).
- d. Selecting “Export Movie or Image Set...” from the “Utilities” pull down menu (see Figure 7 A).

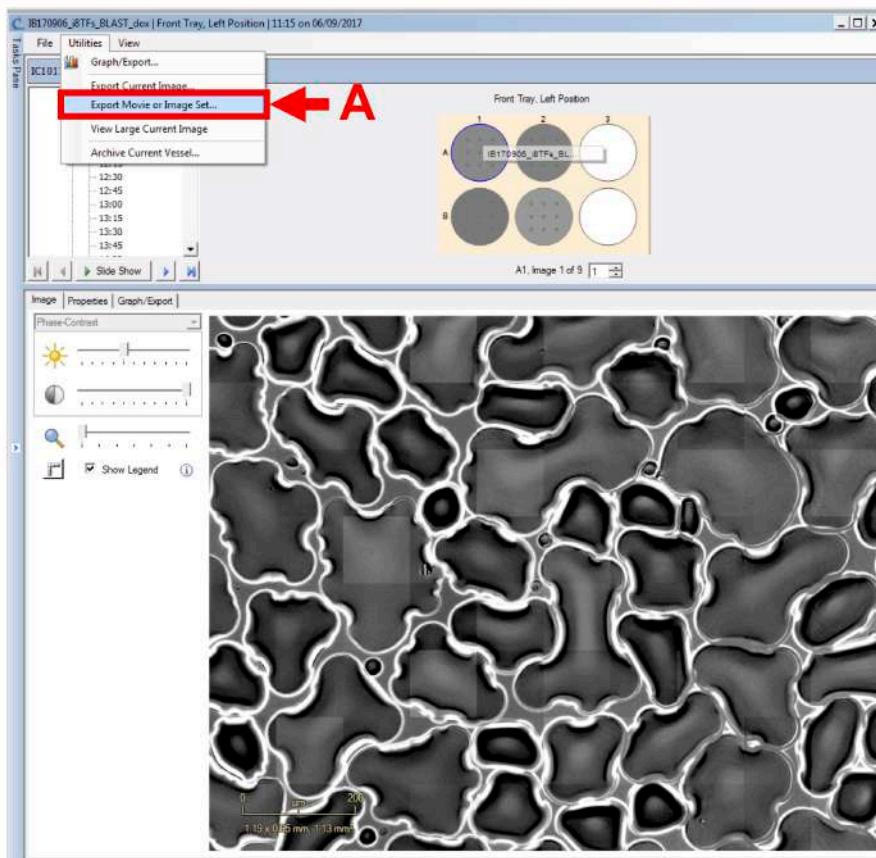


Figure 7. Export image set

- e. In the new window, select the appropriate time frame (see Figure 8 A), the wells corresponding to one condition (see Figure 8 B), all images (see Figure 8 C), select the sequence type labeled “set of individual images” (see Figure 8 D) and export phase-contrast original image (.JPEG).
 - f. Specify the destination folder and the prefix of the files that will be generated (see Figure 8 E).
- Note: Create one folder per condition per cell line.*
- g. Click on “Export ... files” (see Figure 8 F).

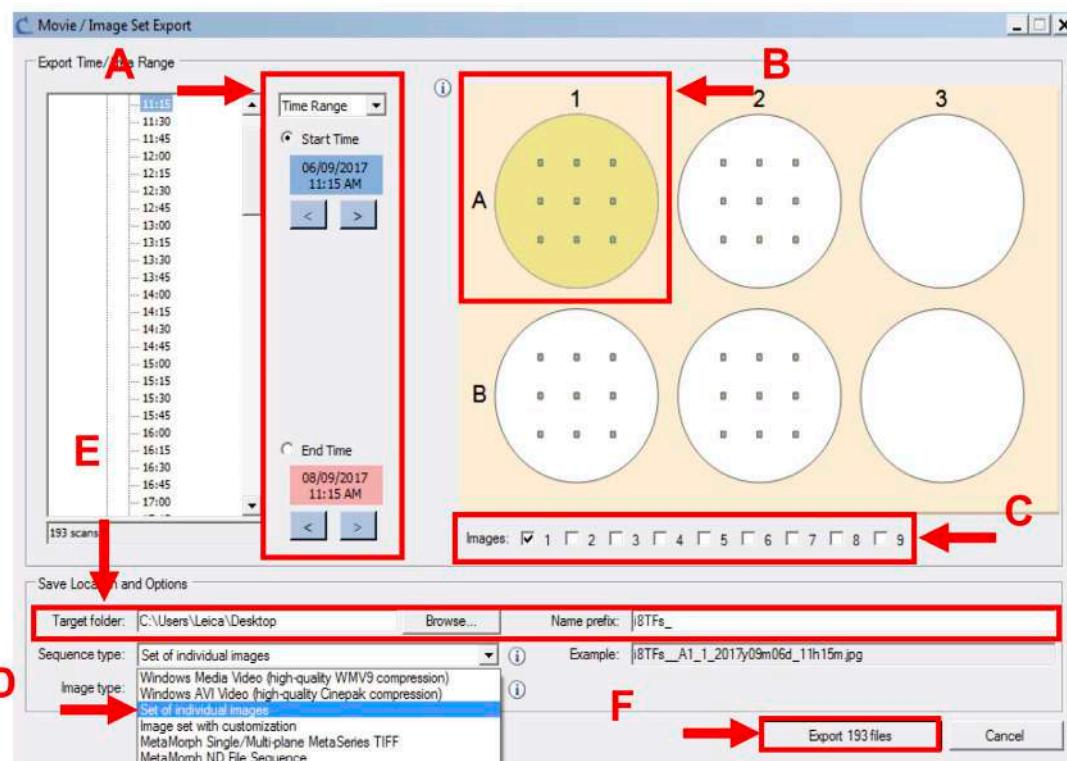


Figure 8. Final step of export image set

- h. Repeat Steps 1a-1h for all the vessels and conditions to be analyzed (see [Supplemental File 1: Example dataset of IncuCyte images for the i8TF cell line](#) for an example dataset).
2. Analyze your images using CellProfiler software to get the number of round cells for each time-point, cell line and condition:
 - a. Install CellProfiler2.2.0, which can be downloaded from here: http://cellprofiler.org/previous_releases/
Note: You need Java as well: <http://cellprofiler.org/releases/>.
 - b. Open CellProfiler and load the CellProfiler pipeline (see [Supplemental File 2: CellProfiler pipeline for round cell counting](#)) using *File > Import > Pipeline from File...* in the main menu of CellProfiler.
 - c. Drag and drop the whole IncuCyte export destination folder into the *File list* area of the *Images* module (see Figure 9 A).
 - d. Click on “Analyze images” on the bottom left to start processing (see Figure 9 B).

Note: The pipeline has been developed to automatically generate an output folder named as the input folder with “-- analyzed” appended. The output folder will be generated in the folder containing the input folder. If you want to modify this, simply change the Output File Location to Default Output Folder in the SaveImages and ExportToSpreadsheet Analysis modules, and change the output folder name by selecting “View output settings” in the Output panel of the main window.

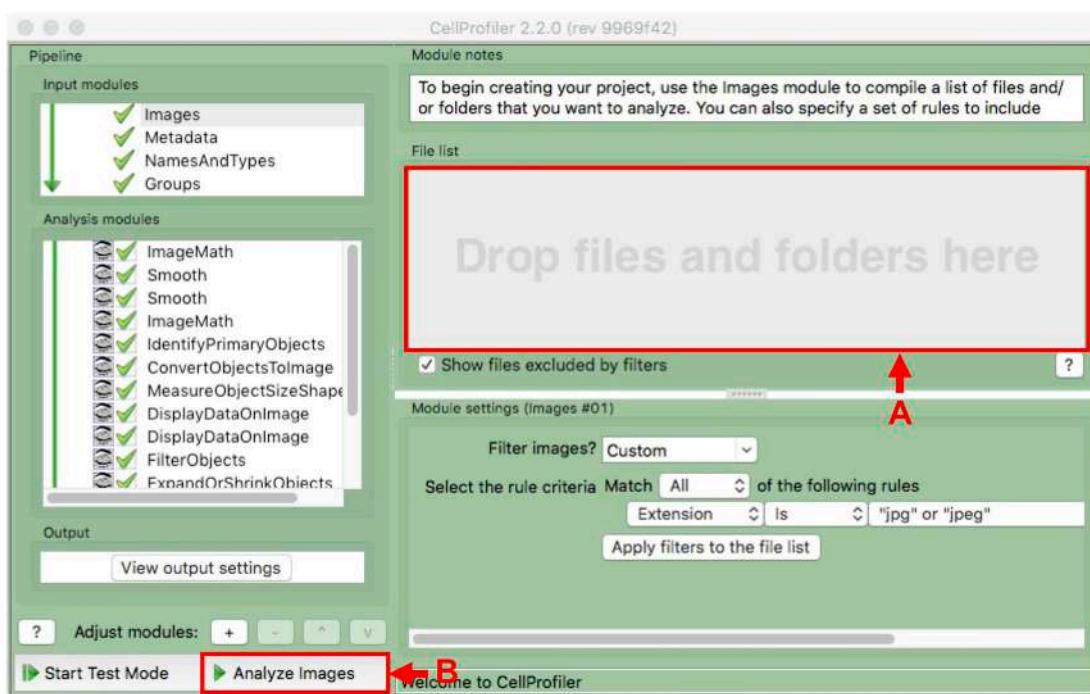


Figure 9. Image module of CellProfiler

- e. Use the Browser to access the output “image.txt” file in the output folder (as an example, see [Supplemental File 3: CellProfiler output files for the example dataset](#)) and open it with Excel or in R to visualize the results as graphs. In the output .txt file, the *Count_RoundCells* column contains the round cell numbers. The *ImageNumber* column contains the order in which the images were analyzed by CellProfiler. The *Metadata_condition* column contains the prefix given while exporting the images from the IncuCyte software. The *Metadata_day* column contains the day in which the images were taken. The *Metadata_filename* column contains the file names of the images analyzed such as exported by the IncuCyte software. The *Metadata_foldername* column corresponds to the input folder. The *Metadata_position* column contains the position of the images inside the well. The *Metadata_time* column contains the time in which the images were taken. The *Metadata_well* column contains the name of the well from which the images were taken. Each row corresponds to one image.
- f. See Figures 10 and 11, and [Supplemental File 3: CellProfiler output files for the example dataset](#) for the analysis output of the example dataset.

Notes:

- i. The *Metadata extraction* described above relies on the IncuCyte file naming scheme. If this naming scheme is changing, e.g., due to version updates by IncuCyte, or because you are using a different microscope, it will not work. In such cases please contact us and we will help you adapting the *Metadata extraction* inside CellProfiler.
- ii. In the output folder, CellProfiler also generates copies of the individual images with all counted round cells marked by a yellow dot (Figure 10).

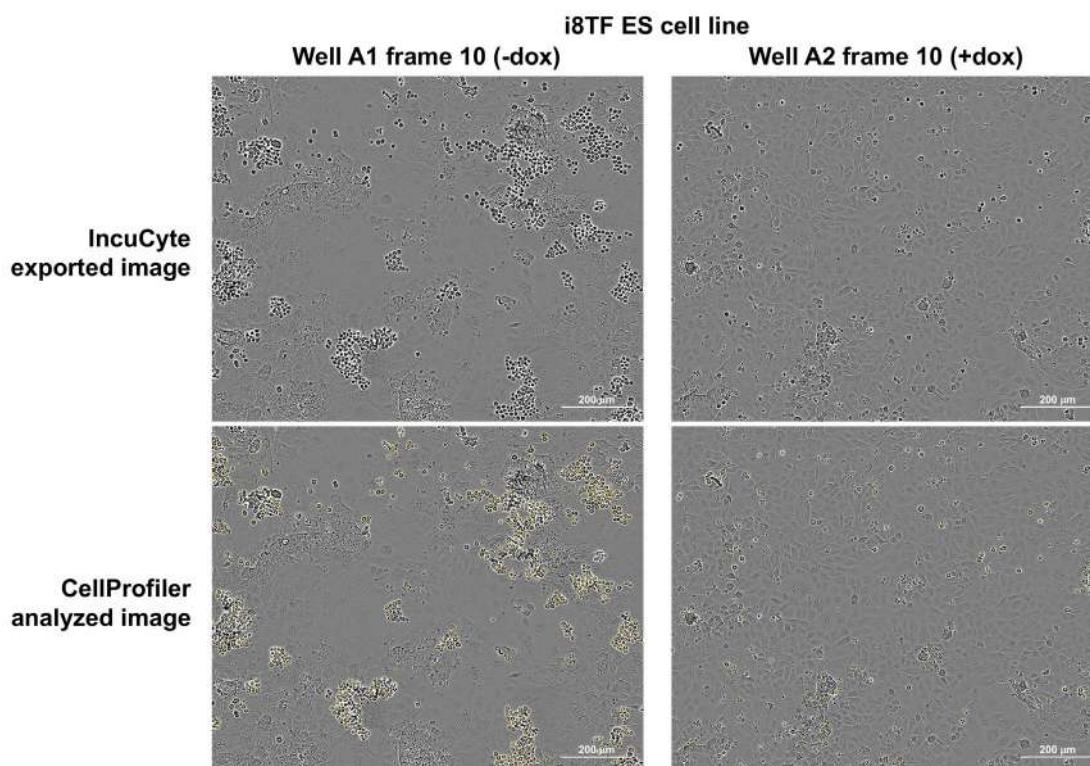


Figure 10. IncuCyte images before and after CellProfiler analysis

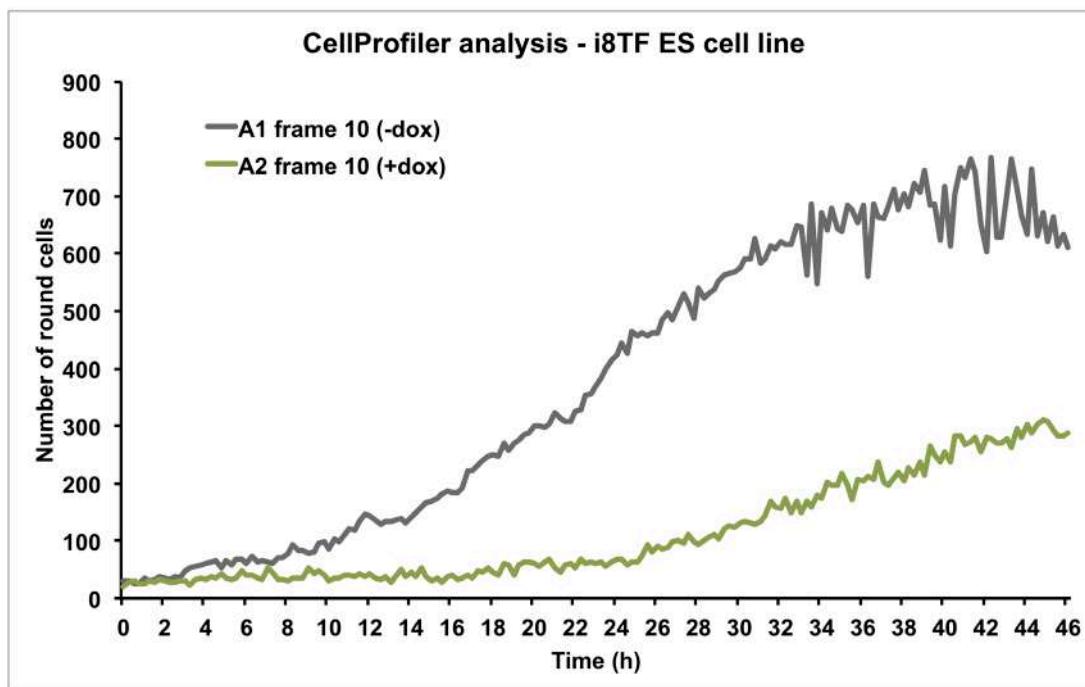


Figure 11. Graph showing the number of round cells over time as calculated by CellProfiler

3. Generate movies from your images using Fiji software to illustrate differences between round cell emergence rates and highlight other morphological variations:
 - a. Install Fiji software, which can be downloaded from here: <http://fiji.sc/Fiji>.
 - b. Open Fiji.
 - c. In your Browser, sort the IncuCyte exported files by Name, select all the files corresponding to the images taken from one frame of one condition for one cell line. By clicking on the last image, drag and drop all the files in Fiji. Let Fiji open the files without changing the order of the opened windows.
 - d. To create a stack, select *Image > Stacks > Images to Stack* and then click on OK in the new window.
 - e. Check the proper alignment of the stack slices by clicking on the *play* icon on the bottom left of the image. If the movie is readable, go directly to Step 3h.
 - f. To align the slices, select *Plugins > Image Stabilizer*. In the new window, insert the following values:
Transformation: Translation
Maximum Pyramid Levels: 1
Template Update Coefficient (0-1): 0.90
Maximum Iterations: 200
Error Tolerance: 0.0000001
Select *Output to a New Stack* and click on OK.
 - g. Check the slice alignment of the stabilized stack by clicking on the *play* icon on the bottom left of the image. If the movie is readable, go to Step 3h otherwise repeat Step 3f until all the slices are properly aligned (up to 3-4 times).
 - h. Save the movie by selecting *File > Save As > AVI...* In the new window, select JPEG Compression and 10 fps as Frame Rate.
 - i. See Videos 1 and 2 based on the example dataset.



Video 1. BL-CFC culture in normal conditions. The video is a 48-h-time-lapse microscopy analysis of BL-CFC culture in absence of doxycycline.



Video 2. BL-CFC culture following over-expression of eight key transcription factors. The video is a 48-h-time-lapse microscopy analysis of BL-CFC culture in presence of doxycycline, i.e., following the over-expression of eight key transcription factors (Runx1, Cbfb, Gata2, Tal1, Fli1, Lyl1, Erg and Lmo2).

Recipes

Note: Prepare solutions 1-9 in advance.

1. 0.1% gelatin solution (stored at 4 °C)

0.2 g of gelatin

200 ml of PBS

Note: Dissolve the powder, sterile filter with Stericup GP 0.2 µm/500 ml, aliquot and store at 4 °C.

2. 10 mg/ml doxycycline stock solution (stored at -20 °C)

10 mg of doxycycline

1 ml of sterile distilled water

Note: Dissolve the powder, aliquot and store at -20 °C. Always freshly thaw an aliquot.

3. 5 mg/ml ascorbic acid stock solution (stored at -20 °C)

0.5 g of ascorbic acid

100 ml of distilled and sterile water

Note: Dissolve the powder, sterile filter with a Stericup GP 0.2 µm/150ml, aliquot and store at -20 °C. Always freshly thaw an aliquot.

4. D4T endothelial cell supernatant (stored at -20 °C)

Prepare D4T endothelial cell supernatant following the procedure described by Choi and colleagues (Choi et al., 1998).

Note: Prepare, aliquot and store at -20 °C. Once thawed, can be stored at 4 °C and used up to one month after.

5. PBS + 0.1% BSA solution

0.05 g of BSA

50 ml of PBS

Note: Dissolve, sterile filter using Millex-GP Syringe Filter, aliquot and store at -20 °C.

6. 10 µg/ml VEGF stock solution (stored at -80 °C)

Dissolve a 10 µg vial in 1 ml of sterile PBS + 0.1% BSA solution

Note: Dissolve, aliquot and store at -80 °C. Once thawed, can be stored at 4 °C and used up to one month after.

7. 10 µg/ml IL6 stock solution (stored at -80 °C)

Dissolve a 10 µg vial in 1 ml of sterile PBS + 0.1% BSA solution

Note: Dissolve, aliquot and store at -80 °C. Once thawed, can be stored at 4 °C and used up to one month after.

8. Conditioned IMDM (stored at 4 °C)

1 bottle of IMDM

5 ml of L-glutamine

5 ml of Penicillin-streptomycin

9. IMDM + 20% FBS (stored at 4 °C)

30 ml of FBS

120 ml of conditioned IMDM

Note: Sterile filter using Stericup™ 150 ml bottle.

10. MTG dilution (to be prepared fresh)

13 µl of MTG

1 ml of conditioned IMDM

Note: Careful, MTG is viscous. Mix well after dilution.

11. BL-CFC culture medium (to be prepared fresh)

14.49 ml of conditioned IMDM

2 ml of FBS

0.2 ml of L-glutamine

0.12 ml of Transferrin

0.06 ml of MTG dilution

0.1 ml of 5 mg/ml acid ascorbic stock solution

3 ml of D4T endothelial cell supernatant

0.01 ml of 10 µg/ml VEGF stock solution

0.02 ml of 10 µg/ml IL6 stock solution

Note: Prepare fresh in a 50 ml Falcon tube and sterile filter using Millex-GP Syringe Filter.

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Competing interests

The authors do not have any conflicts of interests or competing interests.

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Isolation of Neural Stem Cells from the Embryonic Mouse Hippocampus for *in vitro* Growth or Engraftment into a Host Tissue

Oksana Rybachuk^{1, 2,*}, Olga Kopach^{1, 3}, Tetyana Pivneva^{1, 2} and Vitaliy Kyryk²

¹Department of Sensory Signaling, Bogomoletz Institute of Physiology, Kyiv, Ukraine; ²State Institute of Genetic and Regenerative Medicine, Kyiv, Ukraine; ³Queen Square Institute of Neurology, University College London, London WC1N 3BG, UK

*For correspondence: rbk@biph.kiev.ua

[Abstract] For both stem cell research and treatment of the central nervous system disorders, neural stem/progenitor cells (NSPCs) represent an important breakthrough tool. In the expanded stem cell-based therapy use, NSPCs not only provide a powerful cell source for neural cell replacement but a useful model for developmental biology research. Despite numerous approaches were described for isolation of NSPCs from either fetal or adult brain, the main issue remains in extending cell survival following isolation. Here we provide a simple and affordable protocol for making viable NSPCs from the fetal mouse hippocampi, which are capable of maintaining the high viability in a 2D monolayer cell culture or generating 3D neuro-spheroids of cell aggregates. Further, we describe the detailed method for engraftment of embryonic NSPCs onto a host hippocampal tissue for promoting multilineal cell differentiation and maturation within endogenous environment. Our experimental data demonstrate that embryonic NSPCs isolated using this approach show the high viability (above 88%). Within a host tissue, these cells were capable of differentiating to the main neural subpopulations (principal neurons, oligodendrocytes, astroglia). Finally, NSPC-derived neurons demonstrated matured functional properties (electrophysiological activity), becoming functionally integrated into the host hippocampal circuits within a couple of weeks after engraftment.

Keywords: Embryonic neural stem/progenitor cells (NSPCs), Mouse hippocampus, Cell viability, Adherent 2D monolayer cell culture, 3D neuro-spheroids, Organotypic hippocampal tissue

[Background] Multipotent stem cells of neural origin, neural stem/progenitor cells (NSPCs), represent a versatile tool for enabling nerve cell replacement in a number of neurological disorders characterized by the loss of specialized neural subpopulations. The unlimited lifespan and directed NSPC differentiation into the key neural subtypes (neurons, astrocytes, and oligodendrocytes) make these cells the most attractive candidate for emerging stem cell-based therapies for presently incurable brain disorders (Kopach and Pivneva, 2018). Stem cells exist in the developing and mature nervous system and can be isolated from either fetal brain (embryonic NSPCs) or, as established more recently, from mature brain (in adults, both subventricular and subgranular zones in dentate gyrus of the hippocampus, forebrain, cerebellum and olfactory bulb) (Pagano *et al.*, 2000; Roy *et al.*, 2000; Klein *et al.*, 2005; Behnan *et al.*, 2017; Kempermann *et al.*, 2018). Despite that adult stem cells bear expectations for an

advanced translational approach (Kempermann *et al.*, 2018), a limited number of rigorous evidence supports this research avenue at the moment.

Accumulating evidence for the properties of fetal NSPCs and phenotype specifications, confirmed by dedicated clinical trials, retains a focus on this stem cell type. Furthermore, embryonic NSPCs represent an essential tool for developmental biology research. Various methodological approaches were probed to isolate NSPCs from the fetal brains and to maintain cells in conditions determining their high viability, hence, progeny. Such conditions largely dictate phenotyping of differentiating progenitors to acquire the competitive NSPC-derived cells. The two main strategies for preserving the high viability of isolated NSPCs exist in i) cell culture *in vitro* and ii) grafting into a host tissue. A classical 2D culture, an adherently expanded monolayer of cells, has been routinely used for stem cell growth *in vitro* and/or for directed phenotypic differentiation (Conti *et al.*, 2005; Pollard *et al.*, 2006). In parallel, newer developed approach of generating 3D cultures, neuro-spheroids of an assembly of NSPCs at various phenotypic and developmental stages, has been emerged to represent more physiological conditions pertinent to neuronal differentiation rather than glial (Ignatova *et al.*, 2002; Suslov *et al.*, 2002; Makri *et al.*, 2010). This approach can potentially enhance the therapeutic potential of NSPCs *in vitro*. Another alternative—grafting of viable NSPCs into brain tissue—fulfill a strategy for promoting the multi-lineage differentiation of progenitors within a host tissue, regulated by endogenous environment. Such an approach has recently been implemented in the treatment of the ischemia-damaged hippocampal tissue (Tsypykov *et al.*, 2014; Kopach *et al.*, 2018).

Here we provide the detailed protocol that we routinely use for isolation of viable NSPCs from the fetal mouse hippocampus. This protocol was also utilized for engraftment of undifferentiated cells to the hippocampal tissue for monitoring maturation of NSPC-derived cells within a host environment in our previous studies (Kopach *et al.*, 2018). We describe all the details on how to test the viability of the obtained embryonic NSPCs and maintain the cells in multiple approaches for further proposed use.

Materials and Reagents

A. Materials

1. 15 ml and 50 ml centrifuge tubes (Corning, catalog numbers: 430790, 430828)
2. 1.5 ml Eppendorf tubes (Sigma-Aldrich, catalog number: T9661)
3. Glass Pasteur pipette (1 mm diameter) (Fisher Scientific, catalog number: 13-678-6A)
4. 35 mm, 60 mm, and 100 mm tissue culture dishes (CELLSTAR, catalog numbers: 627160, 628160, 664160)
5. Falcon® 40-µm cell strainer (Corning, Falcon®, catalog number: 352340)
6. 0.45-µm sterile filter (Fisher Scientific, Merck™, catalog number: 12279299)
7. 6-well and 24-well tissue culture plates (Greiner Bio-One, catalog numbers: 657160, 662160)
8. 13 mm glass coverslips (Henz Herenz, catalog number: 1051204)
9. SuperFrost® glass slides (VWR, Thermo Fisher Scientific, catalog number: 631-0706)

10. Plastic serological pipettes (5 ml, 10 ml, and 25 ml) (Corning® Costar® Stripette®, catalog numbers: 4487, 4488, 4489)
11. Pipette tips (TipOne, STARLAB, catalog numbers: S1111-3700, S1111-1706, S1111-6701)
12. Millipore® Millicell® cell culture plate inserts (Sigma-Aldrich, catalog number: Z353086)
13. Whatman® qualitative filter paper (Sigma-Aldrich, catalog number: WHA1001110)
14. Scalpel blades (Fisher Scientific, Swann-Morton™, catalog number: 11772724)
15. Scalpel with retractable blade (Eickemeyer®, catalog number: 100504)
16. Parafilm® M (Sigma-Aldrich, catalog number: P7793)

B. Animals

1. Pregnant FVB-Cg-Tg(GFP) 5Nagy/J GFP mice (obtained from the animal facilities of State Institute of Genetic and Regenerative Medicine)
2. Embryos of FVB-Cg-Tg(GFP) 5Nagy/J GFP mice at the developmental stage of E16-E17
3. Pups of FVB mice (8 to 9-days old)

C. Reagents

1. HBBS without calcium and magnesium (Sigma-Aldrich, catalog number: 55021C)
2. Paraformaldehyde (PFA, Sigma-Aldrich, catalog number: P6148)
3. 0.25% trypsin-EDTA (Sigma-Aldrich, catalog number: T4049)
4. 22% Percoll (Sigma-Aldrich, catalog number: 17-0891-01)
5. Neurobasal® medium (Thermo Fisher Scientific, Gibco™, catalog number: 21103049)
6. B-27® supplement (50x) (Thermo Fisher Scientific, Gibco™, catalog number: 17504044)
7. GlutaMAX™ supplement (100x) (Thermo Fisher Scientific, Gibco™, catalog number: 35050061)
8. N-acetyl-L-cysteine (NAC) (Sigma-Aldrich, catalog number: A7250)
9. Penicillin-streptomycin (P/S) solution (100,000 U/ml) (Thermo Fisher Scientific, Gibco™, catalog number: 15140122)
10. FGF-2 (Sigma-Aldrich, catalog number: SRP4037)
11. Matrigel (BD Biosciences, catalog number: 354234)
12. Triton® X-100 (Sigma-Aldrich, catalog number: T8787)
13. Accutase® solution (Sigma-Aldrich, catalog number: A6964)
14. 0.4% Trypan blue (Thermo Fisher Scientific, Gibco™, catalog number: 15250061)
15. Antibodies

a. Primary

- i. Mouse anti-Nestin antibody (1:100) (Santa Cruz Biotechnology, catalog number: sc-23927)
- ii. Rabbit anti-β-tubulin III (1:500) (Sigma-Aldrich, catalog number: T2200)
- iii. Mouse anti-GFAP (1:200) (Sigma-Aldrich, catalog number: AMAB91033)
- iv. Rabbit anti-olig-2 (1:200) (Abcam, catalog number: ab136253)
- v. Mouse anti-NeuN (1:100) (Millipore, catalog number: MAB377)

- b. Secondary
 - i. Donkey anti-mouse Alexa Fluor 555 (1:1,000) (Thermo Fisher Scientific, catalog number: A-31570)
 - ii. Donkey anti-rabbit Alexa Fluor 647 (1:1,000) (Thermo Fisher Scientific, catalog number: A-31573)
 - iii. Nuclei tracer Hoechst 33342 (1:5,000) (Sigma-Aldrich, catalog number: 14533)
- 16. ImmunoHistoMount™ (Sigma-Aldrich, catalog number: I1161)
- 17. 70% Ethanol (Sigma-Aldrich, catalog number: 459836)
- 18. Sodium dihydrogen phosphate monobasic (NaH_2PO_4) (Sigma-Aldrich, catalog number: S0751)
- 19. Disodium phosphate dibasic (Na_2HPO_4) (Sigma-Aldrich, catalog number: S0876)
- 20. Sodium hydroxide (NaOH) (Sigma-Aldrich, catalog number: S8045)
- 21. Minimum Essential Medium (MEM) (Sigma-Aldrich, catalog number: M2249)
- 22. Tris (Sigma-Aldrich, catalog number: 252859)
- 23. Sodium bicarbonate (Sigma-Aldrich, catalog number: 792519)
- 24. HEPES (Thermo Fisher Scientific, catalog number: 15630080)
- 25. D(+)-glucose (Sigma-Aldrich, catalog number: G8270)
- 26. Horse serum (Sigma-Aldrich, catalog number: H1138)
- 27. Phosphate buffered saline (PBS), pH 7.4 (Sigma-Aldrich, catalog number: P3813)
- 28. BD Cell Wash buffer (BD Biosciences, catalog number: 349524)
- 29. Bovine serum albumin (BSA) (Sigma-Aldrich, catalog number: 05470)
- 30. 7-AAD (BD Pharmingen™, catalog number: 559925)
- 31. 0.2 M Phosphate buffer (pH 7.4) (see Recipes)
- 32. 4% Paraformaldehyde (PFA) in 0.1 M phosphate buffer (see Recipes)
- 33. Growth medium (see Recipes)
- 34. Tissue dissecting medium (pH 7.3) (see Recipes)
- 35. Tissue culture medium (pH 7.2) (see Recipes)
- 36. Antibody solution (see Recipes)
- 37. Blocking solution (see Recipes)

Equipment

- 1. Pipettes (Eppendorf, Research® Plus, catalog numbers: EP 3123000012; EP 3123000098; EP 3123000055; EP 3120000062)
- 2. 250 ml Graduated cylinder (Karter Scientific, catalog number: 213I13)
- 3. Dry heat sterilizing cabinet (Grande, model: GIO-072)
- 4. Fine tools for dissection and manipulating embryonic brains
 - a. Dissecting scissors (Dumont, catalog number: 14090-09)
 - b. Small spatula (Dumont, catalog number: 10090-13)
 - c. Forceps (Dumont, catalog number: 91197-00)

- d. Angled forceps (Dumont, catalog number: 00125-11)
5. C-Chip disposable hemocytometer/Neubauer (Labtech International, Heathfield, UK, catalog number: DHC-N01)
6. Refrigerated centrifuge (Thermo Fisher Scientific, model: IEC-CL30R)
7. Water bath (Biosan, model: WB-4MS)
8. Biosafety Cabinet Class II Type A2 (Labconco, model: Purifier Logic)
9. CO₂ incubator (Thermo Fisher Scientific, model: SteriCycle 371)
10. Flow cytometer (Becton Dickinson, model: BD FACSAria cell sorter)
11. Tissue chopper (McIlwain, model: MTC/2E)
12. Alcohol burner (Sigma-Aldrich, catalog number: Z509604)
13. Inverted microscope (Olympus, model: IX71, equipped with camera DP-20)
14. Confocal microscope (Olympus, model: FV1000)
15. Stereo microscope (Olympus, model: SZX7)
16. 4 °C refrigerator (Haier Biomedical, model: HYC-940)

Software

1. QuickPHOTO software (PROMICRA, s.r.o., Czech Republic)
2. FV10-ASW software (Olympus, Japan)
3. BD FACSDiva™ 6.1.2 software (BD Biosciences, USA)

Procedure

A. Set-up (general preparation)

Note: All steps, including harvesting the embryos, are performed in a flow hood.

1. Sterilization of the working area

Expose the biosafety cabinet hood to UV light for 1 h before starting to use it. Wipe all working surfaces and the tools with 70% ethanol.

2. Prepare the Matrigel-coated coverslips for cell cultures

Dissolve Matrigel in a cold Neurobasal medium (10 mg/ml) by pipetting up and down to mix the Matrigel and medium very well. Put a sterile glass coverslip at each well of a 24-well plate. Add the prepared mixture onto each coverslip in a volume of approximately 0.5 ml per coverslip, making sure the surface of each coverslip is well covered. Keep coverslips for 40 min at room temperature to let them be coated with Matrigel. Afterward, remove Matrigel and wash the coverslips with Neurobasal medium 3 times. Avoid letting the Matrigel-coated coverslips become completely dry after washing.

Note: For monolayer cell cultures, prepare the Matrigel-coated coverslips at a 24-well plate. The number of coverslips/wells plated depends on experimental design and density of desired cultures and may need to be adjusted accordingly. Seeding cells onto glass coverslips is also

preferable for the purpose of further immunostaining. Glass coverslips may be prepared in advance. In such a case, seal the 24-well plate containing the Matrigel-coated coverslips very well with parafilm. The Matrigel-coated coverslips should not be kept longer than for 24 h.

3. Polishing and sterilizing glass Pasteur pipettes

Prepare the glass Pasteur pipettes by polishing the pipette tips over flame of an alcohol burner. To obtain pipettes with tips of various sizes, rotate each pipette slowly over flame from few to ten seconds until the glass of pipette tip's edge become polished that can be visually seen (Figure 1). The smallest-sized tips have an internal diameter of approximately 0.2-0.3 mm, the medium-sized—approximately 0.5-0.6 mm, and the large-sized—about 0.9 mm (Figure 1B). Before use, sterilize the flame-polished pipettes at 180 °C for 1-2 h.

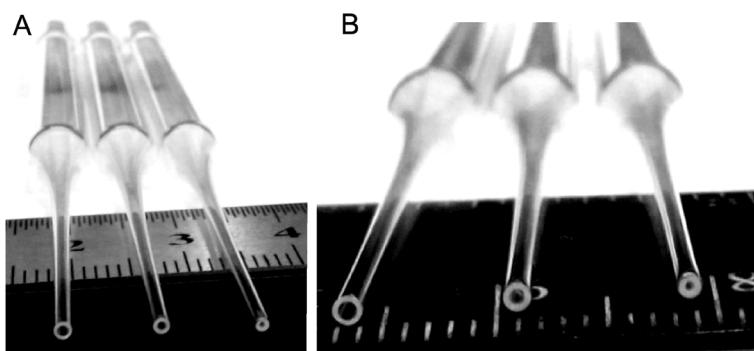


Figure 1. Snapshots of the glass Pasteur pipettes with flame-polished tips of different sizes. The pipettes are used for mechanical dissociation of the cells from the fetal mouse hippocampi, starting using the pipette with the largest-sized internal tip diameter (from left to right on A and B).

B. Harvesting embryos and dissecting the fetal hippocampi

1. Sacrifice a pregnant female mouse at the desired stage (we use E16-E17) according to the procedures approved by your Institute.
2. Wipe the abdominal skin with 70% ethanol and make a longitudinal incision to open the abdominal wall. Use another pair of sterile tools (forceps and scissors) for opening.
3. Cut the uterus and carefully take the embryos out using a stereo microscope to visually control the procedure. If needed, use PBS to wash out the tissue. Place the embryos in a sterile 100 mm Petri dish containing a cold PBS (approximately 10 ml).
4. Dissect out the brains from all embryos and carefully transfer to a 35 mm Petri dish containing 1 ml of HBBS. The dish should be kept on ice to ensure well-chilled medium.
5. Very carefully dissect both hippocampi out of the brain by dissecting first the olfactory bulbs and the cerebellum and splitting then the hemispheres either. For each of the hemisphere, remove the thalamus and very gently pull the hippocampus out of the brain with the help of a spatula.

Note: To prepare a suspension of NSPCs containing approximately 1 x 10⁶ cells, take at least five to six embryos (ten to twelve hippocampi, at the developmental stage E16-E17).

C. Isolating NSPCs

1. Take 0.5 ml of 0.25% trypsin-EDTA to a 15 ml Falcon tube and warm it in a CO₂ incubator (35 °C, for 20-30 min).

2. Enzymatic dissociation of hippocampal cells

Transfer the isolated hippocampi into a warm 0.25% trypsin-EDTA. Incubate in a CO₂ incubator for 5-7 min. After incubation, stop the enzymatic digestion by adding Neurobasal medium in a volume of 1 ml (room temperature). Give a gentle flick to the tube containing the tissue to mix it up.

Note: Each hippocampus may be cut to several parts using a sterile scalpel in order to facilitate enzymatic digestion of the tissue. Also, we recommend shaking the tube over tissue incubation period (2-3 times each minute).

3. Mechanical dissociation of the cells

Pipette the hippocampal tissue up and down using the flame-polished glass Pasteur pipettes. Start to pipette the tissue using the pipette with the largest-sized internal tip diameter first, followed by the medium-sized one. Finally, pipette the dissociated cells using the pipette with the smallest-sized tip. All steps are performed at room temperature.

Note: Use each pipette for up and down pipetting not more than 10 times in order to increase the cell viability upon mechanical dissociation of the cells. Gently apply very small pressure to minimize cell damage while breaking cell aggregates. Avoid bubbles at any step of cell isolation.

4. Take 5 ml of Neurobasal medium and add to the obtained cell suspension, gently mixing while adding the medium.

5. Let the mixture of isolated cells in Neurobasal medium go through a 40-μm cell strainer into a 50 ml centrifuge tube.

6. Centrifuge the obtained suspension at 200 × g for 10 min (room temperature).

7. Aspirate the supernatant, leaving the cell pellet intact. Add 10 ml of Neurobasal medium to the pellet and gently resuspend it.

8. Centrifuge cells at 200 × g for 10 min, as before (Figure 2).

9. Aspirate the supernatant, leaving the cell pellet intact. Add 1 ml of PBS to the pellet and gently re-suspend the cell pellet.

10. Centrifugation in a density gradient

a. Take 1 ml of the obtained cell suspension and transfer to the surface of 22% Percoll solution (total volume: 10 ml).

b. Centrifuge the mixture at 450 × g for 10 min (room temperature). Remove the supernatant, leaving the cell pellet intact (Figure 2).

c. Add 10 ml of Neurobasal medium to the pellet and gently re-suspend it.

11. Centrifuge the obtained NSPCs at 200 × g for 10 min (room temperature). Remove the supernatant, add 1 ml of growth medium to the pellet and gently resuspend it.

Note: Use a 1 ml pipette for mixing and resuspending the cell suspension. This minimizes cell damage and increases the viability of isolated NSPCs.

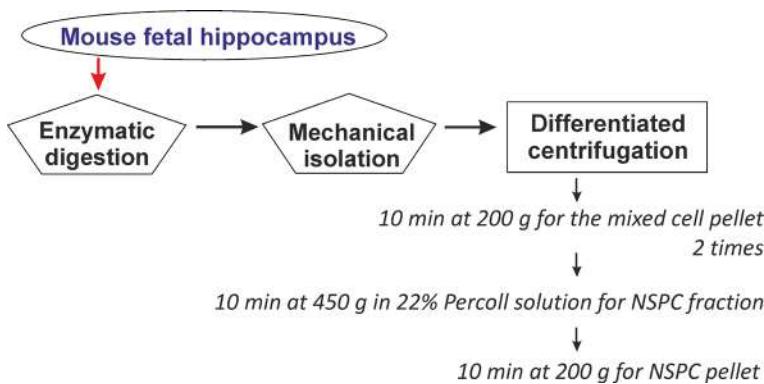


Figure 2. Schematic illustration of the main steps for NSPC isolation from the fetal mouse hippocampus through controlled enzymatic digestion and subsequent mechanical dissociation of the cells, followed by a number of centrifugation/resuspension steps

D. Testing the viability of isolated stem cells

1. Trypan blue staining

Once the suspension of NSPCs has been obtained, perform testing the cell viability using trypan blue. For this, take 20 µl of cell suspension and add 20 µl of 0.4% trypan blue solution to a droplet. Mix the sample well. Load the mixture into a glass hemocytometer very gently. Let the cells fill the chamber of hemocytometer by capillary action. Count the viable cells versus damaged cells (considering those brightly stained with trypan blue). It is advised that the proportion of viable cells shall to get over at least 75%.

Note: For the assessment of cell viability, another dye can be else used. We probed propidium iodide, a commonly used red-fluorescent DNA counterstain, to mark dead cells in a population. This approach requires equipment for detection of the propidium iodide-mediated fluorescence.

2. FACS-based analysis

Take a suspension of the obtained NSPCs (approximately 5×10^5 cells) and resuspend it in 100 µl of BD Cell Wash buffer. Transfer the mixture to a flow cytometer and add 7-AAD (5 µl). Mix carefully. Incubate the cells for 10 min at room temperature. After incubation, perform analysis (we use BD FACSDiva™ 6.1.2 software). Typically, viable cells count up to ~88% (see Figure 3). This confirms the high viability of embryonic NSPCs obtained from mouse hippocampi using the described protocol.

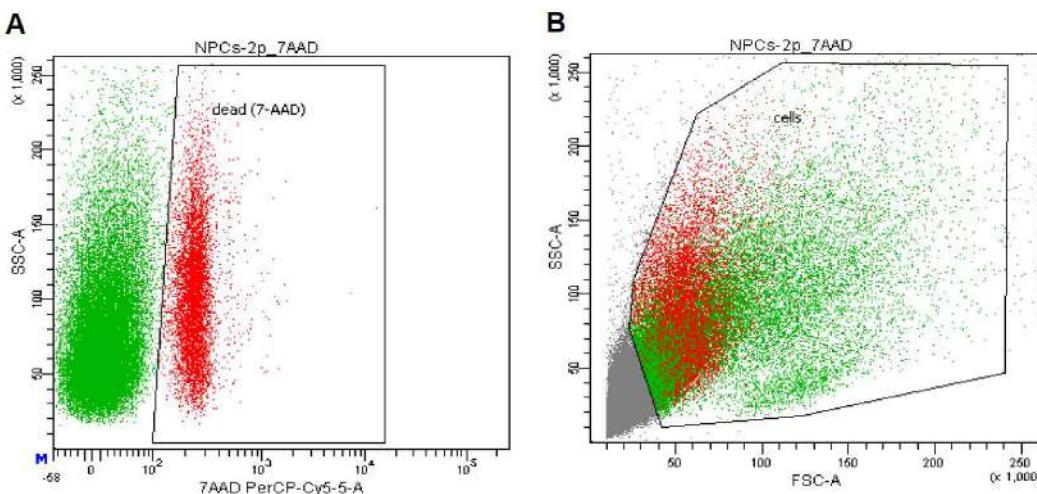


Figure 3. FACS-based analysis of embryonic NSPCs, freshly isolated from the fetal mouse hippocampi. A. Dot plot with gate of 7-AAD-positive non-viable (red) cells to show the proportion of dead cells after isolation. B. Distribution of viable (green) and non-viable (red) cells by morphology on the dot plot FSC vs. SSC. At least 5×10^4 cells were analyzed for each sample using BD FACSDiva™ software.

E. Growing embryonic NSPCs in a 2D culture

1. Take the obtained NSPC suspension (1×10^5 cells/ml) and add 250 μ l of FGF-2 (20 ng/ml) to the cells. We seed the cells on the Matrigel-coated coverslips at a 24-well plate by diluting the mixture to get the total volume of 50 ml.
Note: The total volume depends on the desired cell density (number of coverslips) to grow.
2. To seed NSPCs in a 2D culture, take 0.5 ml of the mixture and transfer the cells onto the Matrigel-coated coverslips at a 24-well plate.
Note: For a 2D culture, seed NSPCs by distributing cells evenly across the coverslip surface, since cell aggregates dramatically influence growth and subsequent differentiation of NSPCs in a monolayer cell culture.
3. Place the plate in a CO₂ incubator (35 °C, 5% CO₂) and incubate for the next 24 h.
4. Next day, check the cell survival using an inverted microscope. At this point, change the growth medium (0.5 ml/well) (see Recipes) by gently aspirating the entire medium from each well containing coverslips with the cells.
5. Change the growth medium every two days. While performing the changes, visually assess cultures for cell growth. We present an example of embryonic stem cells shortly after plating in a 2D culture (see Figure 4A). Undifferentiated cells can be confirmed by immunostaining for nestin, a specific marker of undifferentiated neural stem cells, showing typically up to 97% nestin-positive staining in 2D cultures (see Figure 4B).

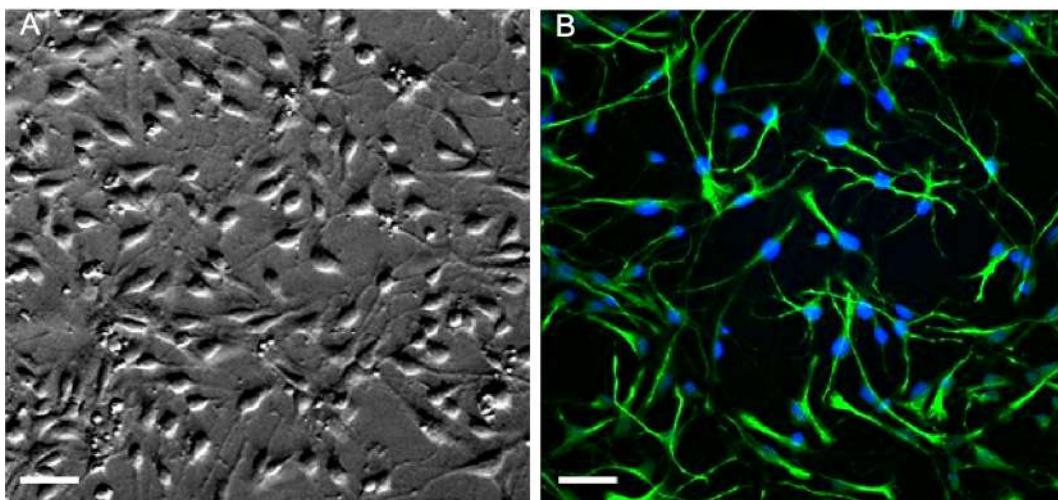


Figure 4. Example images of embryonic NSPCs in a 2D culture. A. Transmitted light image of undifferentiated NSPCs isolated from the fetal mouse hippocampi shortly after plating in an adherent 2D culture (snapshot taken on Day 1 *in vitro*). B. Confocal image of immunostained NSPCs for nestin (green) and Hoechst 33342 (blue) staining. Scale bars, 30 μ m.

F. Passaging NSPCs in a 2D culture

1. Passage the cells once NSPCs reach about 80% confluence.
2. Aspirate all media from each well containing the cells at a 24-well plate. Gently wash the attached cells with a warm PBS (see Recipes).
3. Take 200 μ l Accutase (1x solution, thawed in advance at room temperature) and add it to the cells (each well of the plate). Incubate the cells with Accutase in a CO₂ incubator (at 35 °C) for 5 min.
4. Check under a microscope that cells are detached and then add 1 ml of growth medium to the cells (to dilute/deactivate Accutase).

Note: If cells are not detached after 5 min of incubation with Accutase, return the 24-well plate into a CO₂ incubator (up to 5 min), by checking every minute or two if the cells have detached.

5. Collect the detached cells to a 1.5 ml Eppendorf tube and centrifuge the suspension at 200 x g for 10 min (room temperature). Discard the supernatant and re-suspend the pellet in 1 ml of growth medium.

Note: Perform a check of the NSPC viability for each of the obtained passage (we use trypan blue staining, but another approach can also be used). Also, estimate the density of the obtained suspension before seeding the cells. If the density is higher than 1 x 10⁵ cells/ml, dilute the suspension accordingly (typically, 1:2).

6. Take 0.5 ml of the obtained suspension and transfer the cells onto each of the Matrigel-coated coverslips in a 24-well plate for seeding the next passage of NSPCs.

Note: We recommend seeding NSPCs in a 2D culture in the density at least 3 x 10⁴ cells per coverslip, as we have established experimentally.

G. Generating 3D neuro-spheroids by embryonic NSPCs

1. Formation of the adherent neuro-spheroids (neurospheres)

For the formation of 3D neurospheres take a suspension of NSPCs (8×10^4 cells per coverslip) and resuspend the cells in the culture medium (without supplementing FGF-2). As we have observed routinely, removing FGF-2 from the culture medium boosts spontaneous differentiation of embryonic NSPCs and facilitates the formation of the adherent neuro-spheroids.

2. Transfer cells onto the Matrigel-coated coverslips at a 24-well plate in a volume of 0.5 ml per coverslip. Place the plate in a CO₂ incubator (35 °C, 5% CO₂).

Note: For the formation of adherent neuro-spheroids, coverslips need to be coated with Matrigel. There is no requirement to coat coverslips if experimental design includes formation of floating neuro-spheroids.

3. Neurospheres will become forming within first 24-48 h after plating the cells. There is no need to change the culture medium during that period of time in order to not disrupt the formations. Neurospheres progressively grow over the time in a 3D culture. After 6-7 days in a 3D culture, embryonic NSPCs generate stable neuro-spheroids, which can develop up to 150-200 µm (see Figure 5).

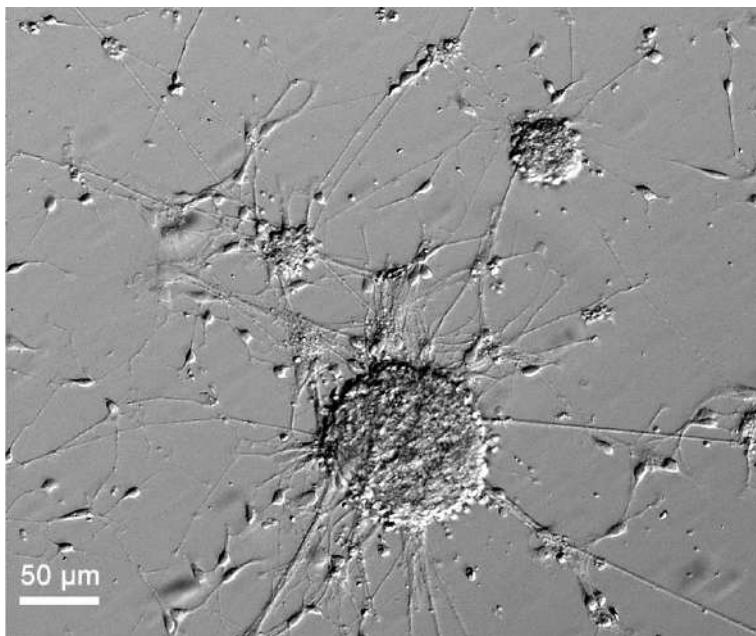


Figure 5. Typical neuro-spheroids generated by embryonic mouse NSPCs after 5 days in an adherent 3D culture. Scale bar: 50 µm.

Note: We recommend not growing embryonic NSPCs in a 3D culture for over 9-10 days since 3D neurospheres facilitate spontaneous cell differentiation. At that time-point, 3D neuro-spheroids can reach above 300 µm that could promote cell death within the formation core. Therefore, it is strongly advised passaging NSPCs before 3D neuro-spheroids reach overgrowth.

H. Immunostaining of NSPCs in 2D or 3D cultures

1. Aspirate all the media from 2D or 3D cultures of embryonic NSPCs. Wash the cells with PBS (pre-warmed to room temperature) to remove dead cells and any debris.

2. Add 500 µl of 4% PFA in 0.1 M phosphate buffer to each well containing the cells. Incubate for 30 min at room temperature.

3. Wash with PBS at least 2 times.

Note: We recommend using ~0.5 ml of the buffer to ensure sufficient washing. At this step, cells may be stored until proceeding with immunostaining (up to a week time). In such a case, seal the plate containing coverslips with the cells hermetically with parafilm to avoid drying and keep it at 4 °C.

4. For blocking unspecific staining incubate the cells (or neuro-spheroids) in blocking solution containing 0.3% Triton X-100 and 0.5% BSA (see Recipes) for 1.5 h at room temperature.

5. Incubate with primary antibodies in antibody solution containing 0.3% Triton X-100 and 0.5% BSA (see Recipes) for overnight at 4 °C. We use 200 µl of antibody solution per well.

Note: Shaking the plate in a slow rate is useful (but not required) during incubation in blocking solution or with antibodies for facilitating the treatment.

6. Wash the cells/neurospheres 3 times in PBS, each for 15 min.

7. Incubate with secondary antibody(ies) in antibody solution containing 0.5% BSA (without Triton X-100) for 1 h at room temperature in the dark. We use 200 µl of the solution per well.

8. Wash 3 times in PBS for 15 min.

9. Incubate with Hoechst 33342 (1:5,000 in PBS) for 3-5 min at room temperature in the dark.

10. Wash 3 times in PBS. Then wash in ddH₂O to rinse out all salts. The cells/spheroids are ready for imaging.

11. Imaging

We carry out imaging of the immunostained cells on a glass slide. For this, mount the coverslips containing a 2D culture (or 3D neuro-spheroids) onto the SuperFrost® glass slides, using a mounting medium (ImmunoHistoMount). Then let the slides air-dry for overnight at room temperature. Keep the samples at 4 °C. With such an approach, the immunostained cell preparations can be stored as long as needed. We present the example images for immunostaining of i) the adherent neuro-spheroids generated by embryonic NSPCs for both nestin and β-tubulin III staining (see Figure 6) and ii) an adherent monolayer of embryonic NSPCs for both nestin and Hoechst staining in a 2D culture (see Figure 4B). Staining for other proteins can also be performed using this method.

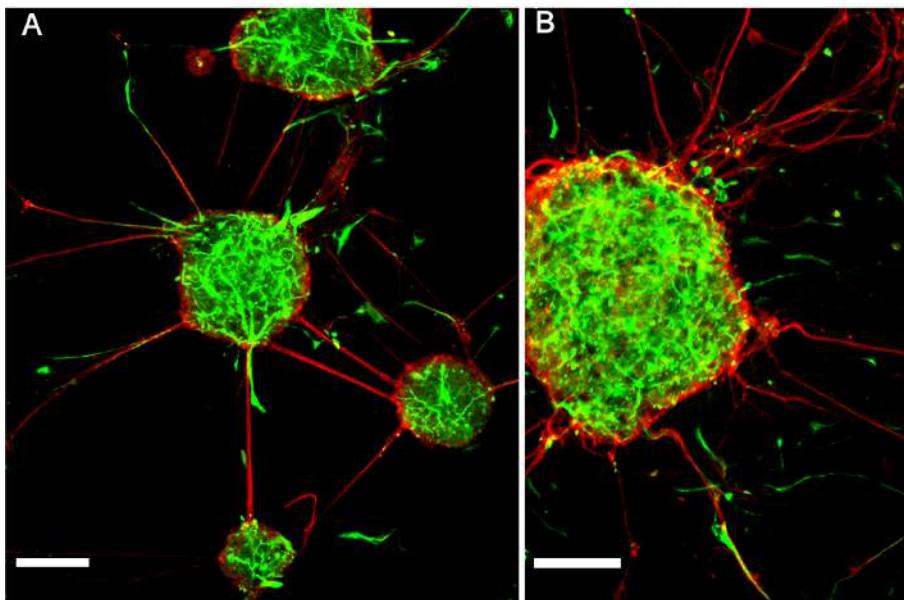


Figure 6. Immunofluorescent staining of neuro-spheroids generated from mouse embryonic NSPCs in a 3D culture. A and B. Confocal images of neuro-spheroids generated by embryonic NSPCs merged for nestin (green) and β -tubulin III (red) staining on Day 5 in a 3D culture. Scale bars, 50 μ m (A) and 40 μ m (B).

I. Engraftment of mouse embryonic NSPCs onto organotypic hippocampal tissue

1. Preparation of the hippocampal slices

Sacrifice pups of FVB mice at P8-P9 according to the procedures approved by your Institute. Decapitate the animals, dissect out the brains and remove both hippocampi into a cold dissecting medium (see Recipes), as we have described above and previously (Rybachuk *et al.*, 2017). Place each hippocampus on a tissue chopper disc, mounted on a tissue chopper (McIlwain), and initiate chopping to cut the tissue to the transverse slices of 350- μ m thick. Flash hippocampal slices into a Petri dish containing a cold dissecting medium and turn slices over to make their medial side faces up. Collect only hippocampal slices those have a well-recognized hippocampal-like morphology.

Note: For ‘good’ slicing the tissue, we recommend to put a filter paper above the chopper disc and place the hippocampus on it to avoid slipping and wobbling the tissue by the blade. Also, remove excess medium around the tissue before initiating chopping.

2. Organotypic hippocampal tissue cultures

Transfer slices using a glass Pasteur pipette with the flame-polished large-sized tip (inner diameter of near 3 mm) onto a surface of 0.4- μ m membrane inserts placed at a 6-well plate. We typically plate four slices on a 0.4- μ m membrane insert. Remove the spare medium using a glass Pasteur pipette of a small-sized tip (inner diameter of around 0.8 mm). Add 1 ml of culture medium (see Recipes) to each well containing slices on membrane insert. Place the plate in a CO₂ incubator (35°C, 5% CO₂). Change the culture medium on Day 2 after plating. Afterward, change the media every two days.

Note: A number of membrane inserts (wells at a tissue culture plate) depend on the amount of slices cut ‘good’ and may be adjusted from a 6-well plate to a 24-well plate.

3. Engraftment of embryonic NSPCs

Take 50 μ l of a suspension of freshly isolated NSPCs and place the cells onto the surface of organotypic hippocampal tissue using a standard pipette (Eppendorf, Research[®] Plus). We grafted embryonic NSPCs onto hippocampal tissue at 7 days post-plating, but any time-point can be used. Any timing needs to be adjusted accordingly to experimental design. Transfer NSPCs by very carefully distributing the cells evenly across the tissue surface, not forming cell aggregates. Also, make sure that NSPCs settle exclusively on the tissue surface. Do not move the plate sharply to prevent the freshly engrafted cells drop down on membrane inserts. At Day 2 after engraftment, change the culture medium to wash out NSPCs not attached to a host tissue.

Note: We recommend engraftment of NSPCs at the density of 0.25×10^5 cells per slice. NSPCs from 2D or 3D cultures may also be engrafted after careful dissociation of the cells to ensure homogeneous distribution across the surface of a host tissue.

4. Growth of embryonic stem cells within a host organotypic tissue.

Maintain organotypic hippocampal tissue with NSPC grafts in a CO₂ incubator (35 °C, 5% CO₂) until use. Change the culture medium every two days.

Note: We recommend changing the culture medium by aspirating it under membrane inserts.

5. Check the growth of differentiating NSPCs within a host tissue. This can be performed at any time-point due to simple tracing of the GFP-labeled NSPCs within the tissue. After 3 days, the grafted NSPCs began to incorporate into a host tissue (see Figure 7). Immunostaining in organotypic hippocampal tissue is performed similarly to the procedure described for cell cultures above and in our previous works (Rybachuk et al., 2017; Kopach et al., 2018).

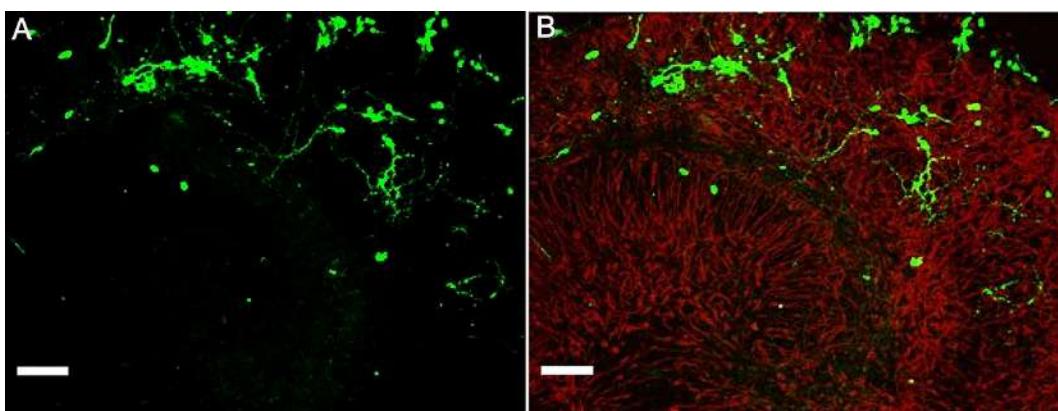


Figure 7. Example of embryonic mouse NSPCs grafted onto a host hippocampal tissue.

A and B. Confocal images of immunostained organotypic hippocampal tissue with GFP-labeled NSPCs (green, A) and merge image of GFP-labeled NSPCs (green) and GFAP (red) staining (B) on Day 3 after engraftment. Scale bars, 100 μ m.

Data analysis

The obtained suspension of embryonic NSPCs can be used to grow and/or differentiation of the cells *in vitro* or within endogenous environment of a host tissue.

1. In a 2D culture, the growth of the cells can be monitored visually and confirmed through immunostaining for nestin (red) and Hoechst 33342 (blue), as shown in Figure 4. See also Kopach *et al.* (2018), Figure 1B, for immunostaining images of NSPC fraction at 1 d *in vitro* and also Figure 6A for statistical analysis of the proportion of nestin-positive NSPCs (undifferentiated progenitors) in different experimental conditions.
2. In 3D cultures, NSPCs can follow a robust multi-lineage differentiation. NSPCs isolated from the fetal mouse hippocampi can generate neuro-spheroids, the formation that enhances cell growth and differentiation and facilitates maintaining the high viability of differentiating progenitors. For the assessment whether neurons or glia are generated from embryonic NSPCs in a 3D culture, use of the cell type-specific markers is required. For visualization of phenotypic distribution within neuro-spheroids, we carried out immunostaining of differentiating progenitors for astrocytic marker GFAP together with neuronal marker NeuN and oligodendrocyte-specific marker olig-2. The example shown are images of phenotypic differentiation of embryonic NSPCs taken on Day 5 of 3D culture (see Figure 8).

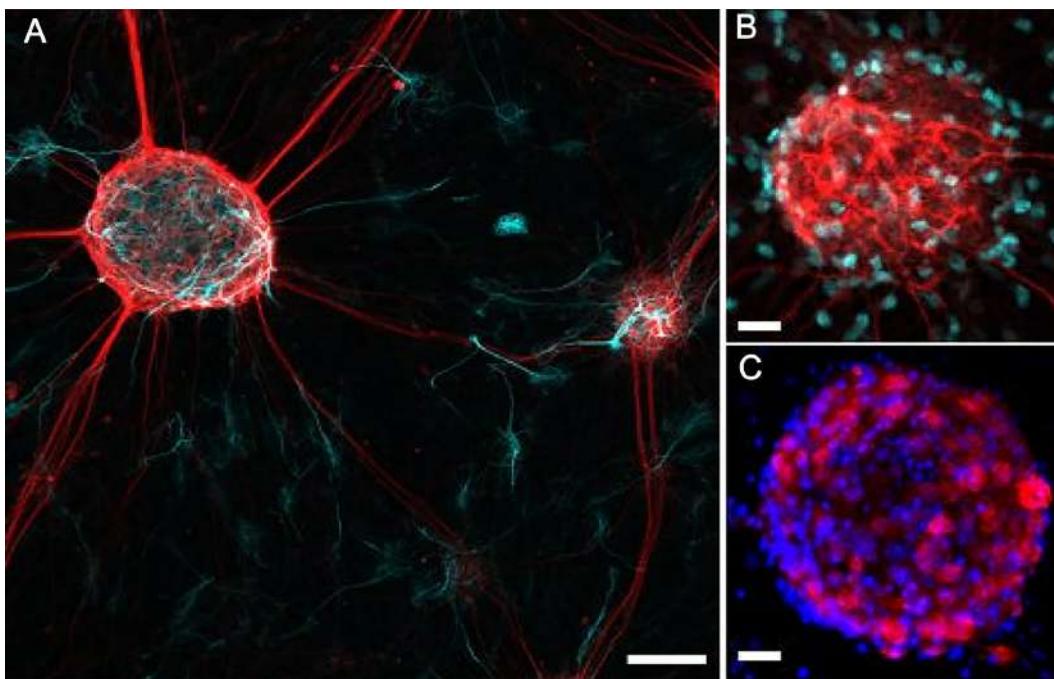


Figure 8. Immunofluorescent staining of 3D neuro-spheroids generated by embryonic mouse NSPCs. A. Confocal image of immunostained neuro-spheroids of embryonic hippocampal NSPCs for GFAP (cyan) and β -tubulin III (red) staining on Day 5 of 3D culture. B. Confocal image of neuro-spheroid generated by embryonic NSPCs for GFAP (red) and olig-2

(cyan) staining. C. Confocal image of the NSPC-generating neuro-spheroid for Hoechst 33342 (blue) and NeuN (red) staining. Scale bars, 50 μ m (A) and 20 μ m (B and C).

3. In a host brain tissue, organotypic hippocampal slices where both morphological layer architecture and signaling pathway assemblies remained preserved for the required period of time (weeks of tissue maintenance), embryonic NSPCs displayed a prompt multi-lineage neurogenesis. Using organotypic hippocampal tissue, we enabled to trace the time-dependent maturation of NSPC-derived hippocampal neurons through monitoring neuronal excitability with electrophysiological approaches. In particular, we recorded the passive membrane properties from differentiating progenitors [see Kopach *et al.* (2018), Figure 1], neuronal firing [see Kopach *et al.* (2018), Figure 3] and synaptic events spontaneously evoked in NSPC-derived neurons [see Kopach *et al.* (2018), Figure 2]. Organotypic hippocampal tissue represents a useful tool to feasibly assess maturation of neurophysiological properties of differentiating NSPCs within endogenous host environment at various time-points after engraftment. Moreover, it is implementable to carry out the direct quantitative comparisons between the functional properties of maturing progenitors versus endogenous principal neurons. Glial lineage of differentiating NSPCs can be further confirmed using immunostaining for the cell type-specific markers (see the description of the protocol in Kopach *et al.* [2018] and Figure 6F, for images of NSPC-derived oligodendrocytes or astroglia at 2 or 3 weeks post-grafting).

Recipes

1. 0.2 M Phosphate buffer (total volume 250 ml; pH 7.4)
 - a. Take 1.56 g sodium dihydrogen phosphate (NaH_2PO_4) and add 50 ml ddH₂O
 - b. Take 5.68 g disodium phosphate (Na_2HPO_4) and add 200 ml ddH₂O
 - c. Mix 200 ml diluted Na_2HPO_4 with 50 ml diluted NaH_2PO_4
 - d. Store 0.2 M phosphate buffer (pH 7.4) at 4 °C
2. 4% Paraformaldehyde (PFA) in 0.1 M phosphate buffer (total volume 100 ml)
 - a. Take 4 g PFA and add it into 50 ml ddH₂O while stirring at 60 °C
 - b. To facilitate dilution of PFA in ddH₂O, add 20 μ l NaOH to the mixture while stirring
 - c. Stir the mixture for approximately 30 min
 - d. Add 50 ml of 0.2 M phosphate buffer to the mixture
 - e. Filter the mixture using a 0.45- μ m filter
 - f. Store 4% PFA at -20 °C if not use it over the next days
3. Growth medium (total volume 50 ml)
 - a. Take 48.5 ml Neurobasal medium and add it to a 50 ml Falcon tube
 - b. Add 1 ml B-27® (50x)
 - c. Add 0.5 ml GlutaMAX™ (100x)
 - d. Add 50 μ l N-acetyl-L-cysteine (NAC) and 0.25 ml P/S

- e. Store the medium at 4 °C if not use immediately
- f. Add 20 ng/ml FGF-2 to the medium just before use it
4. Tissue dissecting medium (total volume 100 ml; pH 7.3)
 - a. Take 50 ml MEM and add it to a Falcon tube
 - b. Add 25 ml HBSS
 - c. Add 60 µg Tris to get its final concentration of 5 mM
 - d. Add 17.5 µg NaHCO₃ (final concentration 2 mM)
 - e. Add 1.26 ml HEPES (final concentration 12.5 mM)
 - f. Add 276.5 µg glucose (final concentration 15 mM)
 - g. Add 1 ml P/S
 - h. Dilute in ddH₂O to get the total volume of 100 ml
 - i. Filter the prepared medium using a 0.45-µm sterile filter
 - j. Store the medium at 4 °C (or freeze if not use it over the next days)
5. Tissue culture medium (total volume 100 ml; pH 7.2)
 - a. Take 50 ml MEM and add it to a Falcon tube
 - b. Add 25 ml HBSS
 - c. Add 30 µg Tris (final concentration 2.5 mM)
 - d. Add 17.5 µg NaHCO₃ (2 mM)
 - e. Add 1.26 ml HEPES (12.5 mM)
 - f. Add 276.5 µg glucose (15 mM)
 - g. Add 1 ml P/S
 - h. Filter the medium through a 0.45-µm sterile filter and store it at 4 °C (or freeze it if not use)
 - i. Add 250 µl/ml horse serum to the medium right before using
 - j. Add 20 µl/ml B-27® supplement (50x) to the medium before using
6. Antibody solution
Dissolve BSA (0.5%) in PBS just before use it
7. Blocking solution
Dissolve BSA (0.5%) and Triton X-100 (0.3%) in PBS before use

Acknowledgments

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Competing interests

Authors declare that there are no conflicts of interest or competing interests.

Ethics

All procedures were used in accordance with the protocols approved by the Animal Care and Use Committee at Bogomoletz Institute of Physiology and State Institute of Genetic and Regenerative Medicine (Kyiv, Ukraine) and were within the European Commission Directive (86/609/EEC) guidelines.

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Differentiation of Human Induced Pluripotent Stem Cells (iPSCs) into an Effective Model of Forebrain Neural Progenitor Cells and Mature Neurons

Scott Bell, Nuwan C. Hettige, Heika Silveira, Huashan Peng, Hanrong Wu, Malvin Jefri, Lilit Antonyan, Ying Zhang, Xin Zhang and Carl Ernst*

Psychiatric Genetics Group, McGill University and Douglas Hospital Research Institute, Department of Psychiatry, Verdun, Montreal, QC H4H 1R3, Canada

*For correspondence: carl.ernst@mcgill.ca

[Abstract] Induced Pluripotent Stem Cells (iPSCs) are pluripotent stem cells that can be generated from somatic cells, and provide a way to model the development of neural tissues *in vitro*. One particularly interesting application of iPSCs is the development of neurons analogous to those found in the human forebrain. Forebrain neurons play a central role in cognition and sensory processing, and deficits in forebrain neuronal activity contributes to a host of conditions, including epilepsy, Alzheimer's disease, and schizophrenia. Here, we present our protocol for differentiating iPSCs into forebrain neural progenitor cells (NPCs) and neurons, whereby neural rosettes are generated from stem cells without dissociation and NPCs purified from rosettes based on their adhesion, resulting in a more rapid generation of pure NPC cultures. Neural progenitor cells can be maintained as long-term cultures, or differentiated into forebrain neurons. This protocol provides a simplified and fast methodology of generating forebrain NPCs and neurons, and enables researchers to generate effective *in vitro* models to study forebrain disease and neurodevelopment. This protocol can also be easily adapted to generate other neural lineages.

Keywords: iPSC, Forebrain, Cortical, NSC, NPC, Neuron

[Background] Induced pluripotent stem cells (iPSCs) are stem cells produced from non-pluripotent source cells and tissues (Shi *et al.*, 2017). Due to their ability to differentiate into a wide range of cell types, they are a promising avenue for improving our understanding of human development and treatment of degenerative diseases (Marchetto *et al.*, 2011). Of particular interest are iPSC-derived models of human forebrain neurons, as these cells are known to mediate higher order brain functions, including consciousness (Baxter and Chiba, 1999), emotion (Morgane *et al.*, 2005), and sleep (Schwartz and Roth, 2008). As a result, deficits in these cells can cause a wide range of neurological disorders, including neurodegenerative diseases like Alzheimer's (Auld *et al.*, 2002) and Huntington's (McColgan and Tabrizi, 2018), as well as neurodevelopmental diseases such as autism (Donovan and Basson, 2017) and epilepsy (Heath, 1976). As there are few effective forebrain models for humans, the discovery of iPSCs spurred a rapid push to develop effective protocols to differentiate iPSCs to forebrain neurons (Srikanth and Young-Pearse, 2014). The first protocols that were developed drew upon previous work using embryonic stem cells (ESCs), which relied upon feeder cell cultures. This complicated the procedure and raised concerns about clinical applications. Later protocols were able to generate

forebrain neurons without using feeder cells (Bell *et al.*, 2017), with some eliminating all animal generated products entirely (Yuan *et al.*, 2015). It can be difficult to make an all-encompassing statement about the protocols currently used to generate forebrain NPCs, due to the multitude of labs currently generating forebrain neurons and the many variables that can be changed and optimized. However, many of the recently most cited published protocols for the generation of forebrain NPCs and neurons can be divided into two kinds, monolayer and embryoid bodies (EBs) protocols. In an EB based protocol, iPSCs are dissociated and plated in suspension in a neural induction media to allow them to form EBs, which gradually aggregate over 5-7 days (Pasca *et al.*, 2011). These EBs are then transferred to a plate that supports cell attachment, enabling the embryoid bodies to attach to the bottom of the plate and spread out into a neural rosette. From this rosette, neural stem cells (NSCs) arise, which can be passaged to form relatively stable neural progenitors cells (NPCs) (Shi *et al.*, 2012). NPCs can then be plated in a neuronal induction media to give rise to mature neurons (Bell *et al.*, 2017). Monolayer based protocols chiefly differ in that iPSC colonies are maintained as a monolayer during neural induction, and develop directly into rosettes without aggregation (Chandrasekaran *et al.*, 2017). Using either approach, generation of NPCs from iPSCs is typically reported to require 21-30 days, with electrically active neurons requiring an additional 30+ days of differentiation from NPCs, for a total time of 50+ days to generate forebrain neurons from iPSCs (Yuan *et al.*, 2015).

This protocol describes a methodology for generating forebrain neurons from iPSCs, where iPSC colonies are induced to form neural rosettes without mechanical dissociation, and neural progenitor cells are purified from immature clusters of neural cells, known as neural rosettes based on differential adhesion. Neural progenitor cells will not attach to non-adherent plates and aggregate together in a floating mass, while other cell types either adhere or float but do not aggregate with NPCs (Bell *et al.*, 2017). This allows rapid purification of NPCs, has the potential for automation and enables the generation of NPC cultures within 14 days of initiation of differentiation. This modification does not appear to negatively influence the fate of the cells, as we observe uniform staining for key neural progenitor cells markers (Zhang *et al.*, 2010; Venere *et al.*, 2012; Zhang and Jiao, 2015). Indeed, we have found that we are capable of recording electrical activity from neurons consistently in as little as five days of differentiation from NPCs. This protocol can be used to generate forebrain neurons simply and effectively for use in investigating neurodevelopment, the etiology of diseases that affect the forebrain, and drug testing.

Materials and Reagents

A. For Cell Culture

1. 6-well plate (SARSTEDT, catalog number: 83.3920)
2. 35-mm dish (SARSTEDT, catalog number: 83.3900)
3. 60-mm dish (SARSTEDT, catalog number: 83.3901.300)
4. 100-mm dish (SARSTEDT, catalog number: 83.3902.300)
5. Petri dishes (Fisher Scientific, catalog number: FB0875713)

6. Coverslips (Fisher, catalog number: 12-545-80)
 7. Liquid nitrogen (PRAXAIR, catalog number: 7727-37-9)
 8. iPSCs, either derived from somatic cells or thawed from a frozen aliquot
 9. TeSR™-E8™ Media (Stem Cell Technologies, catalog number: 05990)
 10. BrainPhys™ Neuronal Medium (Stem Cell Technologies, catalog number: 05790)
 11. Matrigel® (Corning, catalog number: 354277)
 12. KnockOut™ DMEM/F-12 (Thermo Fisher, catalog number: 12660012)
 13. DMSO (Sigma-Aldrich, catalog number: C6164)
 14. StemPro NSC SFM (Thermo Fisher, catalog number: A1050901)
 15. SM1 Neuronal Supplement (Stem Cell Technologies, catalog number: 05711)
 16. N2 Supplement-A (Stem Cell Technologies, catalog number: 07152)
 17. BSA (Gibco, catalog number: 16140071)
 18. Non-Essential Amino Acid (NEAA) (Gibco, catalog number: 11140050)
 19. SB431542 (Stem Cell Technologies, catalog number: SB431542)
 20. Noggin (Gibco, catalog number: PHC1506)
 21. Laminin (Sigma-Aldrich, catalog number: L2020)
 22. Gentle Cell Dissociation Reagent (Stem Cell Technologies, catalog number: 07174)
 23. Epidermal growth factor (EGF) (Sigma, catalog number: E9644)
 24. Fibroblast growth factor (FGF) (Sigma, catalog number: F0291)
 25. Brain-Derived Neurotrophic Factor (GenScript, catalog number: Z03208-25)
 26. Glial-Derived Neurotrophic Factor (GenScript, catalog number: Z02927-50)
 27. Accutase (Sigma-Aldrich, catalog number: SCR005)
 28. DPBS without CaCl₂ and MgCl₂ (Sigma-Aldrich, catalog number: D8537)
 29. Neural Induction Medium 1 (see Recipes)
 30. Neural Induction Medium 2 (see Recipes)
 31. Neural Progenitor Media (see Recipes)
 32. Neuronal Media (see Recipes)
 33. Culture Dish Coating with Matrigel® (see Recipes)
- B. For Immunocytochemistry (ICC)
1. Glass coverslips (Fisher, catalog number: 12-545-81)
 2. Microscope slides (Fisher, catalog number: 12-552-3)
 3. Pipette tips, 1 ml (SARSTEDT, 70.1186)
 4. Pipette tips, 200 µl (SARSTEDT, 70.1186)
 5. Pipette tips, 20 µl (SARSTEDT, 70.1186)
 6. 15-ml conical tube (SARSTEDT, 62.554.002)
 7. Paraformaldehyde (PFA) (Sigma-Aldrich, catalog number: 252549)
 8. BSA (Sigma-Aldrich, catalog number: A2058)
 9. Triton X-100

10. DAPI (Thermo Fisher, catalog number: 62248)
11. Vectashield® (Vector Labs, catalog number: H-1000)
12. Nail Polish (Sally Hansen Insta-Dri Fast-Dry Clear Nail Color)
13. Antibodies

Antibodies for iPSCs:

- a. TRA-1-60 (Embryonic Stem Cell Marker Panel, Abcam, catalog number: ab109884)
- b. SSEA (Embryonic Stem Cell Marker Panel, Abcam, catalog number: ab109884)
- c. Nanog (Embryonic Stem Cell Marker Panel, Abcam, catalog number: ab109884)
- d. OCT4 (Stemcell Technologies, catalog number: 60093)
- e. PAX6 (Stemcell Technologies, catalog number: 60094)

Antibodies for NPCs:

- a. SOX1 (Stemcell Technologies, catalog number: 60095)
- b. Nanog (Embryonic Stem Cell Marker Panel, Abcam, catalog number: ab109884)
- c. Nestin (Stemcell Technologies, catalog number: 60091)
- d. PAX6 (Stemcell Technologies, catalog number: 60094)

Antibodies for Neurons:

- a. Tuj1 (Abcam, catalog number: ab14545)
- b. S100B (Abcam, catalog number: ab52642)
- c. VGLUT1 (Abcam, catalog number: ab77822)
- d. GABA (Abcam, catalog number: ab86186)
- e. GFAP (Abcam, catalog number: ab7260)

Secondary Antibodies:

- a. ALEXA 488 anti-mouse (Invitrogen, catalog number: A-11008)
- b. ALEXA 555 anti-rabbit (Invitrogen, catalog number: A-21422)

14. Coating glass coverslips with Poly-ornithine and laminin (see Recipes)

C. For Electrophysiology

1. Borosilicate pipettes with resistances of 3-6 MΩ (World Precision Instruments, catalog number: 1B150-4)
2. Cell strainer (40 µm) (Sigma, catalog number: CLS431750)
3. Tetrodotoxin (TTX) (Alomone labs, catalog number: T-550)
4. BrainPhys™ Without Phenol Red (Stem Cell Technologies, catalog number: 05791)
5. HEPES (Sigma-Aldrich, catalog number: H3375)
6. KCl (Sigma-Aldrich, catalog number: 793590)
7. Potassium Gluconate (Sigma-Aldrich, catalog number: G4500)
8. EGTA (Sigma-Aldrich, catalog number: 324626)
9. Mg-ATP (Sigma-Aldrich, catalog number: A9187)
10. Creatine phosphate (Sigma-Aldrich, catalog number: CRPHO-RO)
11. Guanosine triphosphate (Sigma-Aldrich, catalog number: G8877)

12. NMDA (Alomone labs, catalog number: N-170)
13. Magnesium Chloride hexahydrate (Sigma, catalog number: M2393)
14. Internal pipette solution (see Recipes)

Equipment

1. Pipettes (Fisher, catalog number: 4680100)
2. Pipette puller (Sutter Instrument, model: P-1000)
3. Osmometer (Advanced Instruments, model: 3320)
4. Bead bath (Lab Armor, model: M706)
5. Fluorescent microscope (Olympus, model: 1X73)
6. Recording chamber with six-channel valve controller (Warner Instruments)
7. Automatic temperature controller (Warner Instruments, model: TC-324C)
8. Micromanipulator (Sutter Instrument, model: MP-225)
9. Microelectrode amplifier Multiclamp 770B (Molecular Devices)
10. Acquisition system Axon digitata 1550A (Molecular Devices)
11. Biological Safety Cabinet Class 2 (Nuaire, Model: NU440400)
12. Incubator (Thermo Fisher, catalog number: 51030287)
13. Centrifuge (Allegra, model: X-12)

Software

1. Clampex 10.5 (Molecular Devices, www.moleculardevices.com)
2. GraphPad Prism 7 (GraphPad, www.graphpad.com)
3. Excel 2016 (Microsoft, <https://products.office.com/en-ca/excel>)

Procedure

A. Differentiation from iPSCs to Forebrain NSCs (Neural induction)

Note: The following steps are described assuming high-quality iPSCs (see Figure 1) are plated on a 60-mm tissue culture dish that is coated with Matrigel® (for more details, see Recipes and Table 2).

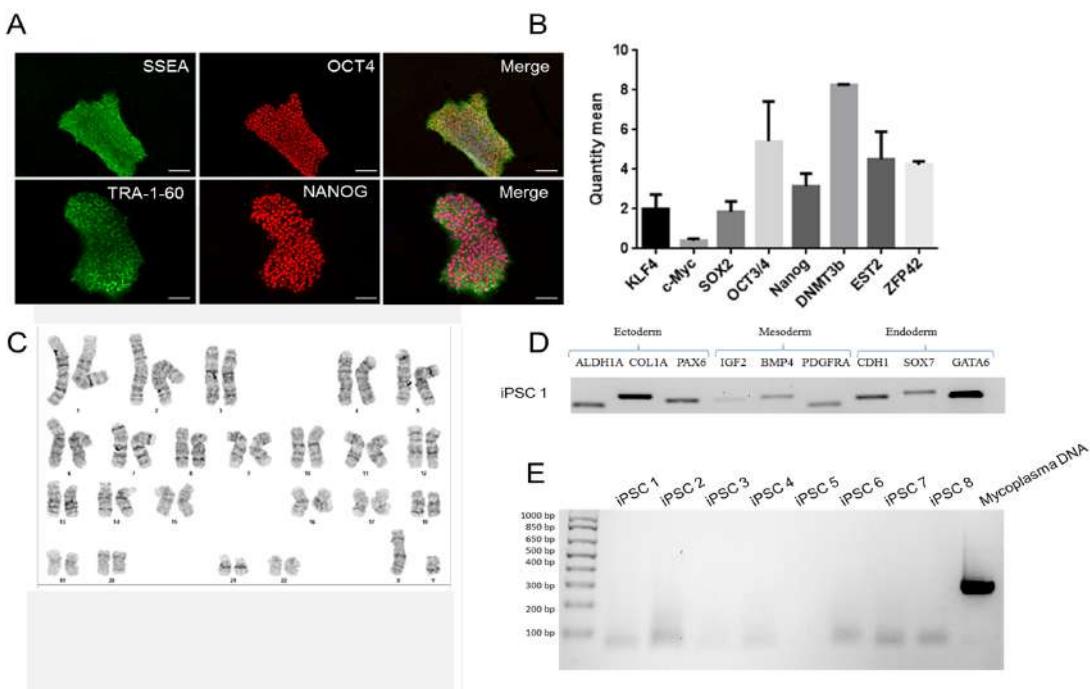


Figure 1. Sample ICC staining for high-quality iPSC cultures. In order for differentiation to proceed effectively, ensure that you begin differentiation with high-quality iPSC cultures. iPSCs should uniformly express pluripotency markers SSEA, OCT4, TRA-1-60, and NANOG (A) when assessed using ICC. Additional pluripotency markers DNMT3b, EST2, and ZFP42 can also be assessed using a qPCR assay (B). iPSCs should be free of karyotypic abnormalities (C), possess the ability to differentiate into all three germ lineages and express characteristic markers of each lineage (D), and test negative for mycoplasma contamination (E). Scale bars represent 100 μ m.

1. Working in a class II biological safety cabinet, use appropriately sized pipettes to plate iPSCs in E8 medium in a 60-mm dish. If starting from a frozen aliquot of iPSC, we recommend plating at least 300,000 cells. This is considered Day 0 of iPSC culture.
2. On Day 1 of iPSC culture (15%-25% confluence), aspirate the spent medium to remove non-attached cells, and check the size of colonies. If colonies are approximately 100-200 μ m in diameter, they are an appropriate size to begin differentiation. Add 3 ml of complete Neural Induction Medium 1, pre-warmed in a bead bath to each plate using a 5 ml pipette. Return the plates to an incubator maintained at 37 °C, 5% CO₂ and atmospheric (~20%) Oxygen. If plates do not contain colonies of sufficient size, add 3 ml of E8 media and check daily until colonies reach the appropriate size.
3. On Day 2 (about 48 h after switching to Neural Induction Medium), change the medium by aspirating old medium from each well. Add 3 ml of pre-warmed complete Neural Induction Medium 1 to each plate.
4. On Day 4 of neural induction, cells will be reaching confluence. **If necessary**, mark any colonies with non-neural differentiation. Remove these unwanted colonies with a 200 μ l pipette tip.

Aspirate the spent medium from each well. Add 3 ml of pre-warmed complete Neural Induction Medium 1 to each plate.

Note: Due to high cell density in the culture from Day 4 onwards, doubling the volume of Neural Induction Medium is very critical for cell nutrition. Also, minimal cell death should be observed from Days 4 to 7 after neural induction. If the color of cells turns yellowish with many floating cells during Days 4 to 7 of neural induction, it indicates that the starting density of iPSCs was too high. In this case, change the Neural Induction Medium every day, remove some colonies and double the volume per well/plate. Ideally, work with these variables to ensure that the media does not continue to turn yellow.

5. On Day 6 of neural induction, cells should be near maximal confluence. Remove any non-neural differentiated cells that can be observed and add 3 ml of complete Neural Induction Medium into each plate.
6. On Day 7 of neural induction, the medium should be switched into Neural Induction Medium 2. Add 3 ml of complete Neural Induction Medium 2 to each plate. The medium should be changed every day for 5 days. For example morphologies, see Figure 2.

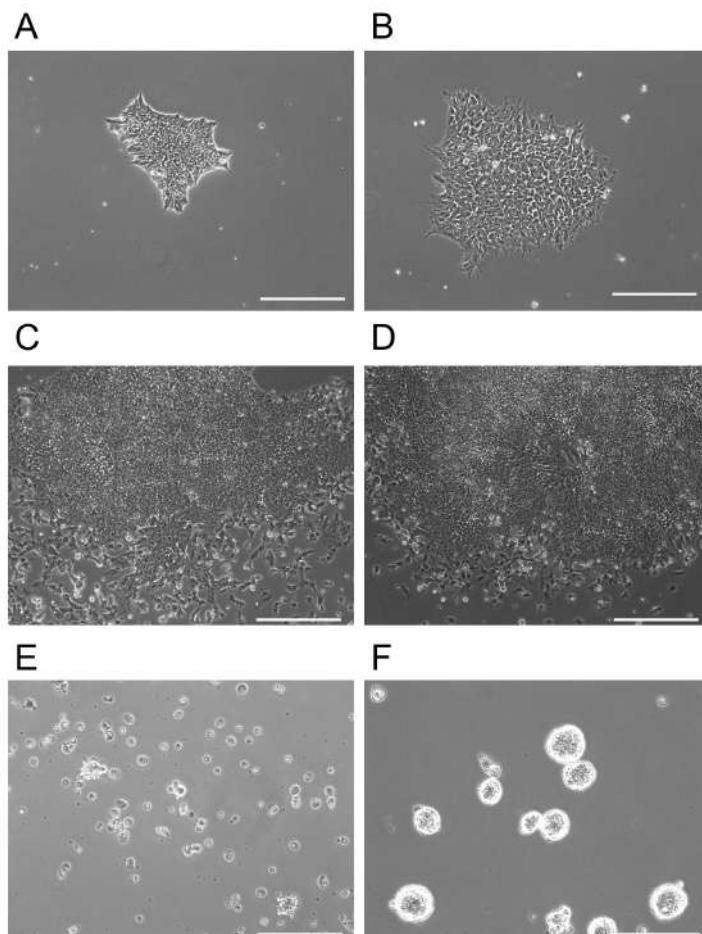


Figure 2. Morphology of iPSCs differentiating into forebrain NPCs. A. Day 0: Showing a single iPSC colony of appropriate size immediately prior to addition of Neural Induction Media

1. B. Day 2: The iPSC colony, which has been treated with Neural Induction Media 1 for 2 days, begins to change cellular morphology and some cells extend processes. C. Day 5: Increased expansion of the colony with some differentiation of outer cells. D. Day 12: Appearance of rosettes in the colony become visible. NSCs are present in high confluence in the middle of these structures. It is at this point that colonies are detached and re-plated on non-adherent plates at D13 for two days. E. D13 immediately after plating on adherent plates. This image shows floating rosette colonies that will continue to proliferate and differentiate in a floating mass. Non-rosette cells either remained on the dish at D12 after chemical release or float as single cells on the non-adherent plates shown in (E). F. At D15, rosette clusters expand in size and are moved to adherent plates. Cell aggregates here are 3-dimensional, but are attached to the plate. Note the purity of the clusters at this point (F). Scale bars represent 130 μ m.

B. Harvest and expansion of NPCs

Note: On Day 12 of neural induction, NPCs are ready to be harvested and expanded.

1. Aspirate the spent Neural Induction Medium from each plate to be passaged.
2. Gently add DPBS without CaCl_2 and MgCl_2 to each plate twice to rinse the cells.
3. Add 1.5 ml of pre-warmed Gentle Cell Dissociation Reagent to each plate and incubate for 5 min at 37 °C until most cells detach from the surface of the culture vessels. Tap plates gently to dislodge cells still attached.
4. Use a pipette to gently rinse the surface of the plates with the Gentle Cell Dissociation Reagent already in the plates to detach any remaining cells.
5. Using a pipette, transfer the cell suspension to a 15-ml conical tube.
6. Add 1 ml of DPBS to each plate to collect residual cells and transfer the cell suspension to the conical tube.
7. Gently pipet the cell suspension up and down 3 times with a 5-ml or 10-ml pipette to break up the cell clumps.
8. Centrifuge the cells at 300 $\times g$ for 5 min.
9. Aspirate the supernatant and re-suspend the cells in pre-warmed Neural Progenitor Cell (NPC) Medium (*i.e.*, 10 ml for all cells from each plate).
10. Plate the cells suspended in NPC Medium onto a 10 cm Petri dish.
11. Culture the cells in a CO_2 incubator for 2 days. During this time, NPCs will form aggregations while floating in NPC medium.
12. Once aggregates have reached an appropriate size of approximately 70-200 μ m, prepare a 10 cm tissue culture dish coated with 5 ml of Matrigel® for at least 1 h. If few aggregates have formed, plate cells in a 60 mm dish instead.
13. Using a 5-ml or 10-ml pipette, take up and pass the NPC medium through a cell strainer to collect NPC aggregations.
14. Reverse the strainer and pass 10 ml of fresh pre-warmed NPC medium through strainer where the cell aggregates are bound so that they are transferred onto the Matrigel®-coated 10 cm plate.

15. Culture the cells in a CO₂ incubator to allow for NPC aggregates to attach to the coated dish and migrate and proliferate.
16. Change medium every 2-3 days until cells reach confluence and are ready for passaging or cryopreservation. Dissociate using warm accutase at 37 °C for five minutes.
17. To assess the purity of NPC culture, fix cells and check for NPC markers using ICC (see Figure 3).
18. To cryopreserve NPCs, freeze in an 80/20 mix of FBS/DMSO. Store at -80 °C for use within a few months, or in liquid nitrogen for long-term storage.

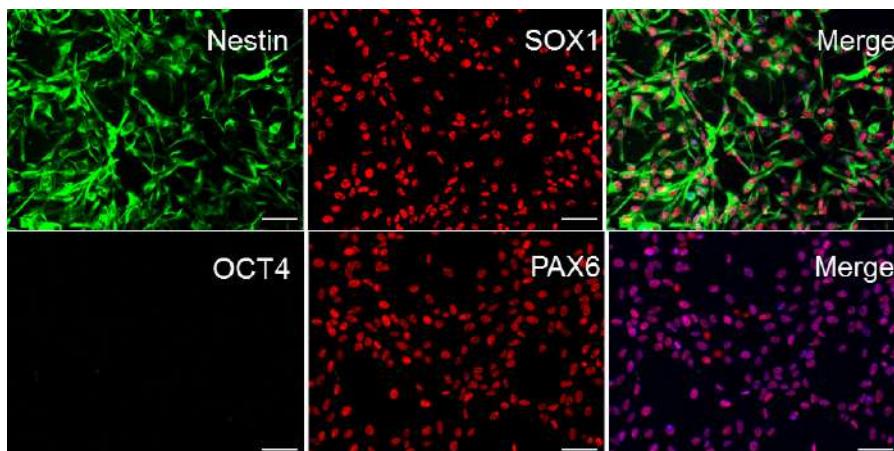


Figure 3. Sample ICC staining for high-quality NPC cultures. In order for differentiation to proceed effectively, ensure that iPSC cultures uniformly express Nestin, SOX1, and PAX6. NPC cultures should have no expression of the pluripotent marker OCT4 (DAPI shown in blue in merge of PAX6 and OCT4; all cells express PAX6, i.e., 100% purity of the culture). Scale bars represent 100 µm.

C. Differentiation of forebrain NPCs into neurons

1. Plate NPCs on a tissue culture dish that is coated with Matrigel®. Wait until cells have achieved 70%-95% confluence before beginning differentiation.
2. Once NPCs have reached desired confluency, aspirate media and replace with an equal volume of Neuronal Media.
3. Every 2-3 days, aspirate half of the media in the plate and replace with fresh Neuronal Media.
Note: As some media will be lost to evaporation, you may need to add a little more media than you remove from the plate in order to keep the media volume stable over time.
4. Continue to change the media until neurons reach the desired stage of development. For example morphologies of developing forebrain neurons, consult Figure 4. For example ICC characterization of forebrain neurons, consult Figure 5.

Notes:

- a. *The purity of your line will be very easily detected during this stage of development. Cell lines that contain a high percentage of NPCs will rapidly polarize and form neuronal*

projections (usually around Days 2-5), whereas lines that contain a high percentage of non NPC cells (Astrocytes, neural crest cells, etc.) will not.

- b. *There is variability in how long neurons in a particular plate will take to reach a certain stage of development depending on line, clone, passage number etc. However, we have found that neurons in good quality cultures consistently achieve the following landmarks by the following number of days into differentiation.*

Day 5 = Cells are post-mitotic

Day 15 = Cells have clearly polarized axons and dendrites, and have clearly detectable electrophysiological properties, such as action potentials

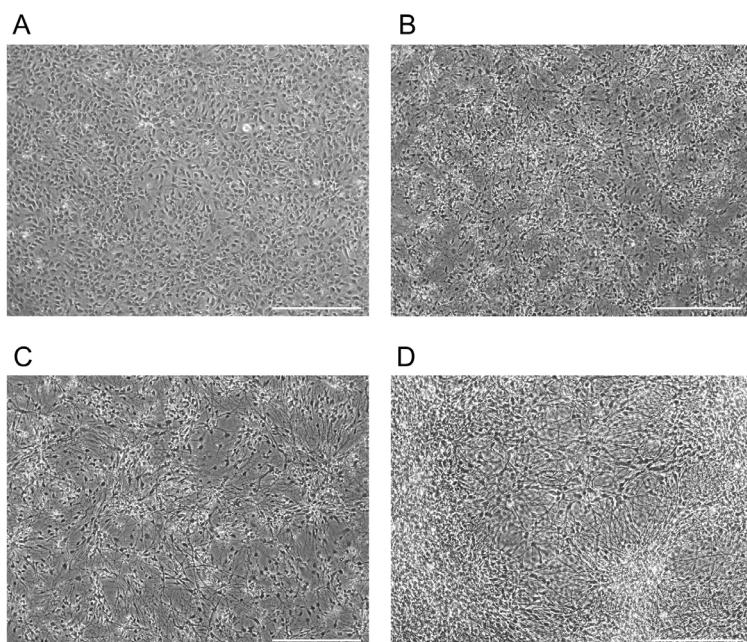


Figure 4. Example morphology for forebrain NPCs differentiating into neurons.
Morphology of a forebrain NPC culture differentiating into neurons. Images taken at D0 (A), D5 (B), D15 (C), D30 (D). Scale bars represent 130 µm.

5. To assess the purity of your neuronal culture, fix cells and check for forebrain markers using ICC. To assess the quality of the neurons produced, ensure that the cells display proper electrophysiological properties (see Data analysis).

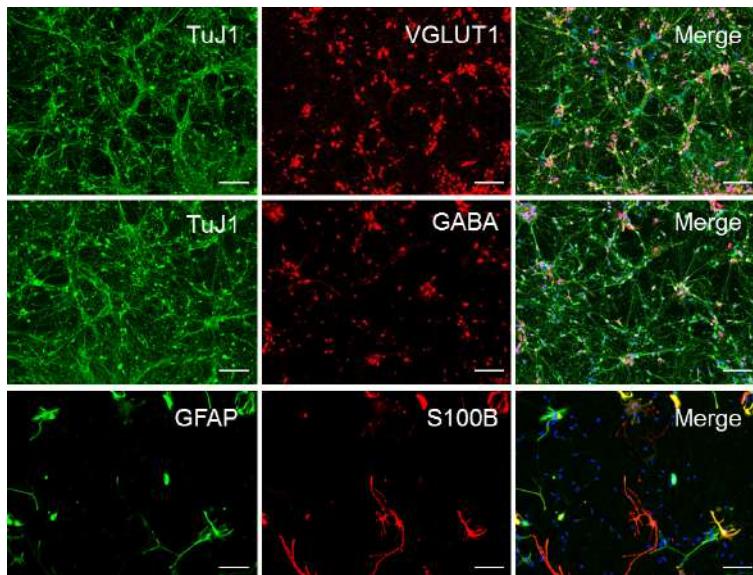


Figure 5. Sample ICC staining for high-quality forebrain neuronal. Representative ICC of forebrain neuronal culture following 30 days of differentiation (D30) from NPCs, demonstrating the relative abundance of glutamatergic, GABAergic, and astrocytic markers in the population. These cultures are approximately 65% glutamatergic, 30% GABAergic, and 5%-10% astroglial. Scale bars represent 50 μ m.

D. Assessment of culture purity using immunocytochemistry

Note: The following steps are described assuming cells are plated on glass coverslips coated with poly-ornithine and laminin (For more details, see Recipes).

1. Fix samples in 4% paraformaldehyde (PFA) diluted in PBS. Incubate at room temperature for 15 min.
2. Wash samples with PBS (3 x 15 min) at room temperature.
3. Permeabilize samples by incubation in PBS + 0.1% Triton X-100 at room temperature for 10 min.
4. Aspirate permeabilization buffer and replace with 5% BSA diluted in PBS to block samples. Incubate at room temperature for 60 min.
5. Prepare working stocks of primary antibodies by diluting in blocking 5% BSA-PBS. See Table 1 for recommended working dilutions and antibodies for different cell types. Coat coverslips in primary antibody solution and incubate overnight at 2-8 °C.

Table 1. Antibodies used in immunocytochemistry

Antibody	Concentration Used
Antibodies for iPSCs	
TRA-1-60	1/100
SSEA	1/100
Nanog	1/100
OCT4	1/100
PAX6	1/500
Antibodies for NPCs	
SOX1	1/1,000
Nanog	1/100
Nestin	1/2,000
PAX6	1/500
Antibodies for Neurons	
Tuj1	1/2,000
S100B	1/200
VGLUT1	1/300
GABA	1/500
GFAP	1/500
Secondary antibodies	
ALEXA 488 anti-mouse	1/2,000
ALEXA 555 anti-rabbit	1/2,000

6. Wash samples with PBS (3 x 15 min) at room temperature.
7. Aspirate PBS and add secondary antibodies diluted in 5% BSA-PBS. See Table 1 for recommended secondary antibodies and dilutions. Incubate coverslips at room temperature, away from light for one hour.
8. Wash samples with PBS (3 x 15 min) at room temperature.
9. If desired, add DAPI diluted in PBS, incubate for 5 min in room temperature.
10. Add a drop of Vectashield® to a glass slide. Carefully use a needle and forceps to transfer the coverslip, cell-side down, to the slide. Seal using nail polish.

E. Assessment of neuronal quality using Electrophysiology

1. Pull pipettes from glass capillaries. Their resistance should range from 3 to 6 MΩ when filled with the internal pipette solution.
2. Transfer individual coverslips containing differentiated human iPSC-derived neurons into a heated recording chamber and continuously perfused (1 ml/min) with BrainPhys Neuronal Medium without phenol bubbled with a mixture of CO₂ (5%) and O₂ (95%) and maintained at 35 °C using an automatic temperature controller.

3. Choose the cells that you will record from.
4. Fill the pipette with the internal pipette solution and place it in the electrode holder. Lower the pipette to place it into the external solution. After compensating offsets, approach the pipette to the chosen cell with the help of the remote micromanipulator to form a high resistance cell-attached seal.
5. Once the seal is formed and the whole cell configuration is established, compensate series resistance at 80%-90%.
6. Wait for 5 to 10 min before starting to record. This allows the cell content to equilibrate with the internal pipette solution.
7. For acquisition, set your filter at 2 kHz and your sampling rate at 20 kHz.
8. Once whole-cell recording had been established, recordings of fundamental neuronal properties, including rheobase, resting membrane potential, action potential parameters and spontaneous postsynaptic currents can be performed. Add NMDA via pipette. Assessed neurons should be held neurons in voltage clamp at -70 mV except when examining changes in the resting membrane potential and rheobase, which should be performed in current clamp. Clampex and GraphPad Prism 7 are recommended software to use to display data. For example electrophysiological recordings obtained from cortical neurons, consult Figure 6.

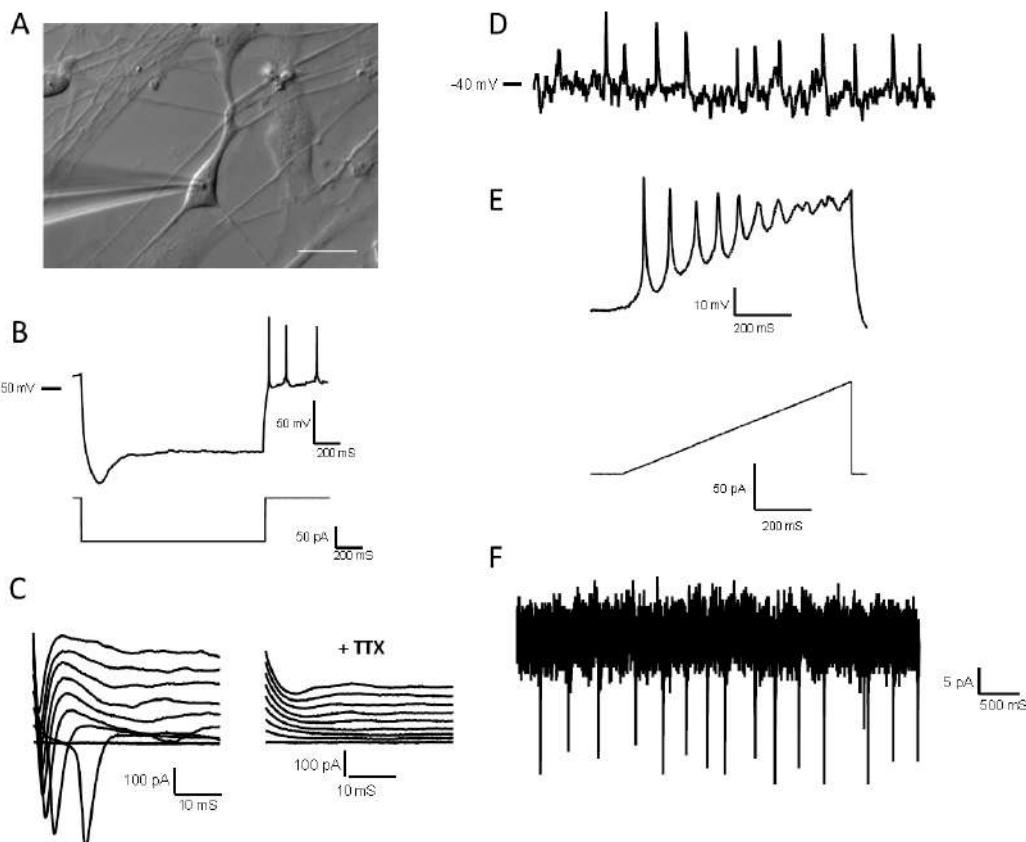


Figure 6. Electrophysiological properties of high-quality forebrain neurons. A. Differential image contrast of a glass microelectrode recording from a single neuron in the whole-cell

configuration. Scale bars represent 20 μm . B. A hyperpolarizing pulse showing a depolarizing sag followed by multiple rebound action potentials. C. Left: Representative traces of voltage clamp recordings showing inward Na^+ currents; Right: Sodium current traces disappear after tetrodotoxin (TTX) 1 μM application. D. Representative current-clamp recording from a spontaneously active neuron with resting membrane potential -40 mV. E. Representative recording showing action potentials fired by forebrain neurons during a current ramp protocol. F. Representative voltage-clamp recording from a neuron with spontaneous synaptic input. All electrophysiological data was obtained from D14 neurons.

Data analysis

Electrophysiological data should be processed with currents should be filtered at 2 kHz and digitized at 20 kHz. Values should be reported correcting for the nominal membrane potential in voltage- and current-clamp recordings for the calculated 10-mV liquid junction potential.

Recipes

A. Media

Note: All media as given as recipes for 50 ml as it is possible to prepare this volume in a single 50 ml tube, suitable for warming in a bead or water bath.

1. Neural Induction Medium 1

DMEM/F12 Medium	47.5 ml
N2 supplement	0.5 ml
B27 supplement	1 ml
BSA	1 mg/ml
NEAA	0.5 ml
SB431542	10 μM
Noggin	200 ng/ml
Laminin	1 $\mu\text{g}/\text{ml}$
	Total: 50 ml

2. Neural Induction Medium 2

DMEM/F12 Medium	47.5 ml
N2 supplement (N2-A from Stem Cell Technologies)	0.5 ml
B27 supplement (SN-1 from Stem Cell Technologies)	1 ml
BSA	1 mg/ml
NEAA	0.5 ml
Laminin	1 µg/ml
	Total: 50 ml

Note: Complete Neural Induction Medium 1 and 2 can be stored at 2-8 °C in the dark for up to 2 weeks. Warm the Neural Induction Medium in a 37 °C water bath for 5-10 min before using. Do not warm the Neural Induction Medium in a 37 °C water bath for longer than 10 min, as this may cause degradation of the medium.

3. Neural Progenitor Media

KnockOut™ DMEM/F-12	47.5 ml
N2 supplement	0.5 ml
B27 supplement	1 ml
EGF	20 ng/ml
FGF	20 ng/ml
Laminin	1 µg/ml
	Total: 50 ml

4. Neuronal Media

BrainPhys Medium	47.5 ml
N2 supplement	0.5 ml
B27 supplement	1 ml
BDNF	20 ng/ml
GDNF	20 ng/ml
Laminin	1 µg/ml
	Total: 50 ml

B. Buffers and solutions

1. Internal pipette solution

5 mM HEPES

2 mM KCl

136 mM potassium gluconate

5 mM EGTA

5 mM ATP-Mg²⁺

8 mM creatine phosphate

0.35 mM guanosine triphosphate

The pH is adjusted to 7.2 with KOH and the osmolality is adjusted with distilled water or concentrated potassium gluconate if needed to between 295 and 298 mOsm

Note: The difference in osmolality between Internal and external solutions should be near 5%.

2. Culture Dish Coating with Matrigel®

- Thaw a frozen aliquot of Matrigel® (250 µl) from -80 °C by placing it in a 4 °C fridge for 1 h
- To create a working solution, dilute the thawed Matrigel® in 25 ml of cold PBS or media

Note: Diluting Matrigel® in DMEM or other media with a strong coloration will make it easier to determine that the whole dish is evenly covered.

- Quickly cover the whole surface of each culture vessel with the appropriate amount matrix solution (Table 2)

- Incubate the culture vessels in a 37 °C, 5% CO₂ incubator for at least 1 h

- The culture vessels are now ready for use. Just before use, aspirate the diluted Matrigel® solution from the culture vessels. Cells can be plated directly onto the Matrigel®-coated culture vessels without rinsing

Note: Coated culture vessels can also be stored at 2-8 °C for up to one week. When storing, seal culture vessels with Parafilm® laboratory film to prevent drying. Before using, warm up the coated culture vessels stored at 2-8°C at room temperature for 30 min.

Table 2. Required volume of Matrigel® matrix solution for coating different culture vessels

Culture Vessel	Approximate surface area (cm ²)	Diluted Matrigel® matrix volume (ml)
6-well plate	9.6 cm ² /well	1 ml/well
35-mm dish	11.8 cm ²	1 ml
60-mm dish	20 cm ²	2 ml
100-mm dish	60 cm ²	5 ml

3. Coating glass coverslips with Poly-ornithine and laminin

- Place glass coverslips in a Petri dish or suspension plate
- Sterilize coverslip by exposing plates to UV radiation for 20 min
- Coat coverslips in 100 µl of 50 µg/ml polyornithine in PBS. Wait two hours
- Aspirate solutions. Wash once using PBS, then coat coverslip in 10 µg/ml laminin diluted in PBS
- Incubate at 37 °C for two hours
- Aspirate solution, coat coverslips in 10% FBS DMEM

- g. The plates are now ready to use. For best results, use within 24 h of preparation. Before plating cells, wash plates once with PBS, as FBS may influence differentiation

Acknowledgments

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Competing interests

Carl Ernst is president of ManuStem.com.

Ethics

All work was approved by the Research Ethics Board of the Douglas Hospital.

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Axon-seq for in Depth Analysis of the RNA Content of Neuronal Processes

Jik Nijssen, Julio Aguila and Eva Hedlund*

Department of Neuroscience, Karolinska Institutet, Stockholm, Sweden

*For correspondence: eva.hedlund@ki.se

[Abstract] Neuronal processes have an RNA composition that is distinct from the cell body. Therefore, to fully understand neuronal biology in health and disease we need to study both somas, dendrites and axons. Here we describe a detailed protocol of a newly refined method, Axon-seq, for RNA sequencing of axons (and dendrites) grown in isolation using single microfluidic devices. We also detail how to generate motor neurons from mouse and human pluripotent stem cells for sequencing, but Axon-seq is applicable to any neuronal cell. In Axon-seq, the axons are recruited through a growth factor gradient, lysed and directly processed to cDNA without RNA isolation. A careful bioinformatic step ensures that any soma-contaminated samples are easily identified and removed.

Keywords: RNA sequencing, Motor neuron, Axons, Stem cells, Microfluidic devices, Bioinformatics

[Background] Neurons are highly polarized cells. Their processes, both dendrites and axons, need to be able to respond to changes in the microenvironment in a manner independent of the soma (Holt and Schuman, 2013). To fully understand neuron biology, it is therefore important to be able to study neurites in isolation, in addition to conducting analysis of the cell bodies. This appears particularly important for neurons where the axon and dendrites constitute the majority of the cellular volume, as is the case for spinal motor neurons where we estimate that they comprise approximately 99% of the cellular volume. To isolate neurites, there are excellent tools such as Campenot Chambers (Boyden, 1962; Campenot, 1977) or microfluidic chambers (Taylor et al., 2005).

However, while neurites are separated out in such devices, it is important to bear in mind that cross-contamination between compartments can still occur. Thus, RNA sequencing of isolated axonal compartments (Minis et al., 2014; Saal et al., 2014; Briese et al., 2016; Rotem et al., 2017) can lead to incorrect results/conclusions when the purity and exclusion of somas is insufficiently examined. To ensure detailed and accurate investigation of motor axonal mRNA composition and its modulation in ALS we developed Axon-seq (Nijssen et al., 2018). This is an application of our LCM-seq method for single-cell spatial RNA-sequencing (Nichterwitz et al., 2016).

In Axon-seq, we use microfluidic devices to separate axons from stem cell-derived motor neurons (mouse and human) from their somas. As motor neurons have axons that traverse far longer distances than their dendrites, we are able to analyze axons alone. This may not be possible for all neuronal subtypes, but the method still allows for analysis of neurites as an entity.

In contrast to previous methods, Axon-seq does not require an RNA isolation step, and it allows for high sensitivity and cost-efficient sequencing from a single microfluidic device.

Notably, Axon-seq effectively eliminates all samples with any somatic cross-contamination, as it uses

a highly stringent and sensitive bioinformatic quality control step that identifies axonal samples containing trace levels of mRNA from undesired cell somas. Here we provide a detailed protocol for Axon-seq which can be applied to any cell containing longer processes.

Materials and Reagents

A. Materials

1. Sterile 6 and 10 cm bacterial dishes (non-adhesive plastic) (Corning, catalog numbers: 351007 and 351029)
2. Pipette tips (0.5-10 µl, 10-100 µl, 100-1,000 µl)
3. Sterile 15 and 50 ml Falcon tubes
4. Parafilm
5. 70 µm cell-strainer filter (VWR, catalog number: 732-2758P)
6. 24 x 32 mm cover glasses (VWR, Menzel Gläser, catalog number: 630-2873)
7. Soft pencil-type brush (Any soft brush type that does not have too strong hairs that could pierce/damage the PDMS-surface of which the microfluidic devices are made. Brushes, from a typical hardware/convenience store, with a diameter of 2-5 mm work best, laboratory grade is not required. Nylon/synthetic hairs are not an issue.)
8. Waterproof marker with fine (1 mm) or very fine (0.3 mm) tip

B. Biological material

1. Motor neurons from mouse embryonic stem cells
2. Motor neurons from human pluripotent stem cells

C. Reagents

1. 70% ethanol
2. Triton X-100 (Sigma-Aldrich, catalog number: X100)
3. PBS (Thermo Fisher, catalog number: 14190136)
4. Poly-L-ornithine, 0.01% solution (Sigma-Aldrich, catalog number: A-004-C)
5. Fibronectin, 1 mg/ml solution (Sigma-Aldrich, catalog number: F1056)
6. Laminin, 1 mg/ml solution (Sigma-Aldrich, catalog number: L2020)
7. TrypLe Express (Thermo Fisher, catalog number: 12604021)
8. Neurobasal (Thermo Fisher, catalog number: 21103049)
9. DMEM/F12 with GlutaMAX (Thermo Fisher, catalog number: 31331028)
10. B27 supplement, custom (Thermo Fisher, catalog number: 0080085SA)
11. Penicillin/Streptomycin (Thermo Fisher, catalog number: 15140122)
12. GlutaMAX supplement 100x (Thermo Fisher, catalog number: 35050061)
13. 2-Mercaptoethanol (Sigma-Aldrich, catalog number: M6250)

14. Ascorbic acid, 100 mg powder (Sigma-Aldrich, catalog number: A4403), prepare as 200 mM stock solution by dissolving the powder in 2.84 ml of nuclease-free dH₂O
15. Y-27632, 10 mg powder (Tocris, catalog number: 1254), prepare as 10 mM stock solution by dissolving the powder in 3.12 ml nuclease-free dH₂O
16. LDN-193189 dihydrochloride, 10 mg powder (Tocris, catalog number: 6053), prepare as 10 mM stock solution by dissolving the powder in 2.09 ml DMSO, then prepare 1 mM working stocks (1:10 diluted) in DMSO
17. SB-431542, 10 mg powder (Tocris, catalog number: 1614), prepare as 10 mM stock solution by dissolving the powder in 2.60 ml DMSO
18. CHIR-99021, 10 mg powder (Tocris, catalog number: 4423), prepare as 3 mM stock solution by dissolving the powder in 7.16 ml DMSO
19. DAPT, 10 mg powder (Tocris, catalog number: 2634), prepare as 10 mM stock solution by dissolving the powder in 2.31 ml DMSO
20. SAG, 1 mg powder (Tocris, catalog number: 4366), prepare as 500 µM stock solution by dissolving the powder in 4.08 ml DMSO
21. All-*trans* retinoic acid, 50 mg powder (Sigma-Aldrich, catalog number: R2625), prepare as 100 mM stock solution by dissolving the powder in 1.66 ml DMSO, then prepare 1 mM working stocks (1:100 diluted) in DMSO
22. Dimethyl sulfoxide (DMSO) (Sigma-Aldrich, catalog number: D2650)
23. Bovine serum albumin (BSA), 7.5% solution in dPBS (Sigma-Aldrich, catalog number: A8412)
24. Glial-derived neurotrophic factor (GDNF), 10 µg powder (Peprotech, catalog number: 450-10), prepare as 10 µg/ml stock solution by dissolving the powder in 1 ml dPBS with 0.1% BSA
25. Brain-derived neurotrophic factor (BDNF), 10 µg powder (Peprotech, catalog number: 450-02), prepare as 10 µg/ml stock solution by dissolving the powder in 1 ml dPBS with 0.1% BSA
26. RNase-inhibitor (Takara, catalog number: 2313A)
27. DTT (part of SuperScript II Reverse Transcriptase kit, Thermo Fisher, catalog number: 18064071)
28. Nuclease-free dH₂O (Thermo Fisher, catalog number: 10977-035)
29. 37% formaldehyde solution (Merck Millipore, catalog number: 818708)
30. Mouse MN medium (see Recipes)
31. N2 medium (see Recipes)
32. B27 medium (see Recipes)
33. Human EB medium (see Recipes)
34. Lysis/harvesting solution (see Recipes)

Equipment

1. Glass beaker (500 ml)
2. Sterile forceps (Dumont, catalog number: 11251-20, or other forceps of similar standard size)

3. Pipettes (0.5-10 µl, 10-100 µl, 100-1,000 µl)
4. Magnetic stirrer
5. Sterile laminar flow hood
6. Humidified incubator (37 °C, 5% CO₂)
7. Suction aspirator
8. Standard Neuron Device (Xona Microfluidics™, SND75/150/450/900)
9. Low-speed orbital shaker (Elmi-tech, DOS-20S)
10. Inverted bright field microscope (> 20x magnification is required to reliably see axons exiting the microgrooves. Examples are Laxco LMI-3000 or Carl Zeiss PrimoVert. Any similar setup is fine.)

Software

1. R (<https://www.r-project.org>)
2. RStudio open source edition, Boston, MA, USA (<https://www.rstudio.com>)

Procedure

A. Storage and washing of microfluidic devices

Commercial microfluidic devices from Xona Microfluidics™ (Standard Neuron Devices) with any groove length can be stored in dH₂O and used multiple times.

Note: The groove length of the devices could affect the proportion of axons versus dendrites that can cross over to the opposite chamber, as could the length of time of culture in the devices. For mouse motor neurons, we used devices with 150 µm length grooves and saw a clear majority of axons being recruited during the 7 days of culture. For human motor axons, we used devices with 450 µm grooves.

1. Place devices in a glass beaker with 500 ml of 1% Triton X-100 in dH₂O for 2 h and use a magnetic stirrer to keep devices agitated.
2. Use a soft brush to gently clean the microgroove surfaces and the chambers of the devices. Transfer devices into dH₂O afterward.
3. Wash 3 x 10 min in fresh dH₂O each time to remove any residues of Triton X-100. Then, sterilize devices in 70% ethanol for a minimum of 10 min.

Note: The washing procedures can be done at any time and devices can be stored in 70% ethanol to be ready when needed. Start the device attachment (below) the day that the coating procedure is to be initiated.

B. Attachment of microfluidic devices to cell culture surface

1. Remove microfluidic devices from 70% ethanol using sterile forceps and allow the devices to air-dry in a sterile laminar flow hood for 20 min. Use a suction aspirator to remove any residual ethanol.

Note: It is very important that devices are entirely dry at this stage, as any remaining liquid will negatively affect device attachment and can cause leakage in later stages.

2. Place 4 drops of 4 μl dH₂O in a sterile 10 cm bacterial dish (Figure 1A).
3. Place sterile glass cover glasses (32 x 24 mm, thickness #1 or #1.5) on top of the water drops to ensure that the cover glasses do not move around within the dish (Figure 1B).
4. Use forceps to pick up one device at a time and gently press it, feature side down, onto the glass coverslip (Figure 1C). Apply gentle force, using the back of the forceps, to the center of the device (above the grooves), and all four corners. A microscope can be used to assess if all the grooves are in the same focal plane and thus attached to the coverslip. Devices attached in this way are stable for at least four weeks (and likely longer). Label the glass coverslips with a waterproof marker in one corner to define the future left-right orientation of the devices. The device forms the ‘ceiling’ for the cells and creates grooves for the axons to grow through. Cells will attach to the glass surface of the coverslip.

Notes:

- a. The final setup of devices (Step B4) is a suggestion. Devices can also be placed directly in tissue culture-grade plastic dishes (i.e., directly onto the plastic, without cover glasses). For imaging purposes, however, placing them on glass coverslips is beneficial.
- b. In addition to the PDMS devices described here, Xona Microfluidics™ also offers pre-bonded devices for cell culture use (XonaChips). While these cost the same as regular devices, they are not optimal for reusing, as can be done with the regular devices (SND150). In the case of pre-bonded devices, the attachment step (Procedure B) can be skipped.

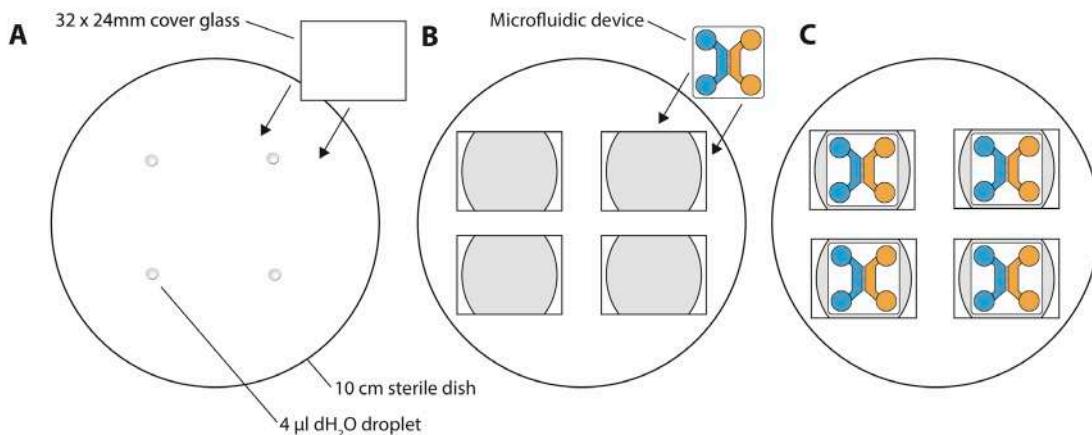


Figure 1. Setup of 4 microfluidic devices in a 10 cm dish. A. First, place 4 drops of 4 μl dH₂O spaced apart evenly in the 10 cm dish. Then place glass cover glasses onto the drops to create a water seal between cover glasses and the plastic dish. B. When cover glasses are attached, place one microfluidic device with feature-side down on each cover glass. Press gently on the top of the device to ensure attachment. C. Final setup of four devices in one 10 cm dish. Other arrangements of devices (in terms of dish size) can be made if desired.

C. Coating of surfaces in microfluidic devices

Day 1:

1. The primary surface coating consists of 0.001% poly-L-ornithine in dH₂O. Prepare 0.5 ml solution per device.
2. Add 75 µl solution into the two bottom wells of each device. Typically, the liquid will quickly fill the chambers through capillary forces. If this does not happen, use the aspirator with a mounted 1 ml pipette tip to gently suck the liquid through from the top. Do not aspirate all the liquid.
3. Once the liquid has flown through, add 150 µl to the top left and 200 µl to the top right compartment. Adding different volumes between the left and right chamber ensures that the coating is forced into the microgrooves over time.
4. Leave the coating on overnight at room temperature and shield it from light (e.g., a drawer works well).

Day 2:

5. Wash the devices twice using 1x PBS. To wash, aspirate the coating from the wells, then add PBS into the wells, 75 µl in the bottom, then 150 µl in the top.
6. The second coating consists of a solution of 2 µg/ml fibronectin and 5 µg/ml laminin in PBS. Add this last coating in a similar fashion to the first—see Steps C2-C4.

D. Specification of motor neurons from mouse embryonic stem cells

Typically, a good starting point for differentiation is a 10 cm dish of stem cells at ~70-80% confluency. Cells are always maintained in a humidified incubator at 37 °C and 5% CO₂.

Day 1:

1. Prewarm TrypLE Express to be used for dissociation at 37 °C (*i.e.*, 2 ml for a 6 cm dish, 4 ml for 10 cm dish) in a tube. In addition, pre-warm a tube with 8 ml DMEM/F12-GlutaMAX (or any other plain MEM-based media).
2. Add the TrypLE Express to initiate the dissociation.
3. When cells have rounded up and are coming off the culture dish, use a pipette to dissociate them fully in the TrypLE Express, then transfer everything into the tube with pre-warmed DMEM.
4. Spin down for 4 min at 200 *x g*, at room temperature.
5. Remove supernatant and resuspend cells in 1 ml of mouse MN media (Recipe 1) and count.
6. Place cells in bacterial (non-adherent) dishes at a density of 0.5-1.5 million cells per ml (the number of cells is line dependent and needs to be tested out), and place dishes on a low-speed orbital shaker at 30 rpm overnight in the incubator.

Day 2:

7. Remove dishes from the shaker and change to fresh mouse MN media. To change media on EBs, pipette the entire volume of media (including EBs) into a tube. Allow the EBs to sink for ~2 min and remove the supernatant. Resuspend EBs in 1 ml fresh media and place into a new culture dish.

Days 3-6:

8. Change to fresh mouse MN media, supplemented with 100 nM all-*trans*-retinoic acid (RA) and 500 nM smoothened agonist (SAG). Repeat for 4 successive days. See Table 1.

Table 1. Factors for motor neuron differentiation from mouse pluripotent stem cells

	Days 1-2	Days 3-6	Day 7 dissociation	Day 8+
Base media	Mouse MN	Mouse MN	Mouse MN	Mouse MN
RA		100 nM	100 nM	
SAG		500 nM		
GDNF			20 ng/ml	20 ng/ml
BDNF			20 ng/ml	20 ng/ml

Day 7:

9. After 4 consecutive days of patterning, cells are ready to be dissociated and seeded into microfluidic devices (see below).
- E. Specification of motor neurons from human pluripotent stem cells

Typically, a good starting point for differentiation is a 10 cm dish of stem cells at ~70-80% confluency. Cells are always maintained in a humidified incubator at 37 °C and 5% CO₂.

Day 1:

The procedures on the first day (EB formation) are the same as described above for mouse ES cells. However, in the end, resuspend the cells in human EB media at a density of 0.5 M-0.75 M cells per ml (this density may require a degree of optimization between cell lines). Supplement the Day 1 human EB media with the required factors for Day 1 (for overview, see Table 2):

5 µM Y-27632 (ROCK-inhibitor)

200 nM LDN-193189

40 µM SB-431542

3 µM CHIR-99021

200 µM ascorbic acid

Table 2. Factors for motor neuron differentiation from human pluripotent stem cells

	Days 1-2	Days 3-9	Day 10 dissociation	Days 11-12	Day 13 onward
Base media	N2/B27	N2/B27	B27	B27	B27
Y-27632	5 µM		5 µM		
SB-431542	40 µM				
LDN-193189	200 nM				
CHIR-99021	3 µM				
Retinoic acid		200 µM	200 nM		
SAG		500 nM			
Ascorbic acid	200 µM	200 µM	200 µM	200 µM	200 µM
DAPT			10 µM	10 µM	
GDNF			10 ng/ml	10 ng/ml	10 ng/ml
BDNF			10 ng/ml	10 ng/ml	10 ng/ml

Days 2-10:

Perform daily media changes at approximate 24 h intervals with the corresponding factors for each day (Table 2).

Note: During the RA-SAG stage of the protocol (days 3-10), it is not absolutely required that media changes follow 24 h intervals, and if necessary (but not recommended) one day of media changes can be omitted. However, it is critical that the dual-SMAD inhibition (using SB-431542 and LDN-193189) during the first two days is 48 h and not shorter.

Day 10:

Cells are ready to be dissociated and seeded into microfluidic devices (see below).

F. Cell dissociation and preparation for seeding

1. Transfer EBs into a sterile 15 ml Falcon tube. Allow the EBs to sink to the bottom for 1-5 min and aspirate the remaining media.
2. Add 1 ml pre-warmed (37 °C) TrypLE Express to the Falcon tube with EBs. The dissociation takes 10-20 min, depending on the size of the EBs.
3. Gently agitate the EBs every 5 min by tapping the tube or gently pipetting up and down using a 1 ml pipette.

Note: If you resuspend EBs by pipette for the first time after 5 min in TrypLE Express, do not pipette them up into the pipette tip as they tend to stick on the inside. Instead, just pipette the liquid and ensure the EBs consequently are agitated slightly in the suspension.

4. Once the EBs are beginning to break apart, pipette them up and down rather vigorously approximately 10 times to finalize the dissociation. Add 9 ml of pre-warmed DMEM to dilute the 1 ml of TrypLE Express.
5. Filter the resulting suspension through a 70 µm cell-strainer filter to remove undissociated clumps/debris, then spin at 200 x g for 4 min, at room temperature.

6. Resuspend the pellet in 1 ml of DMEM/F12-GlutaMAX (or any other plain MEM-based media). Count the cells.
7. Adjust the density to a final concentration of 2.5×10^4 cells per μl (this will ensure that a total of 1×10^5 cells are seeded per microfluidic device later on). To do this, transfer 1 ml of the cell suspension into an Eppendorf tube and spin once more at $200 \times g$ for 4 min. Resuspend cells in the required amount of media (*i.e.*, 1 μl for every 2.5×10^4 cells). At this stage, the cells should be resuspended in the final culturing media, for human cells this is B27 media with the Day 10 factors (see Table 2), for mouse cells this is mouse MN media with Day 7 factors (see Table 1). *Note: The exact composition of the final media can vary based on the type of neurons in culture. However, the addition of ascorbic acid and trophic factors (and ROCK-inhibitor for human cells) is highly recommended to improve survival upon plating in the confined environment of the microfluidic device.*
8. Prepare sufficient final media to fill all devices (0.5 ml per device). In the end, the device will hold media volumes as shown in Figure 2.
9. Take the required amount of media (see Figure 2) for the axonal compartment (top and bottom well) and transfer to a new tube. Add trophic factors to increase the concentration to 50 ng/ml. The higher concentration of trophic factors on the “axonal” side will improve axonal recruitment across the microgrooves.

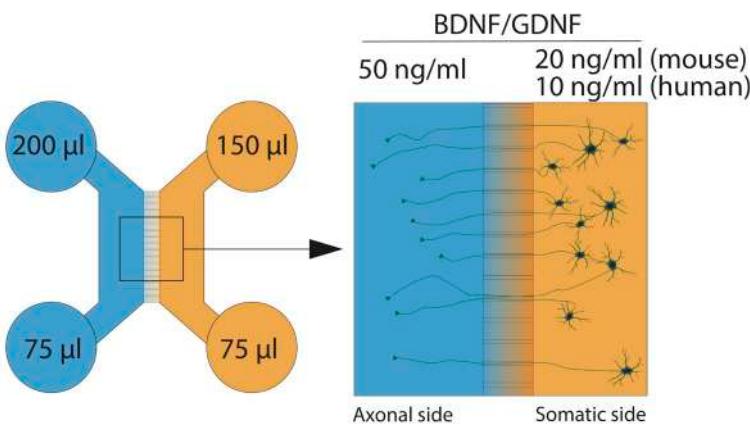


Figure 2. Media volumes and trophic factor concentrations in the microfluidic device at seeding. Note that somatic concentrations of the neurotrophic factors differ between human and mouse MNs. After axons have crossed the microgrooves into the other compartment, trophic factor concentrations can be equalized to 10 ng/ml on both sides, both for mouse and human.

G. Cell seeding

1. Start removing coating from the devices by aspiration (from one 10 cm dish with four devices at a time).

2. Add 4 μ l of the axonal media (containing 50 ng/ml BDNF/GDNF) into the chamber on the axonal side. Pipette close to the chamber entrance on the top-left (in the orientation of Figure 2, see Figure 3A for photograph) and allow capillary forces to pull the liquid into the chamber.
3. Add the cells in the same manner to the other compartment. Once cells are seeded in the chamber, place devices in the incubator and allow the cells to attach for approximately 15-20 min.
4. Start checking devices from 10 min after seeding the cells and onward to avoid excessive evaporation of the media. Once cells appear attached, add the final media amounts to the wells as indicated in Figure 2. Check if the cells remain attached and then place devices in the incubator overnight.

Notes:

- a. *Occasionally cells do not attach well even after 15 min, maybe in part due to the flow between the compartments. If this occurs, we recommend adding only 40 μ l of media to all wells (keeping the right media types for each side), which will avoid excessive flow between compartments. This way, there is minimal flow and evaporation will not be an issue. Then keep the cells like this for an additional hour and finally add media to the recommended amounts in Figure 2.*
- b. *For human cells, the dissociation takes place on Day 10 of the protocol. In addition to everything described here, maintain the corresponding factors in the media for each day as described in Table 2. The trophic factor (BDNF/GDNF) concentrations described there are for the somatic compartment, the axonal compartments receive the trophic factor gradient for the first few days (see below).*

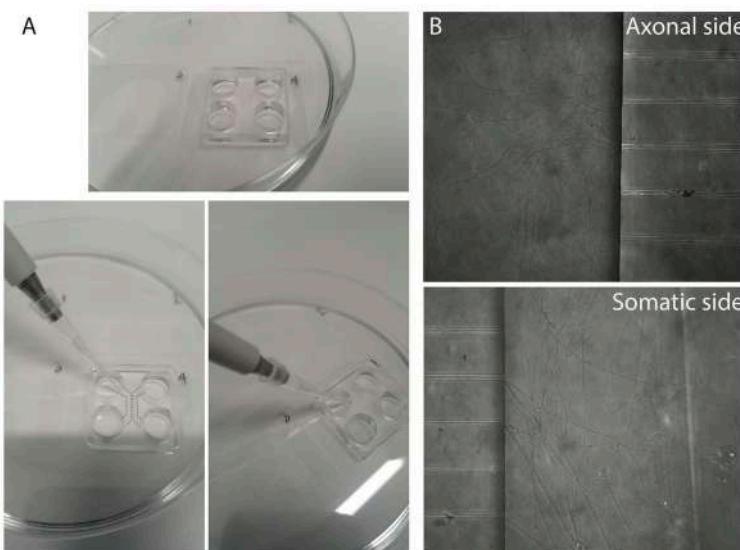


Figure 3. Seeding cells into microfluidic chambers and representative micrographs. A. Example photographs of the angle at which media/cells can be added into the chambers of the microfluidic device. Dashed lines show the location of the chambers, and chamber entrances.

B. Micrographs of a good culture in the axonal compartment and the somatic compartment, where clusters of neuron somas are visible.

H. Culturing devices

1. After seeding neurons into microfluidic devices, change media daily to ensure that the trophic factor gradient stays intact. A bright field microscope can be used to keep track of the axonal growth.

Note: When changing media, do NOT aspirate media out of the chambers as this will forcibly remove all cells/axons, only change media in the wells.

2. Once axons can be seen crossing several microgrooves into the axonal compartment (*i.e.* > 10 grooves with axons), the trophic factor gradient can be removed, and both compartments instead receive 10 ng/ml of GDNF and BDNF. At this point, media can be exchanged every other day. See Figure 3B for a successful micrograph of a microfluidic device with growing axons.

I. Compartmental harvesting for RNA sequencing

1. Prepare the lysis/harvesting solution. Prepare ~70 µl solution per microfluidic device, or less if only one compartment is harvested (final recommended lysis volumes are 50 µl for the somatic compartment and 10 µl for the axonal compartment). The lower volume for axons ensures that a higher concentration of RNA is present in the final solution, which aids in downstream applications.

2. The lysis solution (see Recipes) is made up as a 2% Triton X-100 solution in nuclease-free dH₂O. First, cool this solution on ice, then supplement with:

0.5 µl RNase-inhibitor per 100 µl (final concentration of 0.2 U/µl)

1 mM dithiothreitol (DTT), required for RNase-inhibitor enzymatic activity

Note: Always keep lysis solution on ice during preparation and use.

3. Prepare nuclease-free Eppendorf tubes. Label them according to the samples that are being collected, and pre-cool them on ice.

4. Have dry ice ready to snap-freeze harvested samples immediately following the collection.

5. Optionally, before starting the harvesting, take bright field images along the grooves. If somatic contamination is detected in the downstream bioinformatics analysis of axon samples, these images can be scrutinized for evidence of any cross-contaminating somas.

6. Harvest one device at a time. Begin by washing both compartments once with pre-warmed (37 °C) PBS. Use at least 100 µl PBS.

7. Remove PBS from the axonal wells and add 10 µl lysis solution to the top of the chamber, as if seeding cells (the vastly higher volume of PBS on the somatic side prohibits any lysis solution from flowing through the grooves and accidentally lyse somas).

8. Wait for 10 s, then pipette up and down ~5 x in the bottom well, and extract as much solution as possible from the bottom of the chamber (can be > 10 µl). Transfer solution into a prepared Eppendorf tube and snap-freeze on dry ice.

9. Add at least 100 μ l PBS to the axonal side again.
10. Repeat Steps I7 and I8 above for the somatic side, but instead use 50 μ l lysis solution. When extracting the solution, try to extract as much as possible ($> 50 \mu$ l). Snap-freeze on dry ice.
11. After collection and snap-freezing, samples for RNA sequencing can be stored at -80 °C until further used.

Note: For detailed instructions, protocols and reagents for the preparation of RNA sequencing libraries without RNA-extraction, see Nicterwitz et al., 2018. To generate axonal and somatodendritic sequencing libraries, standard laboratory practices related to handling of RNA must be considered. Namely, keep the samples cold (4 °C) and surfaces clean. Work with RNase/DNAse-free disposables and reagents certified for molecular biology grade. Consider establishing a dedicated working station (bench) and a thermocycler exclusive for library preparation purposes. Due to the low amount of material (RNA) per device, comparable to that of single cells, we employed the Smart-seq2 protocol, an established methodology for single-cell transcriptomics. Briefly, in the first step, samples are quickly thawed from -80 °C and 10 μ l of axonal lysate (5 μ l of somatodendritic lysate) are subjected to reverse transcription using an oligo-dT primer and a template switch LNA-oligo. In the second step, an enrichment PCR with 18 cycles is introduced to amplify the amount of cDNA and samples purified using magnetic beads. At this point, the quality and quantity of the cDNA libraries can be measured by bioanalyzer using a dsDNA high sensitivity chip (for representative examples, see Figure 4). Finally, cDNA libraries are fragmented (Nextera XT Kit, Illumina) and barcoded with Illumina indexes for RNA sequencing.

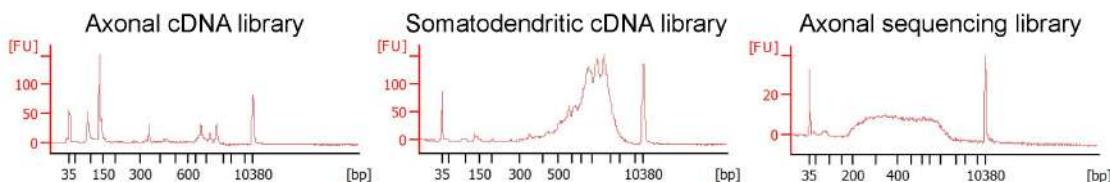


Figure 4. Typical bioanalyzer profiles for RNA sequencing using Axon-seq. Axonal cDNA samples have a distinct profile compared to somatodendritic samples. An example of ready to sequence axonal sequencing library is also shown after fragmentation reaction and barcoding.

J. Fixation of cells in devices for staining purposes

1. Remove all media from the wells and wash both compartments once with PBS.
2. Add a solution of 4% formaldehyde in PBS. When adding the formaldehyde, maintain similar volumes in all wells as used during media changes (see Figure 2) to ensure a flow both through the compartments and across the microgrooves.
3. Leave the devices to fix at room temperature for 30 min.
4. Wash 3 times with PBS, dispose of formaldehyde waste.
5. Store devices in PBS (~200 μ l in all wells) until further use. If not used within a few days, wrap the 10 cm dish with parafilm.

Note: Either before or after performing the desired staining method (immunocytochemistry/FISH/RNAscope), devices need to be removed along with coverslips from the 10 cm plastic dish.

6. Using a 1 ml pipette, add a thin line of H₂O around the edges of the cover glass. This will loosen it from the underlying surface and cause it to float after a few seconds/minutes.

Data analysis

Bioinformatic quality control

1. After RNA sequencing has been performed, a bioinformatic quality control (QC) step should be performed to exclude axonal samples with (traces of) soma contamination.

Note: The following is a simplified version of the bioinformatic QC that is conducted using the programming language R (R Core Team, 2018). More in-depth knowledge of bioinformatics and R will be required to perform these analyses, but what follows is a general guide to exclude any soma-contaminated samples.

- a. Ensure that the RNA sequencing data is processed to both a count table (raw read counts) and an RPKM table with normalized values (reads per kilobase per million mapped reads).
- b. As a general QC guideline, samples with too few mapped reads or detected genes should be excluded. To perform this, extract the total number of counts (= mapped reads) per sample from the count table, as well as the total number of genes with counts.
- c. Exclude samples with fewer than 2.5×10^5 mapped reads.
- d. Exclude samples that have fewer than 2,500 genes with any counts.

2. After this general QC, the data can be inspected for soma-contamination of axonal samples.

Briefly, there are four indicators to investigate:

The number of detected genes in axonal samples

2D visualization plots (PCA/umap)

Sample correlations in a correlation heatmap

Hierarchical clustering based on all expressed genes (or a subset)

- a. Obtain the number of detected genes in the axonal samples, and plot these as a dot plot. As a rough guideline, axon samples contain fewer than ~8,000 genes. Investigate any outliers in this distribution. Soma-contaminated samples will have a strong increase in the number of detected genes.
- b. Using the base R function *prcomp*, or alternatively the package *umap* (Konopka, 2018), plot a 2D visualization of your samples (first 2 principal components for *prcomp*, or *n_components = 2* for *umap*). Investigate the plot. Typically, axon and soma samples will separate strongly in 2D space. Contaminated axon samples will be significantly shifted towards the somatic samples (Figure 5).
- c. Using the base R function *corr*, calculate the correlation between samples based on all expressed genes. This correlation matrix can be plotted as a heatmap using the function

- pheatmap* from the package carrying the same name. Typically, axonal samples have a low correlation to somatic samples. However, contaminated samples will have an increased overall correlation to all somatic samples.
- d. Using *pheatmap* (Kolde, 2019), a heatmap with corresponding hierarchical tree clustering can be generated based on all expressed genes in the dataset. Where axon and soma samples normally cluster in distinct branches, contaminated samples can cluster together with soma samples.

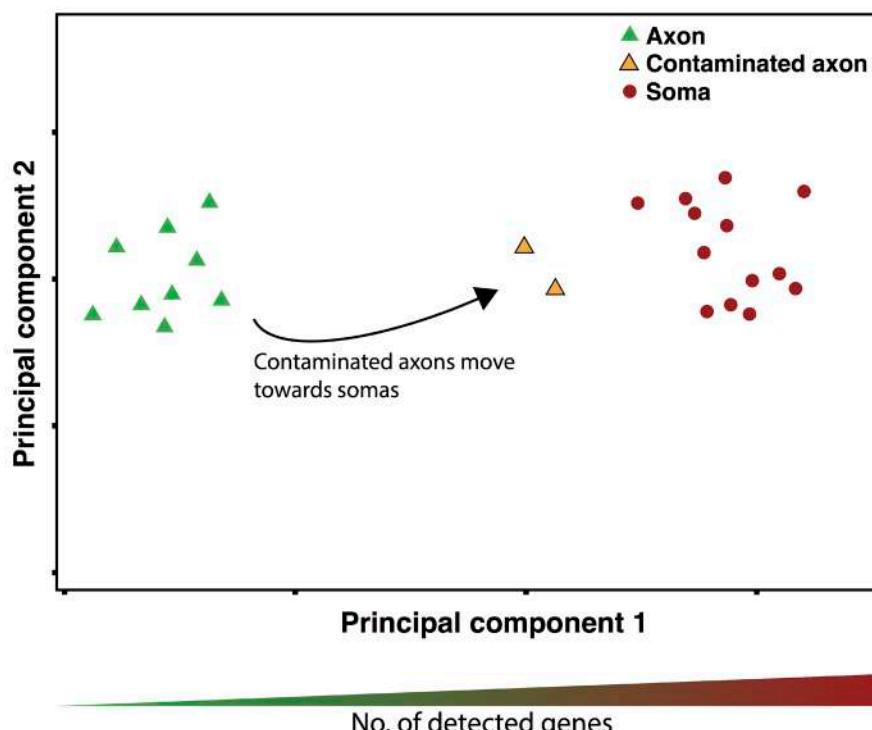


Figure 5. Example PCA-plot showing the shift of contaminated axon samples towards the somatic samples. They additionally have an increase in the number of detected genes per sample.

3. Using these four measures, one can obtain an idea of samples that are possibly soma contaminated. Note that cutoffs need to be set for each of the measures, and the decision to exclude samples depends on the severity of the contamination.

Recipes

1. Mouse MN medium (500 ml)
240 ml Neurobasal
240 ml DMEM/F12-GlutaMAX
10 ml B27 supplement, custom (stock 50x, add the full supplement bottle)
5 ml Penicillin/Streptomycin

- 5 ml GlutaMAX supplement
- 0.91 ml 2-Mercaptoethanol
- 2. N2 medium
 - 1 bottle (500 ml) of DMEM/F12-GlutaMax
 - 5 ml N2 supplement (stock 100x, add the full supplement bottle)
 - 5 ml Penicillin/Streptomycin
- 3. B27 medium
 - 1 bottle (500 ml) of Neurobasal
 - 10 ml B27 supplement, custom (stock 50x, add the full supplement bottle)
 - 5 ml Penicillin/Streptomycin
- 4. Human EB medium
 - 50/50 mixture of N2 medium and B27 medium (above)
- 5. Lysis/harvesting solution (~70 µl per microfluidic device)
 - 2% Triton X-100 in nuclease-free dH₂O. Cool on ice, then supplement with:
 - 0.5 µl RNase-inhibitor per 100 µl (final concentration of 0.2 U/µl)
 - 1 mM dithiothreitol (DTT), required for RNase-inhibitor enzymatic activity

Acknowledgments

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This protocol is based on our previous study published in Stem Cell Reports in 2018: Nijssen *et al.* (2018).

Competing interests

The authors declare that there are no financial or non-financial competing interests.

Ethics

All work was carried out according to the Code of Ethics of the World Medical Association (Declaration of Helsinki) and with national legislation and institutional guidelines. The use of human stem cell lines was approved by the regional ethical review board in Stockholm, Sweden (Regionala Etikprövningsnämnden, Stockholm, EPN).

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Isolation and Culture of Single Myofiber and Immunostaining of Satellite Cells from Adult C57BL/6J Mice

Shujie Chen^{1, #}, Hongrong Ding^{1, #}, Xiangping Yao^{1, 2, #} and Liwei Xie^{1, 2, *}

¹State Key Laboratory of Applied Microbiology Southern China, Guangdong Provincial Key Laboratory of Microbial Culture Collection and Application, Guangdong Open Laboratory of Applied Microbiology, Guangdong Institute of Microbiology, Guangdong Academy of Sciences, Guangdong 510070, China;

²Zhujiang Hospital, Southern Medical University, Guangdong 510282, China

*For correspondence: xielw@gdim.cn

#Contributed equally to this work

[Abstract] Myofiber isolation followed with *ex vivo* culture could recapitulate and visualize satellite cells (SCs) activation, proliferation, and differentiation. This approach could be taken to understand the physiology of satellite cells and the molecular mechanism of regulatory factors, in terms of the involvement of intrinsic factors over SCs quiescence, activation, proliferation and differentiation. Single myofiber culture has several advantages that the traditional approach such as FASC and cryosection could not compete with. For example, myofiber isolation and culture could be used to observe SCs activation, proliferation and differentiation at a continuous manner within their physiological “niche” environment while FACS or cryosection could only capture single time-point upon external stimulation to activate satellite cells by BaCl₂, Cardiotoxin or ischemia. Furthermore, *in vitro* transfection with siRNA or overexpression vector could be performed under *ex vivo* culture to understand the detailed molecular function of a specific gene on SCs physiology. With these advantages, the physiological state of SCs could be analyzed at multiple designated time-points by immunofluorescence staining. In this protocol, we provide an efficient and practical protocol to isolate single myofiber from EDL muscle, followed with *ex vivo* culture and immunostaining.

Keywords: Myofiber isolation and culture, Muscle satellite cells, Immunofluorescence staining

[Background] Satellite cells are considered as an adult stem cell because they maintain self-renew and remarkable postnatal regenerative potential of skeletal muscle (Collins *et al.*, 2005). SCs are located between the basal lamina and the plasma lemma of myofibers (MAURO, 1961). Here, to isolate the single myofiber, EDL muscle is digested with the collagenase to release the connective tissue and completely dissociate the connective tissue between fibers. In 1986, it was reported that the proliferation of satellite cells on single muscle fibers were isolated from adult rats and were cultured in cell culture plate (Bischoff, 1986). It was further developed into a tissue culture system that reliably permits isolation of intact, living, single muscle fibers with associated satellite cells from predominantly fast- and slow-twitch muscles of rat or mouse (Bekoff and Betz, 1977a and 1977b; Rosenblatt *et al.*, 1995). There are two main approaches to study SCs: single myofiber culture and primary myoblast culture prepared from mononucleated cells dissociated from whole muscle (Danoviz and Yablonka-Reuveni, 2012). Even though, primary myoblast could be split and passaged multiple times, these primary myoblasts retain in proliferation or differentiation states. Freshly isolated single myofiber allows satellite cells to stay

beneath the basal lamina at quiescent state, followed with activation by either internal environment or external factors. There are several improvements for myofiber isolation and culture in recent years (Danoviz and Yablonka-Reuveni, 2012; Pasut *et al.*, 2013; Gallot *et al.*, 2016; Lim *et al.*, 2018). However, the difficulty of myofiber isolation and *ex vivo* culture prevent further application of this reliable and practical method. In the current protocol, we optimized reagents used for single myofiber isolation and improved procedures to make it even more simple and easy to repeat from hand to hand. Since satellite cells are sensitive to various factors, we describe a relatively simple, detailed and efficient approach to isolate and culture single myofiber. Based on our protocol, state and function of satellite cells could be analyzed from mice with different genotypes. Different manipulation such as transfection, and drug treatment on myofibers, followed with the downstream procedures including but not limited to myofiber transplantation and immunostaining could be performed. These additional manipulations could not be performed in these approaches such as FASC or cryosection.

Materials and Reagents

1. Pipette tips (Jet Biofil, catalog number: PMT950200)
2. 15 ml tube (TrueLine, catalog number: TR2011)
3. 60 mm Petri Dish, Sterile (Jet Biofil, catalog number: 62060-60)
4. 24-well Plates, Sterile (Corning, Costar, catalog number: CLS3524-100EA)
5. Glass Pasteur Pipettes (Thermo Fisher, catalog number: 1367820A) (Figure 1e-g)
6. Rubber bulbs (Thermo Fisher, catalog number: 1951F15) (Figure 1h)
7. Diamond Pen (XGE, used to cut glass Pasteur pipettes) (Figure 1d)
8. 1 ml Transfer Pipette, sterile (Jet Biofil, catalog number: 25001)
9. Syringe Filters (PTFE, 0.22 µm, 30 mm, Sterile) (Jet Biofil, catalog number: 29525)
10. Microscope glass slide (CITOTEST, catalog number: 70179000)
11. Microscope cover slide (24 x 50 mm) (CITOTEST, category number: 10212450C)
12. Adult C57BL/6J mice at 8-10-week old
13. Glycine (Sangon Biotech, catalog number: A610235, storage temperature: room temperature)
14. Phosphate buffered saline (PBS, Sigma-Aldrich, catalog number: P5368-10PAK, storage temperature: room temperature)
15. Triton X-100 (Sangon Biotech, catalog number: A600198, storage temperature: room temperature)
16. Tween-20 (Sangon Biotech, catalog number: A100777, storage temperature: room temperature)
17. Horse serum (short term storage: 4 °C; long term storage: -20 °C) (Thermo Fisher, catalog number: 16050122)
18. Fetal bovine serum (short term storage: 4 °C; long term storage: -20 °C) (Trinity, catalog number: 010101)
19. Gibco™ Sodium Pyruvate 100 mM Solution (Life Technologies, catalog number: 11360070, storage temperature: 4-8 °C)

20. Gibco™ Penicillin-Streptomycin, liquid (Life Technologies, catalog number: 15140-122, storage temperature: 4-8 °C)
21. Ethylene Diamine Tetraacetic Acid (EDTA, Solarbio, catalog number: E8040, storage temperature: 4-8 °C)
22. Gibco™ Dulbecco's modified Eagle medium (Life Technologies, catalog number: C11995500BT, storage temperature: 4-8 °C)
23. Bovine Serum Albumin (IgG-Free, Protease-Free, Jackson ImmunoResearch, catalog number: 000-001-162, storage temperature: 4-8 °C)
24. Fluoroshield™ with DAPI (Sigma-Aldrich, catalog number: F6057, storage temperature: 4-8 °C)
25. Primary antibody anti-MyoD (Sigma-Aldrich, catalog number: M6190, storage temperature: 4-8 °C)
26. Primary antibody anti-Pax7 (mouse) (Developmental Studies Hybridoma Bank, catalog number: pax7, storage temperature: 4-8 °C)
27. Secondary antibodies:
 - a. Goat anti-Rabbit IgG2a Alexa Fluor® 488 (Thermo Fisher Scientific, catalog number: A-21131, 4-8 °C)
 - b. Goat anti-Mouse IgG Alexa Fluor® 546 (Thermo Fisher Scientific, catalog number: A-21123, 4-8 °C)
28. Collagenase Type 1 (Worthington Biochemical, catalog number: LS004194, storage temperature: -20 to -30 °C)
29. Paraformaldehyde (Sigma-Aldrich, catalog number: P6148, storage temperature: 4-8 °C)
30. Goat serum (Thermo Fisher, catalog number: 31872, storage temperature: -20 to -30 °C)
31. EDTA (Solarbio, catalog number: E8040)
32. Chicken embryo extract (C3999, Biomol)
33. Washing media (see Recipe 1)
34. Collagenase solution (see Recipe 2)
35. 4% Paraformaldehyde (see Recipe 3)
36. Culture media (see Recipe 4)
37. Blocking buffer (see Recipe 5)

Equipment

1. Pipettes (Thermo scientific, Finnpipette F3)
2. Surgical instruments (including a pair of scissors, a pair of fine scissors and a tweezer) (Figure 1a, 1b and 1c)
3. Cell culture hood (AIRTECH, model: BSC-1004IIA2)
4. Mice dissecting table
5. 4 °C fridge
6. Centrifuge (Thermo Fisher, catalog number: 75002420)

7. Water bath (DK-8AX)
8. Stereoscopic microscope (Mshot, OLYMPUS SZ61)
9. Heating pad (DeiuxHeart Mat, model: MDH10)
10. CO₂ incubator (Thermo Fisher Scientific, model: 371)
11. Laser scanning confocal microscope (Carl Zeiss, model: LSM 700)

Procedure

A. Preparation

1. Cut the Glass Pasteur Pipettes (Figure 1e) to a small curved (Figure 1f) and a large (Figure 1g) pipette with a diamond pen (Figure 1d). Polish the terminal with an ethanol flame to smooth the edge. Coat pipettes with undiluted horse serum.
2. Coat two 6-cm cell culture dishes (2 ml for each dish) and a 24-well plate (400 µl for each well) with undiluted horse serum until the entire bottom of plates was covered, followed with incubation for 1 min under a cell culture hood, and then transfer the horse serum back to the original tube and store in a 4 °C fridge.
3. Uncover the plates and air-dry under a cell culture hood for 1 h.
4. Cover the plates and keep them at room temperature. It is better to coat the plate on the same day of the experiment.
5. Set the temperature of a water bath to 37 °C.
6. Prepare washing media (following Recipe 1 and then keep it in a 37 °C water bath). In general, 50 ml washing media is enough for up to 200 myofiber isolation from one EDL muscle.
7. Prepare collagenase solution (following Recipe 2 and then keep it in a 37 °C water bath). Each EDL requires ~1 ml diluted collagenase solution (final concentration: 0.2% w/v) in a 15 ml tube.
8. Thaw the 4% paraformaldehyde (following Recipe 3).
9. Plug in the heating pad.

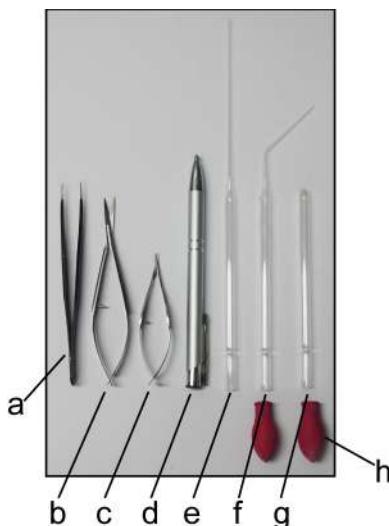


Figure 1. Instruments used for EDL muscle dissection and myofiber isolation. a. Tweezer;

b. Scissor; c. Fine scissor; d. Diamond pen; e. Glass Pasteur Pipette; f. Small curved pipette; g. Large pipette; h. Rubber bulbs.

B. EDL Muscle Dissection (Video 1)



Video 1. Procedures to isolate EDL muscle. This video was made at Guangdong Institute of Microbiology according to guidelines from the Guangdong Institute of Microbiology on Animal Care and approved by the Animal Research Ethics Board of Guangdong Institute of Microbiology under protocol [GT-IACUC201704071].

1. Anesthetize the experimental mice with CO₂.
2. Cut the skin below the ankle.
3. Cut through the skin along the inner leg.
4. Expose the muscle fascia and patella by pulling the skin up (Figure 2A).
5. Tore the tibialis anterior (TA) muscle fascia with a tweezer.
6. Tear off vastus lateralis over the knee, expose and cut off the origin of TA and extensor digitorum longus (EDL) muscle (Figure 2B).
7. Separate the tendon of TA muscle using a tweezer along the inner margin of the shank. Snip the tendons with the fine scissor (Figure 2C). Gently pull it up to separate the TA muscle from the bone and expose the entire EDL muscle (Figure 2D).
8. Cut the tendon of EDL muscle by snipping two-thirds of the lower ankle, Use one tip of the forceps to separate EDL muscle gently (Figure 2E).
9. Place the EDL muscle in a 15 ml tube with the prewarmed collagenase solution and place it in a 37 °C water bath to incubate for 60 to 80 min until myofibers are loose but not in crimped (it depends on the efficacy of collagenase and conditions of mice).
10. Dissect the other EDL muscle following Steps B1-B9.

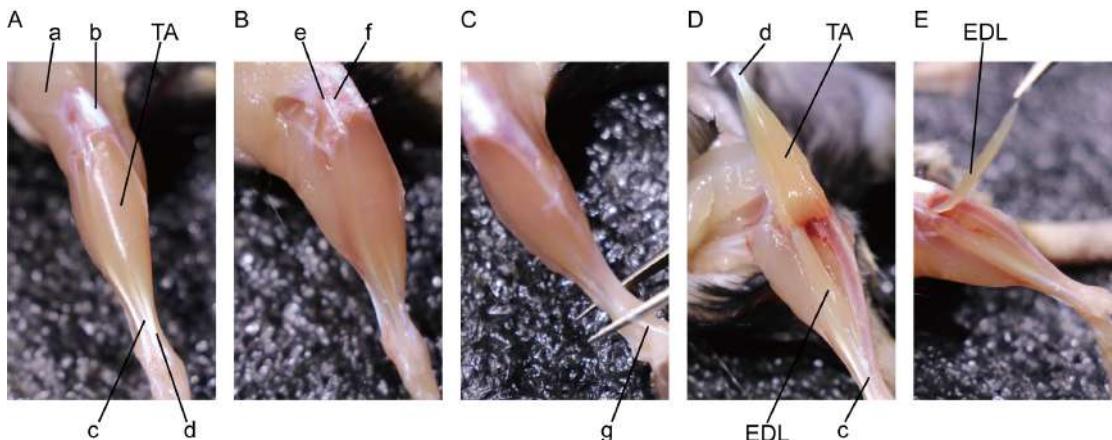


Figure 2. Procedures to isolate the EDL muscle. Pictures presented here depict (A) TA muscle after the skin was removed, (B) Origin of TA and EDL muscle, (C) Distal tendon of TA muscle, (D) EDL muscle after TA muscle was removed, (E) EDL muscle. a. vastus lateralis; b. patella; c. tendon of EDL muscle; d. tendon of TA muscle; e. origin of TA muscle; f. origin of EDL muscle; g. tendon of TA muscle.

C. Single Myofiber Isolation (Video 2)



Video 2. Procedures to isolate myofiber from EDL muscle. This video was made at Guangdong Institute of Microbiology according to guidelines from the Guangdong Institute of Microbiology on Animal Care and approved by the Animal Research Ethics Board of Guangdong Institute of Microbiology under protocol GT-IACUC201704071.

1. Transfer the digested EDL muscle to a horse serum coated 6-cm Petri dish with 5 ml prewarmed washing medium in it using the prepared big Glass Pasteur Pipette.
2. Place the 6-cm Petri dish on the heating pad. Inhale an appropriate amount of solution from the dish. Hold the Petri dish with the right hand to adjust the position of dish. Flush the EDL muscle continuously to rotate it along its long pivots until myofibers are naturally released from EDL muscle.

3. When a single muscle fiber is released, use a small bent glass Pasteur pipette to transfer it into a well of 24-well plate covered with 200 μ l washing media. Repeat Steps C1 and C2 until the number of myofibers meets your requirement. It is recommended that the myofibers be collected within 1-h.
4. Wash each well with 500 μ l washing media three times before adding 500 μ l culture media (following Recipe 4).
5. Place the 24-well plate into a CO₂ incubator at 37 °C.

D. Fixation

1. When myofibers are cultured for 0/24/48/72-h or other different designated length of time, remove as much culture media as possible (leave the minimum amount of culture media to avoid myofibers' exposure to the air).
2. Fix myofibers with 500 μ l 4% PFA for 10 min at room temperature.
3. Aspirate the fixation buffer, and add 500 μ l 50 mM glycine to incubate for 10 min to quench the fixation at room temperature.
4. Wash myofibers with PBS for 3 times at room temperature. The fixed myofibers can be stored in a 4 °C fridge for up to a week before immunostaining (Figure 3).

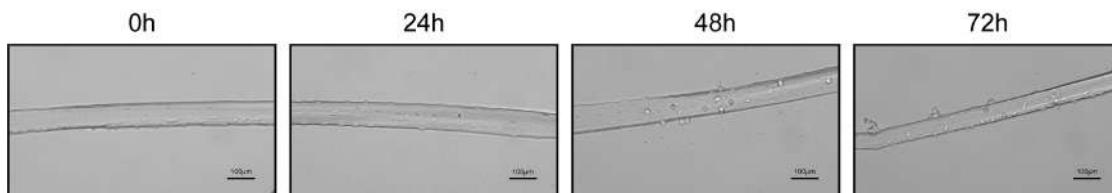


Figure 3. Images of isolated single myofiber after cultured for 0/24/48/72 h. Scale bars = 100 μ m.

E. Immunostaining

1. Permeabilize myofibers with Triton X-100 (0.5% v/v) diluted in PBS for 10-min, followed by a 5-min wash in PBS for 3 times.
2. Prepare blocking buffer as Recipe 5. Centrifuge the blocking buffer at 16,000 $\times g$ for 10 min in case there was any residue precipitate from goat serum. Incubate fibers with 400 μ l blocking solution for 1 h at room temperature.
3. Incubate with 200 μ l blocking buffer containing primary antibodies of Pax7 (1:100) or MyoD (1:300) overnight at 4 °C.
4. After night incubation with primary antibody, wash myofibers 3 times for 5-min with PBS containing 0.5% Tween 20 to remove any unbound antibody.
5. Incubate with 200 μ l blocking buffer containing appropriate secondary antibody (1:500) for 1-h at room temperature protected from light.
6. After 1-h incubation with secondary antibody, wash 3 times at 5 min per wash with PBS containing 0.5% Tween 20.

7. Transfer each fiber to a glass slide and drain the remaining liquid.
8. Mount the glass slide with DAPI-containing mounting medium. Cover with a cover slide and dry the glass slide for at least 10 min before imaging.
9. Observe myofibers under a fluorescent microscope and capture images of immunostained myofibers (Figure 4).

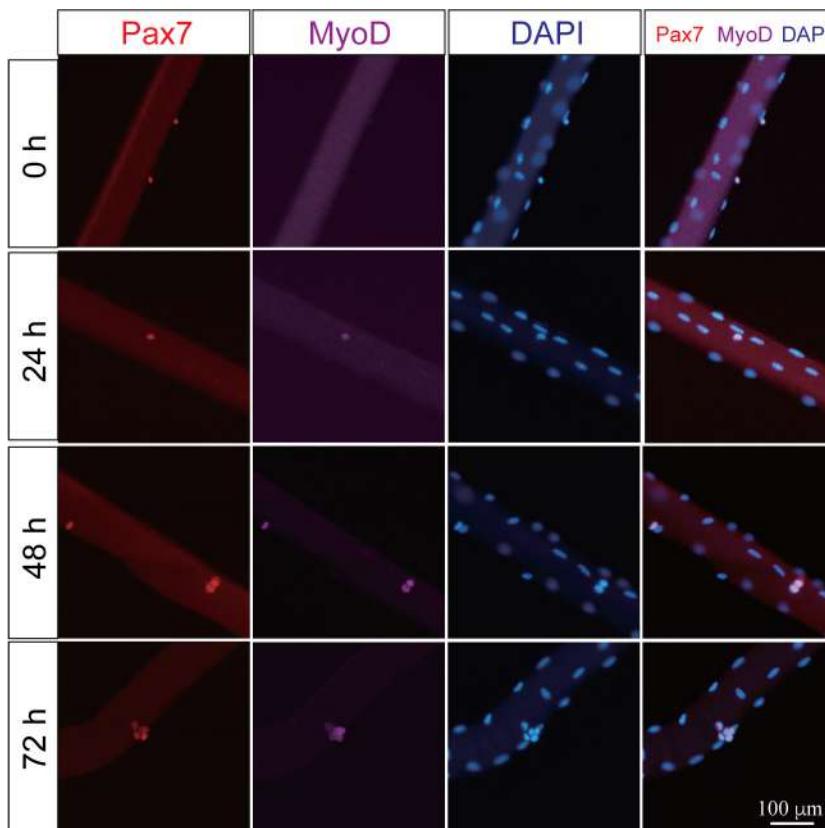


Figure 4. Representative images of single myofiber culture harvested at T0, 24, 48 and 72-h. Single myofiber with satellite cells or clusters were fixed and immunostained with antibodies against Pax7 or MyoD. Red for Pax7⁺ satellite cells. Purple for MyoD⁺ satellite cells. Blue for nucleus. Scale bar = 100 μ m.

Data analysis

Based on our protocol, around ~200 myofibers could be isolated from each EDL muscle. The average number of Pax7⁺ satellite cells on each myofiber at T0 is 6-8 and the MyoD⁺ satellite cells could be barely detected at T0. After 24-h culture, the number of MyoD⁺ satellite cells is increased upon satellite cells activation. Proliferated satellite cells could be observed at 48-h timepoint. The satellite cells cluster could be observed after 72-h culture (Figure 4).

Notes

1. Avoid bubbles when coating plates with horse serum.
2. Do not let tweezers or scissors touch the EDL muscle.
3. When using the bent plastic dropper to transfer muscle fiber, single myofiber should be inhaled along one end of the muscle fiber to avoid snapping the muscle.
4. When transferring myofibers, insert the nozzle below the liquid level to prevent the muscle fibers from being exposed to air.
5. Avoid bubbles when covering glass slips.

Recipes

1. Washing media
DMEM supplemented with:
10% fetal bovine serum
2% sodium pyruvate
1% Penicillin-Streptomycin
2 mM EDTA
Filter the solution through a 0.2 µm filter (optional, to remove any debris from serum)
2. Collagenase solution
DMEM supplemented with 0.2% Collagenase Type 1
3. 4% paraformaldehyde
 - a. Dissolve 4 g paraformaldehyde powder in 100 ml PBS.
 - b. Heat the solution to 65 °C until paraformaldehyde completely dissolved
 - c. Filter the solution through a 0.2 µm filter and store at -20 °C
4. Culture media
DMED supplemented with 20% fetal bovine serum
1% chicken embryo extract
1% Penicillin-Streptomycin
Filter the solution through a 0.2 µm filter
5. Blocking buffer
PBS supplemented with:
3% BSA
0.5% Tween 20
5% goat serum (added right before the blocking procedure)

Acknowledgments

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Author contribution: LW. X. designed the experiment and collected the grant for present study. LW. X., SJ. C., HR. D. and XP. Y. developed and optimized the methodology. SJ. C. collected and

analyzed the data. S.J. C. drafted the manuscript and L.W. X. proofread the manuscript.

Competing interests

The authors declare that there is no conflict of financial or research interest.

Ethics

C57BL/6J mice were raised at the SPF animal facility of Guangdong Institute of Microbiology (GDIM) in a 12/12 dark-light cycle with ad libitum free access to food and water. The animal protocol was proved by the Institute Animal Care Use Committees of GDIM (Permission #: GT-IACUC201704071).

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Co-culture of Human Stem Cell Derived Neurons and Oligodendrocyte Progenitor Cells

Stephanie Dooves^{1, #}, Aishwarya G. Nadadur^{2, #}, Lisa Gasparotto¹ and Vivi M. Heine^{1, 3, *}

¹Pediatric Neurology, Emma Children's Hospital, Amsterdam UMC, Amsterdam, Neuroscience, Vrije Universiteit Amsterdam, The Netherlands; ²Department of Functional Genomics, Center for Neurogenomics and Cognitive Research, Amsterdam Neuroscience, Vrije Universiteit Amsterdam, The Netherlands; ³Department of Complex Trait Genetics, Center for Neurogenomics and Cognitive Research, Amsterdam Neuroscience, Vrije Universiteit Amsterdam, The Netherlands

*For correspondence: vm.heine@amsterdamumc.nl

#Contributed equally to this work

[Abstract] Crosstalk between neurons and oligodendrocytes is important for proper brain functioning. Multiple co-culture methods have been developed to study oligodendrocyte maturation, myelination or the effect of oligodendrocytes on neurons. However, most of these methods contain cells derived from animal models. In the current protocol, we co-culture human neurons with human oligodendrocytes. Neurons and oligodendrocyte precursor cells (OPCs) were differentiated separately from pluripotent stem cells according to previously published protocols. To study neuron-glia cross-talk, neurons and OPCs were plated in co-culture mode in optimized conditions for additional 28 days, and prepared for OPC maturation and neuronal morphology analysis. To our knowledge, this is one of the first neuron-OPC protocols containing all human cells. Specific neuronal abnormalities not observed in monocultures of Tuberous Sclerosis Complex (TSC) neurons, became apparent when TSC neurons were co-cultured with TSC OPCs. These results show that this co-culture system can be used to study human neuron-OPC interactive mechanisms involved in health and disease.

Keywords: Human iPSC, Neuron, Oligodendrocyte, Co-culture, Myelin, Neuron-glia interaction

[Background] The human brain consists of an immense complex organization of cells that we are only recently starting to identify, and that cannot be studied in animal models. The human brain also contains a high white matter content, which is suggested to account for higher brain functions, such as social and cognitive learning (Maldonado and Angulo, 2015; Almeida and Lyons, 2016; Kougoumtzidou *et al.*, 2017). Single cell expression studies in animals indicate that oligodendrocytes form heterogeneous populations of cells (Marques *et al.*, 2016). This supports the notion that oligodendrocytes fulfill more complex functions than solely isolating axons. As the human comprises of higher diversity of neurons compared to rodent brain, and considering the role of white matter in complex functions in learning and cognition, we could expect an even more complex diversity of oligodendrocyte lineage cells in the human brain. Therefore, we need to identify oligodendrocyte-neuron crosstalk in the human brain. Next to the classic white matter disorders, such as multiple sclerosis and the leukodystrophies (van der Knaap and Bugiani, 2017), white matter abnormalities are consistently found in psychiatric disorders (Haroutunian *et al.*, 2014). Increasing evidence shows that crosstalk between neurons and

oligodendrocytes is important for proper neural network functioning (Bergles *et al.*, 2000; Velez-Fort *et al.*, 2010; Maldonado and Angulo, 2015) and myelin formation (Almeida and Lyons, 2016; Kougioumtzidou *et al.*, 2017). Therefore, to study the involvement of neuron-oligodendrocyte interactions in the normal and diseased brains, we are in need of human-based model systems. As current assays mostly involve non-human cells (Cui *et al.*, 2010; Hill *et al.*, 2014; Clark *et al.*, 2017; Pang *et al.*, 2018; Treichel and Hines, 2018), we developed human induced pluripotent stem cell (iPSC)-based co-culture models to study crosstalk between human neurons and human oligodendrocyte progenitor cells (OPCs). The presented co-culture method was used to study neuron-OPC interactions in Tuberous Sclerosis Complex (TSC) (Nadadhur *et al.*, 2019), a genetic multisystem disorder that shows both grey and white matter abnormalities in the brain. Although some neuronal abnormalities were present in mono-cultures of TSC neurons, in the presence of OPCs increased axonal density and hypertrophy became apparent (Nadadhur *et al.*, 2019). This suggests that specific neuronal phenotypes can only be studied when oligodendrocytes are present. Vice versa oligodendrocyte maturation is highly dependent on neuronal signaling. Therefore these culture systems can be applied to study multiple processes in health and disease in which complex neuron-oligodendrocyte interactions are involved, and provide prospects for the development of drug screening platforms for all-human cells, *e.g.*, patient iPSCs. To conclude, this novel human neuron-OPC co-culture model can be used to study neuron-OPC crosstalk in health and disease.

Materials and Reagents

1. 12-well plate (VWR, catalog number: 665180)
2. 10 µl filter tips (Thermo Fisher, catalog number: 11977714)
3. 100 µl filter tips (Thermo Fisher, catalog number: 11953466)
4. 1000 µl filter tips (Thermo Fisher, catalog number: 11973466)
5. 6-well plate (VWR, catalog number: 734-2323)
6. 18 mm coverslips (VWR, catalog number: 631-0153)
7. 5 ml pipette (VWR, catalog number: 606180)
8. 10 ml pipette (VWR, catalog number: 607180)
9. 15 ml tube (VWR, catalog number: 525-0400)
10. Microscope slide (VWR, catalog number: 631-0108)
11. Syringe needle (BD Biosciences, catalog number: 300400)
12. 4',6-Diamidino-2-Phenylindole (DAPI) (Sigma-Aldrich, catalog number: D9542-5MG)
13. Accutase (Merck-Millipore, catalog number: sf006)
14. Anti-MAP2 antibody (Abcam, catalog number: AB5392)
15. Anti-MBP antibody (Covance, catalog number: SMI-99P)
16. Anti-Olig2 antibody (Merck-Millipore, catalog number: AB9610)
17. Anti-SMI312 antibody (Eurogentec, catalog number: SMI-312P-050)
18. Arabinosylcytosine (AraC) (Merck-Millipore, catalog number: 251010)

19. β -mercaptoethanol (Thermo Fisher Scientific, catalog number: 21985023)
20. B27 with vitamin A (Thermo Fisher Scientific, catalog number: 17504-044)
21. B27 without vitamin A (Thermo Fisher Scientific, catalog number: 12587-010)
22. Basic fibroblast growth factor (bFGF) (Peprotech, catalog number: 100-18B-50ug)
23. Brain-derived neurotrophic factor (BDNF) (Peprotech, catalog number: 450-02)
24. Biotin (Sigma-Aldrich, catalog number: B4501-100MG)
25. Bovine Serum albumin (BSA) (Sigma-Aldrich, catalog number: A9418)
26. Cyclic adenosine monophosphate (cAMP) (Sigma, catalog number: D0260-5MG)
27. Defined Trypsin Inhibitor (DTI) (Thermo Fisher Scientific, catalog number: R007100)
28. DMEM/F12 with Glutamax (Life Technologies, catalog number: 21331-020)
29. DMEM/F12 without L-glutamine (Life Technologies, catalog number: 21331-046)
30. Dimethylsulfoxide (DMSO) (Sigma-Aldrich, catalog number: D2650)
31. Dorsomorphin (Tocris Bioscience, catalog number: 3093/10)
32. Epidermal growth factor (EGF) (Peprotech, catalog number: AF-100-15-500ug)
33. Ethylenediaminetetraacetic acid (EDTA) (Invitrogen, catalog number: 15575-038)
34. Fetal Bovine Serum (FBS) (ThermoFisher Scientific, catalog number: 16140063)
35. Fluoromount G (Southern Biotech, catalog number: 0100-01)
36. Glial-cell derived neurotrophic factor (GDNF) (Peprotech, catalog number: 450-10)
37. Geltrex (Life Technologies, catalog number: A1413302)
38. Glutamax (Thermo Fisher Scientific, catalog number: 35050-038)
39. Goat anti-rat/mouse/rabbit/chicken/guinea pig Alexa Fluor antibodies (Life Technologies)
40. Heparin (Sigma-Aldrich, catalog number: H3393-50KU)
41. HEPES (Thermo Fisher Scientific, catalog number: 15630-056)
42. Human Sonic Hedgehog (hSHH) (Peprotech, catalog number: 100-45-500ughSHH)
43. Insulin-like growth factor 1 (IGF1) (Peprotech, catalog number: 100-11-100ug)
44. Insulin (Sigma-Aldrich, catalog number: I9278)
45. KCl (Sigma-Aldrich, catalog number: P5405-250gr)
46. KH₂PO₄ (Sigma-Aldrich, catalog number: P5379)
47. L-glutamine (Thermo Fisher Scientific, catalog number: 25030-024)
48. Mouse laminin (mLaminin) (Sigma-Aldrich, catalog number: L2020-1mg)
49. N2 supplement (Thermo Fisher Scientific, catalog number: 17502-048)
50. Na₂HPO₄ (Sigma-Aldrich, catalog number: S7907-500gr)
51. NaCl (VWR, catalog number: S9888-1Kg)
52. Neurobasal medium (Thermo Fisher Scientific, catalog number: 21103-049)
53. Neurotrophin 3 (NT3) (Peprotech, catalog number: 450-03-100ug)
54. Noggin (Peprotech, catalog number: 120-10C)
55. Non-essential amino acids (NEAA) (Thermo Fisher Scientific, catalog number: 11140-035)
56. Normal goat serum (NGS) (Life Technologies, catalog number: 16210-064)
57. Penicillin/streptomycin (Pen/Strep) (Sigma-Aldrich, catalog number: P0781)

58. Paraformaldehyde (PFA) 16% (Electron Microscopy Sciences, catalog number: 15710-S)
59. Poly-L-ornithine (PLO) (Sigma-Aldrich, catalog number: P3655-100mg)
60. Rock Inhibitor (RI) (Y27632; Selleckchem, catalog number: S1049)
61. Retinoic Acid (RA) (Sigma-Aldrich, catalog number: R2625-100MG)
62. SB431542 (Selleckchem, catalog number: S1067)
63. Triiodothyronine (T3) (Sigma-Aldrich, catalog number: T6397-100MG)
64. TeSRE8 (Stem Cell Technologies, catalog number: 5940)
65. Triton X-100 (Sigma-Aldrich, catalog number: T8787-100ml)
66. TrypLE (Life Technologies, catalog number: 12563-029)
67. Valproic acid (VPA) (Sigma-Aldrich, catalog number: P4543-10G)
68. Vitamin C/Ascorbic Acid (Sigma-Aldrich, catalog number: A4544-25G)
69. N1 supplement (Sigma-Aldrich, catalog number: N6530-5ML)
70. Neuroglia co-culture medium (see Recipes)
71. Neural Maintenance Medium (NMM) with Vitamin A (see Recipes)
72. Neural Maintenance Medium (NMM) without Vitamin A (see Recipes)
73. N2 medium (see Recipes)
74. NB medium (see Recipes)
75. Blocking Buffer (see Recipes)
76. PBS (see Recipes)
77. PLO/mLaminin coating (see Recipes)
78. Geltrex coating (see Recipes)

Equipment

1. Pipette controller (BD Biosciences, model number: Falcon Express)
2. Incubator (Binder, model number: 9140-0044; 5% CO₂, 20% O₂)
3. Tabletop Centrifuge (Eppendorf, model number: centrifuge 5810)
4. -80 °C freezer (Thermo Fisher Scientific; model number ULT1786-6-V49)
5. Bright field microscope (Zeiss, model number: Axiovert 40C.)
6. Fluorescent microscope (Leica Microsystems, model number: Leica DM6000B)

Software

1. Columbus 2.5 online software (Perkin Elmer)
2. Leica Application Suite Advanced Fluorescence (Leica)

Procedure

A. Neuronal differentiation

Neurons are differentiated according to previously published protocols (Shi *et al.*, 2012; Nadadur *et al.*, 2017), shortly:

1. hiPSCs are passaged onto Geltrex-coated 12-well plates in 1 ml TesRE8 medium with 10 µM RI per well.
2. Refresh all medium every day.
3. After 2 days (or when cells are 100% confluent), add 1 ml neural maintenance medium (NMM) with vitamin A + 1 µM Dorsomorphin + 10 µM SB431542.
4. Refresh all medium every day until Day 12.

Note: Between Days 8 and 12 a uniform neuroepithelial sheet should appear (see Figure 1).

5. Prepare a PLO/mLaminin coated 6-well plate as described in the Recipes.
6. Collect neuroepithelial rosette cells (NES cells) by manually cutting them (see description in note) and plate them in the 6-well plate.

Note: Prepare fresh pre-warmed NMM with vitamin A + 1 µM Dorsomorphin + 10 µM SB431542 + 10 µM RI. Remove the medium from the 12-well plate, wash the wells with PBS once and add 1 ml of the freshly prepared medium. Sterilize the microscope area with 70% Ethanol and cut the rosettes using a 10 µl tip to mark their borders and to lift them up. Collect the floating rosettes with a 5 ml pipette and move them to the PLO/mLaminin coated 6-well plate with NMM with vitamin A + 1 µM Dorsomorphin + 10 µM SB431542 + 10 µM RI. All rosettes from 1 well of a 12-well plate are plated in 1 well of a 6-well plate.

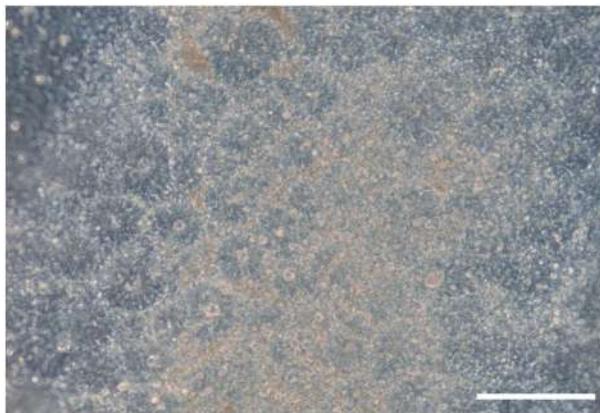


Figure 1. Brightfield picture showing neural rosette structures that form between Days 8 and 12. Scale bar = 200 µm.

7. On Day 13, change the medium to 2 ml pre-warmed NMM with vitamin A + 20 ng/ml bFGF + 20 ng/ml EGF.
8. Every day remove 1 ml of medium and add 1 ml pre-warmed NMM with vitamin A + 40 ng/ml bFGF + 40 ng/ml EGF. When confluent, passage the cells with TrypLE in a 1:2 or 1:3 ratio as described in the note below.

Notes:

- a. Cells can be kept in NES cell stage for 1-4 passages.

- b. *Growth factor concentration is double when only half the medium is changed: together with the medium left in the well the concentration will be similar to the concentration in Step A7.*
- c. *TrypLE passage: Remove all medium from the well and add 300 µl pre-warmed TrypLE. Swirl it around the well and incubate for 2 min at room temperature (RT). Add 600 µl of pre-warmed DTI or medium to stop the reaction and collect the cells into a tube containing 5 ml NMM. Spin down at 300 x g for 5 min at RT and re-suspend the pellet in desired medium.*
9. To start the neuronal induction, plate the NES cells after a TrypLE passage in a PLO/mLaminin-coated 12-well plate in pre-warmed 1 ml NMM + 20 ng/ml bFGF + 20 ng/ml EGF per well.
10. When cells reach 80%-90% confluence, switch half of the medium (500 µl/well) to pre-warmed N2 medium + 800 ng/ml hSHH.

Note: This is considered Day 1 of the differentiation protocol.

11. Until Day 4, refresh half of the medium (500 µl/well) daily with pre-warmed N2 medium + 800 ng/ml hSHH.
12. On Day 5, switch half of the medium (500 µl/well) to pre-warmed NB medium + 40 µM VPA.
13. Until Day 7, refresh half of the medium (500 µl/well) daily with pre-warmed NB medium + 40 µM VPA.
14. Prepare PLO/mLaminin-coated 12-well plates before Day 8 as described in the Recipes.
15. On Day 8, passage the neural progenitors with accutase 1:2 or 1:3 to a new well (see note for explanation). Plate cells in pre-warmed 1.5 ml NB medium + 20 ng/ml BDNF + 10 ng/ml GDNF + 10 ng/ml IGF1 + 1 µM cAMP.

Accutase treatment: Remove all medium, add 300 µl of accutase per well of a 12-well plate and return the plate to the incubator for 5-7 min. By gently tapping the plate, the cells will visibly come off. At this point, collect the cells in a tube, add 5 ml fresh pre-warmed medium, spin at 300 x g for 5 min at RT and re-suspend the pellet in the desired medium. Resuspend carefully; do not break clumps into single cells.

16. Until Day 18 refresh medium three times a week by removing 1 ml of medium and adding 1 ml of pre-warmed NB medium + 30 ng/ml BDNF + 15 ng/ml GDNF + 15 ng/ml IGF1 + 1.5 µM cAMP.
17. On Day 12, prepare a 12-well sandwich plate as described in the note, Figure 2 and Video 1. Coat the plate with Geltrex as described in the Recipes. Plate 25,000 primary rat astrocytes in each well containing pre-warmed DMEM/F12 medium with Glutamax + 10% FBS + 1x NEAA + 1x Pen/Strep.

Note: Sandwich plate preparation: Take a 12-well plate and heat up a sterile syringe needle in flame. Punch in 4 corners of each well to make uniform small bumps in the plastic bottom of the well. These bumps will help suspend a coverslip on them, separated by a small distance from the cells in the bottom of the well. See Figure 2 and Video 1.



Video 1. Sandwich plate

18. Before Day 18, prepare the desired amount of 12-well plates containing 18 mm diameter coverslips and coat with PLO/laminin as described in the Recipes.
Note: Coverslips can be stored by sealing the plate tightly with parafilm for maximum 1 week at 4 °C.
19. On Day 18, make a single-cell suspension of the neurons using accutase as described in the note. Plate 1.5 million cells per 12-well plate on the pre-coated PLO/mLaminin coverslips in pre-warmed 1 ml NB medium + 20 ng/ml BDNF + 10 ng/ml GDNF + 10 ng/ml IGF1 + 1 µM cAMP.
Note: Remove all medium, add 300 µl of accutase per well of a 12-well plate and return the plate to the incubator for 10-15 min. By gently tapping the plate, the cells will visibly come off. Dissociate the cells in the accutase using a 1000 µl pipette very slowly and pipet into a 15 ml tube with 5 ml of neurobasal medium. Spin at 300 x g for 5 min at RT. Remove supernatant and very gently resuspend the pellet in 1 ml of NB medium and try breaking the clumps of cells very gently using a 1000 µl pipette (pipet up and down maximum 3-4 times). Then leave the tube for 2-5 min to let the big clumps settle down at the bottom. Take the supernatant into another tube; thrash the pellet (the big clumps will not have mature cells but mainly proliferating precursor cells).

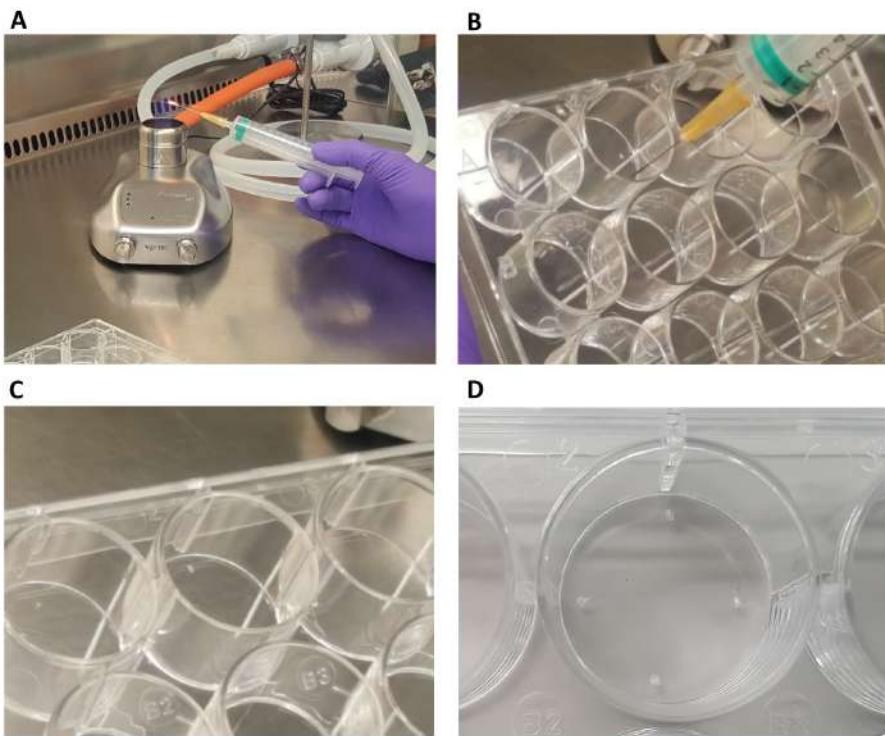


Figure 2. Sandwich plate preparation. A. Take a 12-well plate and a sterile syringe needle. Flame the needle until it is hot. With the hot needle, make a bump on 4 sides of the well (B-D). The astrocytes will be plated on the bottom of the plate, and a coverslip with neurons can be inverted on top. The coverslip will lean on the bumps. See also Video 1.

20. On Day 19, turn the coverslips with adhered neurons upside down over the bumps on the astrocyte plates.

Note: By now, the astrocytes should have reached 80% confluence in order to support the neurons.

21. Change half of the medium (500 μ l/well) twice a week with pre-warmed NB medium + 40 ng/ml BDNF + 20 ng/ml GDNF + 20 ng/ml IGF1 + 2 μ M cAMP.

22. On the day before the second refresh (Day 24/25), add 1 μ M AraC to cultures to stop proliferation.

23. Next day (Day 25/26) refresh half of the medium (500 μ l/well) with pre-warmed NB medium + 40 ng/ml BDNF + 20 ng/ml GDNF + 20 ng/ml IGF1 + 2 μ M cAMP.

24. Keep cultures until Day 37 (see Figure 3), while refreshing medium twice a week as described in Step 21.

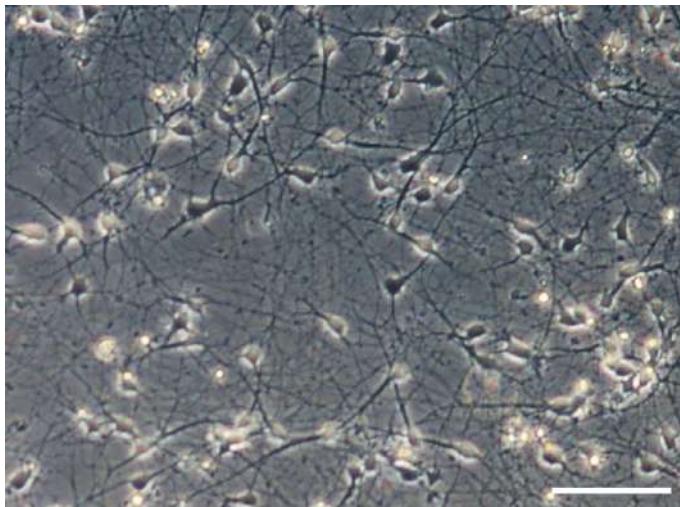


Figure 3. Brightfield picture of Day-37 neurons prior to co-culture showing neuronal network formation in sandwich culture. Scale bar = 100 μm .

B. OPC differentiation

OPCs are generated according to previously published protocol (Izrael *et al.*, 2007), shortly:

1. hiPSCs are passaged with EDTA and plated on an anti-adherent 6-well plate in 3 ml pre-warmed NMM with Vit A + 20 ng/ml EGF + 4 ng/ml bFGF + 10 μM RI + 20 ng/ml T3 per well for embryoid body (EB) formation (see Figure 3).

Note: Use 2 wells of iPSCs for 1 well of EBs.

2. The next day (Day 1), refresh $\frac{2}{3}$ of the medium: swirl cells to the middle of the wells and carefully aspirate 2 ml of medium. Add 2 ml of pre-warmed NMM with Vit A + 20 ng/ml EGF + 4 ng/ml bFGF + 10 μM RI + 20 ng/ml T3.
3. On Day 2, refresh $\frac{2}{3}$ of the medium as described in Step B2 with pre-warmed NMM with Vit A + 20 ng/ml EGF + 4 ng/ml bFGF + 10 μM RA.
4. Repeat every other day.
5. On Day 10, plate EBs on Geltrex-coated plate as described in the note. If EBs become very dark, or decrease in density, plate EBs sooner than on Day 10, but not before Day 4. In our experience, Day 8 is mostly ideal (see Figure 4).

Note: Plate EBs on geltrex coated plate by collecting the medium with EBs in a 15 or 50 ml tube. Leave the tube for 2-3 min to let the EBs settle. Carefully aspirate most of the medium, and resuspend EBs in the appropriate amount of desired medium (1 ml for a 12-well plate; 2 ml for a 6-well plate). On Days 4-9, EBs are plated in pre-warmed NMM with Vit A + 20 ng/ml EGF + 4 ng/ml bFGF + 10 μM RA + 20 ng/ml T3. If EBs are plated on Day 10, NMM with Vit A + 20 ng/ml EGF + 20 ng/ml T3 should be used instead.

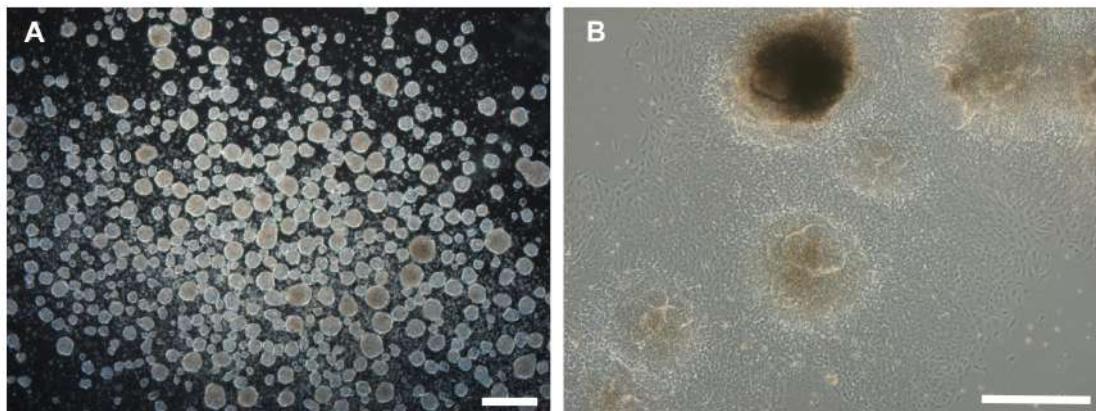


Figure 4. Brightfield pictures showing embryoid body formation (A) and outgrowth of cells from embryoid bodies after plating on a geltrex-coated plate (B). Scale bars = 500 μm .

6. On Day 10, switch all medium to pre-warmed NMM with Vit A + 20 ng/ml EGF + 20 ng/ml T3.
7. From now on: refresh $\frac{1}{3}$ of the medium every other day and split cells when they reach 90% density to a new Geltrex-coated plate using accutase.
Note: Add RI to medium after passaging to help the cells recover.
8. On Day 18, change medium to NMM without Vit A + 20 ng/ml EGF; keep refreshing and splitting cells as described in Step B7.
9. On Day 37, change medium to NMM without Vit A + 5 ng/ml bFGF + 5 ng/ml EGF + 1 $\mu\text{g}/\text{ml}$ mLaminin + 50 $\mu\text{g}/\text{ml}$ Vit C; keep refreshing and splitting cells as described in Step B7.
10. On Day 39, change medium to NMM without Vit A + 5 ng/ml bFGF + 5 ng/ml EGF + 1 $\mu\text{g}/\text{ml}$ mLaminin + 50 $\mu\text{g}/\text{ml}$ Vit C + 50 ng/ml noggin; keep refreshing and splitting cells as described in Step B7.
11. On Day 42, change medium to NMM without Vit A + 1 $\mu\text{g}/\text{ml}$ mouse laminin + 50 $\mu\text{g}/\text{ml}$ Vit C + 50 ng/ml noggin; keep refreshing and splitting cells as described in Step B7.
12. Keep OPC cultures in the medium of Step B11 until Day 65 (see Figure 5).

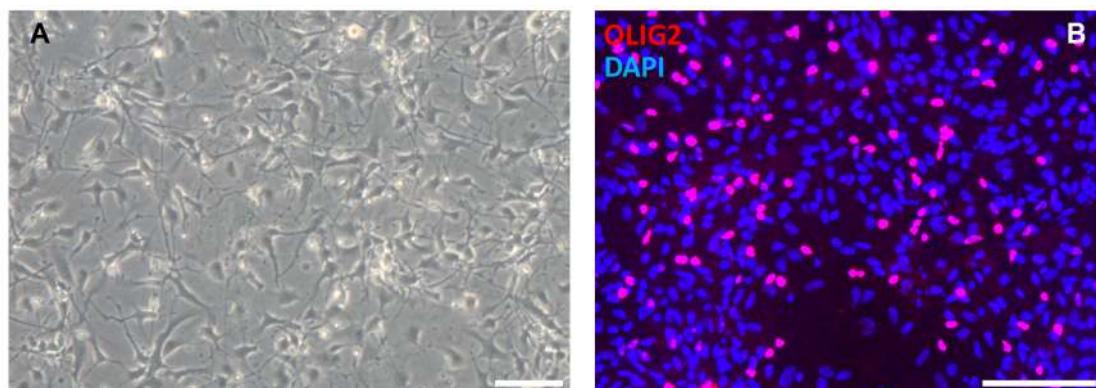


Figure 5. OPC differentiation. At the end of the OPC differentiation protocol, cells show a glial morphology (A, Day 45) and express OPC markers like OLIG2 (B, Day 65). Scale bars =

100 μ m.

C. Neuron-OPC co-culture

1. At day 1 of co-culture, move the Day 37 neurons and plate Day 65 OPCs on top:
 - a. Take a new 12-well plate.
 - b. Pick the Day 37 neuronal coverslips up with a forceps from the rat astrocyte plate and plate in a new 12-well plate (with neurons on top, so flip coverslip compared to how it was on astrocyte plate).

Note: No coating is necessary for the new plate as the neuronal coverslips already contain the coating.
 - c. Add 1 ml of pre-warmed neuroglia co-culture medium.
 - d. Get a plate with Day 65 OPCs.
 - e. Remove medium and wash plate once with PBS.
 - f. Remove PBS and add 750 μ l accutase to every well (6-well plate).
 - g. Incubate the plate for 5-10 min at 37 °C.
 - h. Check if cells are detaching from the plate by gentle tapping; if not, incubate longer.
 - i. When cells are detaching, collect cell suspension in a 15 ml tube with 9 ml pre-warmed DMEM/F12.
 - j. Spin down 5 min at 300 $\times g$ at RT.
 - k. Resuspend cells in 1 ml of pre-warmed neuroglia co-culture medium.
 - l. Remove 10 μ l of cell suspension for cell count.
 - m. Plate 4 $\times 10^5$ OPCs per coverslip on a 12-well plate.
2. Refresh $\frac{1}{2}$ of the medium twice a week: remove 500 μ l of medium and add 500 μ l of new pre-warmed neuroglia co-culture medium.
3. At Day 28 of co-culture, fix cells with 4% PFA for 15-20 min and use coverslips for immunostaining immediately (see Figure 6).

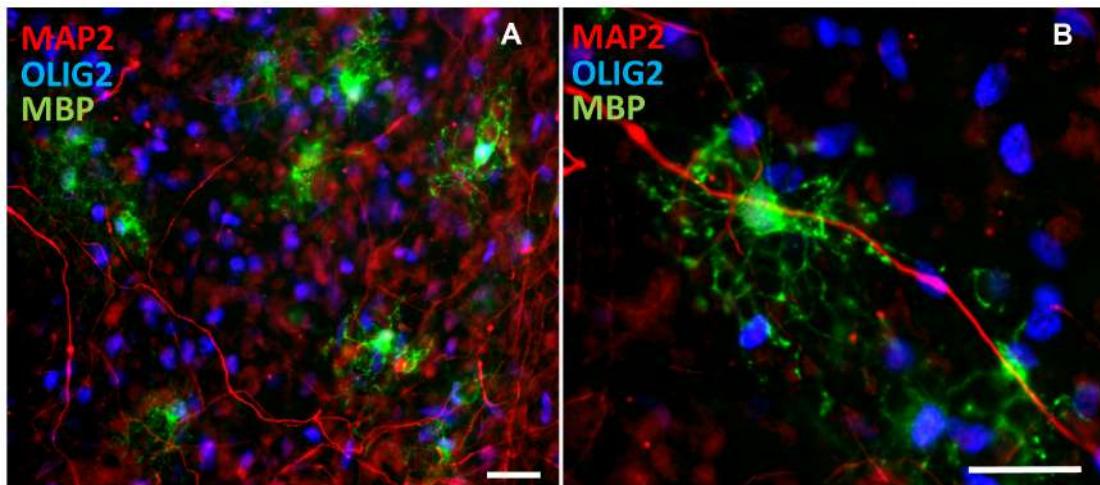


Figure 6. Immunostaining of co-cultures. (A) shows many OLIG2-positive OPCs and MBP-positive mature oligodendrocytes with MAP2-positive dendrites. (B) shows oligodendrocytes (MBP-positive) closely interacting with dendrites (MAP2-positive) Scale bars = 25 μ m.

D. Immunostaining

1. Wash plate 6 x 5 min with PBS.
2. Remove PBS.
3. Add 300 μ l blocking buffer per well.
4. Incubate for 1 h at RT.
5. Remove blocking buffer.
6. Add primary antibodies diluted in 300 μ l blocking buffer.
7. Incubate for 1 h at RT, then overnight at 4 °C.
8. Wash 6 x 5 min with PBS.
9. Add secondary antibodies diluted 1:1,000 in 300 μ l blocking buffer.
10. Incubate for 2 h at RT.
11. Wash 6 x 5 min with PBS.
12. Remove PBS.
13. Add DAPI 1:1000 diluted in PBS.
14. Incubate for 2 min at RT.
15. Wash 1 x with PBS.
16. Mount coverslips with Fluoromount G upside down on a microscopical slide.

Data analysis

1. Coverslips can be stained with markers for OPC maturation (for example OLIG2, MBP) and for markers of neuronal morphology (for example MAP2, SMI312). In our experience, for a control line there will be about 10% OLIG2 $^{+}$ cells, 1-2% MBP $^{+}$ cells and about 40% of the cells in the culture are MAP2 $^{+}$ neurons.

Note: OLIG2 and MBP do not label all human oligodendrocyte lineage cells.

2. Cell properties can be analyzed by automated software packages, for example Columbus 2.5 online software (see <https://www.perkinelmer.com/nl/product/image-data-storage-and-analysis-system-columbus> for more information about the Columbus software and Figure 7 for example pictures of analysis) To analyze neuronal morphology, algorithms for morphology, soma recognition and co-localization can be used. More precisely, based on MAP2 and nuclear staining the dendritic density and average dendritic density per neuron can be determined. Similar analysis based on SMI312 staining can be used to analyze axonal density.
3. OPC maturation can be assessed using immunostaining for mature oligodendrocyte markers like MBP. Because different cell lines might grow at different speeds, and have different efficiencies in oligodendrocyte generation, it is best to correlate the number of MBP-positive to the number of OLIG2- positive OPCs.
4. The correct statistical analysis depends on the research question of interest. For example, this also depends on the culture set-up, *i.e.*, when culturing 1 control oligodendrocyte line with neurons from different lines of patients and controls, vs. all combinations of control/patient oligodendrocytes with control/patient neurons. See Nadadhur *et al.* (2019) for how we performed statistical analysis.



Figure 7. Example of cell analysis in Columbus. Fluorescent images can be imported into Columbus (A) which has preprogrammed settings to find nuclei (B) and trace neurites (C). The output includes parameters as the maximum neurite length, total neurite length and number of extremities per cell.

Notes

As some variation is common in iPSC differentiation protocols, we recommend some quality checks before the start of a neuron-OPC co-culture.

1. Proper OPC differentiation can be verified by checking for OLIG2 expression around Days 57-60 of the differentiation protocol.
2. Proper neuronal differentiation can be verified by staining for MAP2/beta-III tubulin/NeuN in the cultures on Day 37. If GFAP expression is observed, it is advised to discard cultures, as glia contamination could interfere with mixed co-cultures from patient and control iPSCs. See

Nadadhur *et al.* (2017) for more glial free neuronal culture images.

Recipes

1. Neuroglia co-culture medium

480 ml DMEM/F12

480 ml Neurobasal

10 ml N1 supplement

20 ml B27 supplement

10 ml NEAA

60 ng/ml T3

100 ng/ml Biotin

10 ng/ml NT3

1 µg/ml mLaminin

20 ng/ml BDNF

1 µM cAMP

2. Neural Maintenance Medium (NMM) with Vitamin A

482 ml DMEM/F12 + glutamax

482 ml Neurobasal

10 ml B27 supplement with vitamin A

5 µg/ml Insulin

5 ml Glutamax

5 ml N2 supplement

5 ml NEAA

10 ml Pen/Strep

10 µM β-mercaptoethanol

3. Neural Maintenance Medium (NMM) without Vitamin A

482 ml DMEM/F12 + glutamax

482 ml Neurobasal

10 ml B27 supplement without vitamin A

5 µg/ml Insulin

5 ml Glutamax

5 ml N2 supplement

5 ml NEAA

10 ml Pen/Strep

10 µM β-mercaptoethanol

4. N2 medium

48 ml DMEM/F12 without L-glutamine

500 µl 100x N2

- 500 µl Non-essential amino acids (10 mM stock)
- 500 µl L-glutamine (200 mM stock)
- 2 µg/ml Heparin
- 500 µl Pen/Strep
5. NB medium
- 475 ml Neurobasal
- 10 ml B27
- 18 mM HEPES
- 1.25 ml Glutamax
- 5 ml Pen/strep
6. Blocking Buffer
- 95 ml PBS
- 5 ml NGS
- 300 µl Triton X-100
- 0.1 g BSA
7. PBS
- 8 g NaCl
- 0.1 g KCl
- 1.44 g Na₂HPO₄
- 0.24 g KH₂PO₄
- 800 ml H₂O
8. PLO/mLaminin coating
- Add to plate 20 µg/ml PLO in PBS (0.5 ml for 12-well plate; 1 ml for 6-well plate)
 - Incubate at 37 °C for 4 h or O/N
 - Remove PLO
 - Wash 3 times with PBS
 - Add to plate 20 µg/ml mLaminin (same amounts as for PLO)
 - Incubate at 37 °C for at least 2 h or O/N
 - Remove mLaminin
 - Add medium and cells
9. Geltrex coating
- Dilute Geltrex 1:1 with cold DMEM/F12
 - Store aliquots at -80 °C
 - Dilute 1:50 with cold DMEM/F12
 - Add to plate appropriate amount (0.5 ml for 12-well plate; 1 ml for 6-well plate)
 - Incubate at 37 °C for at least 1 h
 - After remove Geltrex, immediately add medium with cells

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Competing interests

The authors declare no conflict of interest.

Ethics

All experiments were exempt from approval of Medical Ethical Toetsingscommissie (METC), Institutional Review Board of the VU medical center.

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3D Organoid Formation from the Murine Salivary Gland Cell Line SIMS

Harleen K. Athwal^{1, 2} and Isabelle M. A. Lombaert^{1, 2, *}

¹Biointerfaces Institute, University of Michigan, Ann Arbor, MI 48109, USA; ²School of Dentistry, Department of Biologic and Materials Sciences, University of Michigan, Ann Arbor, MI 48109, USA

*For correspondence: lombaert@umich.edu

[Abstract] Salivary glands consist of multiple phenotypically and functionally unique cell populations, such as the acinar, ductal, and myoepithelial cells that help produce, modify, and secrete saliva (Lombaert *et al.*, 2011). Identification of mechanisms and factors that regulate these populations has been of key interest, as salivary gland-related diseases have detrimental effects on these cell populations. A variety of approaches have been used to understand the roles different signaling mechanisms and transcription factors play in regulating salivary gland development and homeostasis. Differentiation assays have been performed with primary salivary cells in the past (Maimets *et al.*, 2016), however this approach may sometimes be limiting due to tissue availability, labor intensity of processing the tissue samples, and/or inability to long-term passage the cells. Here we describe in detail a 3D differentiation assay to analyze the differentiation potential of a salivary gland cell line, SIMS, which was immortalized from an adult mouse submandibular salivary gland (Laoide *et al.*, 1996). SIMS cells express cytokeratin 7 and 19, which is characteristic for a ductal cell type. Although adult acinar and myoepithelial cells were found *in vivo* to preserve their own cell population through self-duplication (Aure *et al.*, 2015; Song *et al.* 2018), in some cases duct cells can differentiate into acinar cells *in vivo*, such as after radiation injury (Lombaert *et al.*, 2008; Weng *et al.*, 2018). Thus, utilization of SIMS cells allows us to target and analyze the self-renewal and differentiation effects of ductal cells under specific *in vitro* controlled conditions.

Keywords: Salivary gland, Epithelial, SIMS, Differentiation assay, Mouse cell line

[Background] *In vitro* differentiation assays are a remarkable tool that enables us to analyze the potency of cells without relying on *in vivo* models. Cells in these assays can be manipulated for gene expression to, for instance, analyze their differentiation ability. While previous protocols have described the differentiation potential of primary salivary gland cells *in vitro*, our protocol is optimized for the use of the SIMS cell line (Laoide *et al.*, 1996; Maimets *et al.*, 2016). SIMS cells express cytokeratin 7 and 19, which is characteristic for a ductal cell type. Although adult acinar and myoepithelial cells were found *in vivo* to preserve their own cell population through self-duplication (Aure *et al.*, 2015; Song *et al.* 2018), in some cases duct cells can differentiate into acinar cells *in vivo*, such as after radiation injury (Lombaert *et al.*, 2008; Weng *et al.*, 2018). Thus, utilization of SIMS cells allows us to target and analyze the self-renewal and differentiation effects of ductal cells under specific *in vitro* controlled conditions. Our recent work shows that SIMS cells have the ability to differentiate into unique populations of acinar, myoepithelial, and duct cells in 3D differentiation conditions, that normally produce, modify and secrete saliva *in vivo* (Lombaert *et al.*, 2011; Athwal *et al.*, 2019). This protocol describes the workflow necessary

for the generation and analysis of 3D differentiated SIMS cells. SIMS cells are cultured in a 3D matrigel/collagen matrix over a period of 7-9 days. Matrigel is a solubilized basement membrane preparation extracted from the Engelbreth-Holm-Swarm (EHS) mouse sarcoma, which is rich in several growth factors and extracellular matrix proteins, such as laminin-1, collagen IV, and heparin sulfate proteoglycans (Patel *et al.*, 2016). Matrigel has been shown to induce differentiation of stem/progenitor cells and outgrowth of already differentiated cell types by causing polarization of cells embedded in or on top of it. For SIMS grown in this 3D matrix, organoid formation post single cell culture is apparent around day 5 of culture. These organoids are also stained either via cryosectioning or in 3D to analyze for differentiation markers and cell populations. Alterations, such as cell transfections, transductions and/or media changes, to this condition can easily be made to address specific research questions (Athwal *et al.*, 2019). The media components were adjusted using literature from both existing salivary and mammary gland differentiation protocols, which deemed necessary to induce differentiation of the SIMS cells *in vitro*. Here we show the robust use of this cell line to address specific research questions without having to rely on primary salivary gland cells.

Materials and Reagents

1. 500 ml filter units with 0.22 μ m filter (Fisher, catalog number: 974102)
2. 24-well tissue culture plates (Thermo Fisher, catalog number: 142475)
3. T75 tissue culture flasks (Thermo Fisher, catalog number: 156499)
4. 2 ml pipettes (Fisher, catalog number: 13-678-25C)
5. 10 cm dishes (Thermo Fisher, catalog number: 150464)
6. 15 ml Falcon tubes (Corning, catalog number: 35296)
7. 1 ml pipettes (Fisher, catalog number: 13-678-25B)
8. Forceps (Fisher Scientific, catalog number: XX6200006P)
9. Razor blade (Fisher Scientific, catalog number: 12-640)
10. Glass slides (VWR, catalog number: 89500-466)
11. SIMS cells (available per request)
12. Sterile DMEM medium + 4.5g/L high glucose + L-glutamine without sodium pyruvate (Invitrogen, catalog number: 11965-092)
13. Sterile 1x PBS-Ca⁺²-Mg⁺² free (Thermo Fisher, catalog number: 110010031) or 1x HBSS-Ca⁺²-Mg⁺² free (Thermo Fisher, catalog number: 14175079)
14. Penicillin-Streptomycin (Thermo Fisher, catalog number: 15140122)
15. Sterile USDA-approved FBS (Sigma-Aldrich, catalog number: F2442-500ML)
16. 0.25% Trypsin-EDTA (Sigma-Aldrich, catalog number: T4049-100ML)
17. Epidermal growth factor (EGF) (Sigma-Aldrich)
18. Glutamax (Gibco/ThermoFisher, catalog number: 35050061)
19. Fibroblast growth factor-2 (FGF2) (R&D, catalog number: 3139FB025)
20. N2 (Thermo Fisher, catalog number: 17502048)

21. Dexamethasone (VWR, catalog number: 80056-298)
22. Triiodothyronine (Sigma-Aldrich, catalog number: 709719)
23. Retinoic Acid (Thermo Fisher, catalog number: AA4454077)
24. Hydrocortisone (Sigma-Aldrich, catalog number: H0888)
25. Cholera toxin (Sigma-Aldrich, catalog number: C8052)
26. Calcium, 99%, Granular (Fisher, catalog number: AC201180050)
27. Growth factor reduced Matrigel (BD Biosciences, catalog number: 354230)
28. Collagen Type I, Rat Tail (Millipore, catalog number: 2790888)
29. 4% PFA (VWR, catalog number: AAJ61899-AK)
30. Trypan blue 0.4% (Thermo Fisher, catalog number: 15250061)
31. O.C.T. Compound (Fisher scientific, catalog number: 23-730-571)
32. SIMS subculture media (see Recipes)
33. Differentiation media (see Recipes)

Equipment

1. Water bath (Thermo Scientific, catalog number: TSGP02)
2. Vacuum pump aspirator (standard)
3. CO₂ incubator (standard)
4. Cryotome (standard)
5. Brightfield microscope (standard)
6. Confocal laser scanning microscope (standard)

Software

1. ImageJ software (<http://imagej.net>, available online for download)

Procedure

A. SIMS subculture

1. Pre-warm cell medium, 1x PBS-Ca⁺²-Mg⁺² free or 1x HBSS- Ca⁺²-Mg⁺² free and 0.25% trypsin-EDTA in a water bath at 37 °C.
2. Aspirate off old medium with vacuum aspirator or use 10 ml pipettes to manually aspirate from the T75 cell culture flask.
3. Wash twice with 10 ml of sterile 1x PBS/HBSS-Ca⁺²-Mg⁺², aspirate off completely-either manually with a 2 ml pipette or with an aspirator and sterile fresh 2 ml glass pipette.
4. Add 3 ml of pre-warmed 0.25% Trypsin-EDTA per T75 flask.
5. Incubate plate with cells at 37 °C for 5-7 min. Tap and gently shake the plate to dislodge cells.

6. Neutralize trypsin by adding fresh ~3-5 ml culture medium using a 10 ml pipette. Pipette gently up and down several times to re-suspend cells and transfer to a 15 ml Falcon tube.
7. Centrifuge at 100 $\times g$ for 3 min, wash 2 times with 1x PBS-Ca⁺²-Mg⁺² free, and take up the cells in desired amount of culture medium.
8. Plate up to 1 million cells to get 95% confluence in 4-5 days in a T75 tissue culture flask.

Note: Cell viability (~90-95%) is determined by trypan blue cell count.

B. Organoid differentiation culture

1. Resuspend SIMS cells in SIMS medium at 0.4 $\times 10^6$ cells/ml.
2. Thaw out growth factor reduced matrigel matrix on ice for 10 min before culturing cells. Both collagen and matrigel need to remain on ice until they are resuspended with cells for plating.
3. Add 50 μ l of cell solution (10,000 cells) to 100 μ l of 60:40 ratio of Type I rat tail collagen to growth factor reduced Matrigel on ice. Mix gently while slowly pipetting up and down using a 200 μ l pipette.

Notes:

- a. *Aliquot collagen first then Matrigel. Do not mix with pipette until you add cells to avoid bubbles.*
- b. *Avoid fast aspiration or suspension of aliquoted matrix mix to avoid bubble formation.*
4. Deposit cell/collagen/matrigel mix in the center of 24-well tissue culture plate on ice and incubate for 20 min at 37 °C.
5. Add up to 1 ml differentiation medium.
6. Change medium every 2-3 days.
7. Cultures are maintained up to 14 days as the cells reach maximum confluence within the matrix, and the matrix starts breaking down.
8. Organoid formation can be seen starting Day 5 of culture (Figure 1).

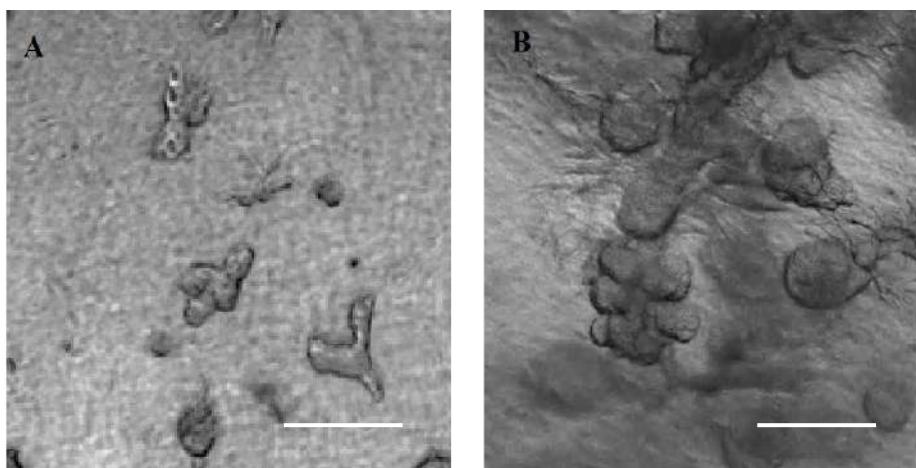


Figure 1. Collagen-matrigel embedded SIMS cells expand and form organoids by Day 5 (A) and 7 (B). Scale bars, 200 μ m (4x magnification).

C. Organoid fixation

1. Wash matrix with ice cold 1x PBS-Ca⁺²-Mg⁺² free gently up to 3 times.
2. Cells can be fixed at any timepoint using 2% PFA in 1x PBS-Ca⁺²-Mg⁺² free for 20 min on ice or at 4 °C.
3. Rinse the matrix with ice cold 1x PBS-Ca⁺²-Mg⁺² free 3 times.
4. Incubate matrix in 1x PBS-Ca⁺²-Mg⁺² free for 10 min. Repeat this 3 times for a total of 30 minutes.
5. Using forceps, gently remove the matrix from the tissue culture plate.
Note: You can cut the matrix into 2-3 pieces using a fine blade if you want multiple cryo blocks.
6. Embed with OCT (a full description of embedding can be found in Campbell *et al.*, 2011).
Note: Matrix will likely curl up in OCT if it is poured too quickly. Gently pour, and fix any curling using your forceps.
7. Optimal cutting thickness is between 12 and 16 µm.
8. Sections can be post fixed with 4% PFA, ice cold acetone, or acetone/methanol for staining optimization.

D. 3D matrix staining

To conserve the 3D morphology, staining is performed on 1 mm³ pieces of the organoids embedded in matrix.

1. Post fixation, small pieces of matrix including organoids are cut using a sharp scalpel blade and pointed tweezers. Treat each chunk separately in a plastic dish or 24-well plate.
2. Matrix can be treated again with 2-4% PFA, acetone/methanol, or methanol treatments if necessary.
3. Matrix is treated like tissue slides for staining. An example is described in Athwal *et al.*, 2019.
4. Mount each sample on glass slides with 1-2 spacers depending on the size of the matrix.
5. Capture images using a confocal microscope (Figure 2).
6. This staining can also be performed on cryosections of OCT embedded organoid matrix (Athwal *et al.*, 2019). A complete description of the primary and secondary antibodies used in Figure 2 can be found in above-mentioned manuscript.

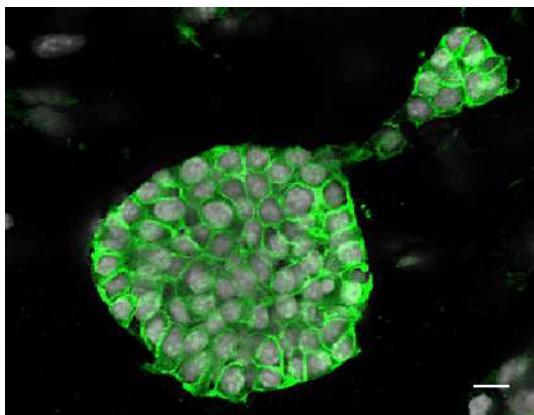


Figure 2. 1 mm³ collagen/matrigel matrix with 3D grown SIMS organoids were stained with E-cadherin post-fixation with 4% PFA. Scale bar, 10 µm.

Data analysis

Brightfield images are taken starting Day 3 until the end of the experiment. Organoid diameter can be quantified using ImageJ. Each experiment is repeated 3 times with n = 5 images per day for quantification. Details on statistical analysis can be found in our previous manuscript (Athwal *et al.*, 2019).

Notes

1. Cell death is noticed by Day 14 as cells become confluent within the matrix.
2. Serial passage of cells dissociated from organoids has not been performed with SIMS cell organoids.
3. Increase the volume of collagen/matrigel if cell number is increased.
4. Transduced or transfected SIMS cells are selected prior to the 3D culture assay.

Recipes

1. SIMS subculture medium (Table 1)

Table 1. SIMS subculture medium

Reagent	Stock	Final	Volume
Sterile DMEM medium + 4.5 g/L high glucose + L-glutamine without sodium pyruvate	100%		455 ml
Pen-Strep	100%	1%	5 ml
FBS	100%	10%	50 ml

2. Differentiation medium

Table 2. SIMS differentiation medium

Reagent	Original Concentration	Final Concentration	Volume Added
SIMS complete medium			50 ml
Glutamax	100x	1x	500 µl
ITS	100x	1x	500 µl
N2 Supplement	100x	1x	500 µl
EGF	200 µg/ml	20 ng/ml	5 µl

FGF2	25 µg/ml	20 ng/ml	40 µl
Dexamethasone	3.85 mM	1 µM	13 µl
Triiodothyronine	29.7 µM	2 nM	3.4 µl
Retinoic Acid	10 mM --> 1 mM	0.1 µM	5 µl
Hydrocortisone	1 mg/ml	0.4 µg/ml	20 µl
Cholera toxin	100 µg/ml	8.4 ng/ml	4.2 µl
Calcium	50 mM	0.8 mM	0.8 µl

Notes:

- a. Make 100 µl aliquots of growth factor reduced matrigel and store at -20 °C.
- b. Make 100 µl aliquots of rat tail collagen and store at 4 °C.

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Competing interests

There are no conflicts of interest.

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Differentiation of Mouse Embryonic Stem Cells to Neuronal Cells Using Hanging Droplets and Retinoic Acid

Jeroen Witteveldt* and Sara Macias

Institute of Immunology and Infection Research, School of Biological Sciences, University of Edinburgh, Edinburgh, EH9 3FL, UK

*For correspondence: Jeroen.witteveldt@ed.ac.uk

[Abstract] Controlled differentiation of embryonic stem cells is an essential tool in stem cell research. In this protocol, we describe a simple differentiation protocol involving the induction of embryoid body formation in mouse embryonic stem cells (mESC) using hanging droplets, followed by differentiation into a neuronal lineage.

Keywords: Embryonic stem cells, Embryoid bodies, Retinoic acid, Hanging droplets, Differentiation, Neuronal

[Background] One of the major achievements in embryonic stem cell research has been the ability to direct the differentiation of stem cells into specific developmental lineages and ultimately terminally differentiated cells. To preserve pluripotency of mESCs in *in vitro* culture, cells are grown in the presence of the Leukemia inhibitory factor (LIF) cytokine, which promotes self-renewal and inhibits differentiation. A common method to direct stem cell differentiation into all three germ layer lineages is to remove LIF from the culture medium and forcing the formation of complex three-dimensional cell aggregates termed embryoid bodies (EBs) (Itskovitz-Eldor *et al.*, 2000). Differentiation in these embryoid bodies is considered to be disorganized, however mESC culture in the absence of LIF and in the presence of certain stimuli can result in more specific cell types, including insulin-producing cells, hematopoietic cells and neuronal cells. Differentiation of ESCs into neural lineage involves the manipulation of Fibroblast Growth Factor and Wnt signaling in the absence of Bone morphogenetic protein signaling activity (Abranches *et al.*, 2009). Common methods involve co-culture of ESCs on stromal feeder cells or conditioned medium or direct manipulation of the essential signaling pathways which can be quite involved and time-consuming. A more straightforward method is by incubating ESCs with the natural metabolite of vitamin A, retinoic acid which acts on nuclear receptors to induce transcription of specific target genes. Retinoic acid has been shown to play a directing role in neural development in early stages of central nervous system development and is considered the most important extrinsic inductive signals for neural differentiation. In this protocol, we describe an efficient method to produce mouse-derived EBs and consequent neuronal differentiation using hanging droplets and retinoic acid (Jones-Villeneuve *et al.*, 1983; Wobus *et al.*, 1991).

Materials and Reagents

1. Pipette tips
2. Pipettes
3. Petri dish (145 mm, Greiner Bio-One, catalog number: 639161; 90 mm, SLS select, catalog number: SLS2002)
4. Cell culture dish (100 mm, Corning, catalog number: 430167)
5. Flask (25 cm², Corning, catalog number: 430639; 75 cm², Corning, catalog number: 430641U)
6. 15 ml tube (Sarstedt, catalog number: 62547004)
7. 50 ml tube (Sarstedt, catalog number: 62554002)
8. Cryo tube
9. Mouse Embryonic Stem cells (mESC) (v6.5, Novus Biologicals, catalog number: NBP1-41162)
Note: Be sure to check local guidelines regarding the use of embryonic stem cells.
10. DMEM (High glucose, pyruvate, Gibco, catalog number: 41966)
11. Dulbecco's phosphate buffered saline (Sigma-Aldrich, catalog number: D8537)
12. MEM Non-Essential amino acids (Gibco, catalog number: 11140035)
13. Trypsin-EDTA (0.25%, Gibco, catalog number: 25200056)
14. 2-Mercaptoethanol (50 mM, Gibco, catalog number: 31350010)
15. Gelatin (0.1% in Ultrapure water, Millipore, catalog number: ES-006-B)
16. Fetal bovine serum (Gibco, catalog number: 10500064)
17. L-Glutamine (200 mM, Gibco, catalog number: 25030024)
18. Penicillin/Streptomycin (Gibco, catalog number: 15140122)
19. Retinoic acid (Sigma-Aldrich, catalog number: 2625-50mg)
20. Leukemia inhibitory factor (LIF, Stemcell Technologies, catalog number: 78056)
21. Liquid nitrogen
22. mESC medium with LIF (see Recipes)
23. mESC medium without LIF (see Recipes)
24. 0.05% Trypsin (see Recipes)

Equipment

1. Incubator (37 °C, 5% CO₂, Thermo Scientific, model: BB15)
2. Centrifuge (Eppendorf, model: 5810R)
3. Tissue-culture hood (Nuaire Labgard, Class II)
4. Hemocytometer (Neubauer chamber or equivalent method)
5. Inverted microscope (Olympus, model: CKX41)
6. Non-inverted microscope (Olympus, model: BX43)
7. Water bath

Procedure

A. Coating of plates

1. Add enough 0.1% Gelatin to cover the bottom of a tissue-culture treated flask or plate (for example, 1.5 ml of 0.1% Gelatin for a T25 flask).
2. Incubate for at least 10 min at room temperature or 37 °C.
3. Carefully remove the 0.1% Gelatin and add mouse embryonic stem cell (mESC) medium (see Recipes).

Note: Washing plates is not necessary when carefully removing gelatin.

B. Recovery of cells from liquid nitrogen

1. Prepare suitable plate as described above.
 2. Collect cryo tube with mESCs from liquid nitrogen and quickly thaw in a water bath at 37 °C.
 3. Slowly add culture medium with LIF to cells in cryo tube and transfer to a 15 ml tube with approximately 10 ml of medium with LIF.
- Note: Slowly adding medium to ESCs prevents damage due to sudden changes in osmotic pressure.*
4. Pellet cells for 5 min at 200 x g and remove supernatant
 5. Resuspend cells in appropriate amount of medium with LIF and add to gelatin-coated plate.

C. Maintaining and splitting mESCs

1. Remove mESC medium from flask or plate and wash cells with PBS.

Note: See Figure 1 for example of v6.5 cells ready for splitting.

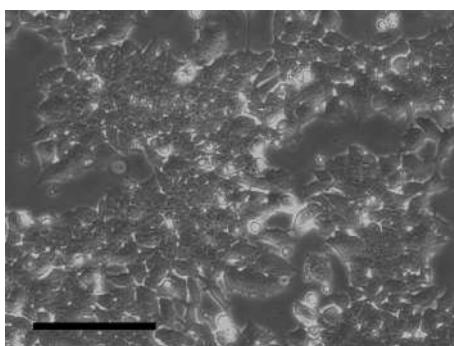


Figure 1. Confluent v6.5 mESCs. Scale bar = 50 µm.

2. Remove PBS carefully and add enough 0.05% Trypsin (see Recipes) to cover cells (for example, 1 ml of 0.05% trypsin in a T25 flask).
3. Incubate at 37 °C. Usually 3-5 min is enough for all cells to dissociate.

Note: Do not leave mESCs in trypsin for too long as it will harm the cells.

4. Transfer the required number of cells to a new flask with the appropriate amount of mESC medium containing LIF. Trypsin can be removed by centrifugation, but this is not necessary for v6.5 cells.

Note: Take care when pipetting mESCs with small (non-serological) pipette tips, they can be easily damaged.

5. Split mESCs every 2-3 days, avoid letting mESCs getting over-confluent.

Note: Avoid culturing mESCs for more than 10 passages.

D. Preparing embryoid bodies (EBs) using hanging droplets (Day 1, see also Video 1 for Steps B5-B7)

1. Trypsinize mESCs as described above, be sure to have single cells.

2. Resuspend in mESC medium without LIF.

3. Carefully count the cells using a hemocytometer or any other preferred method.

4. Adjust concentration to approximately 2×10^4 cells/ml using mESC medium without LIF.

Note: Slight deviations from this concentration is not a problem.

5. Using a multichannel pipette, place 20 μ l droplets of the cell suspension on the inside of a plastic Petri dish lid (not tissue-culture treated and not gelatin-coated). We aim for approximately 100 droplets on a 90 mm plate but depending on how many EBs are required, the size of the dish can be easily scaled up or down.

Note: Avoid touching the surface of the Petri dish with the pipette tip as this tends to make the droplets flatter and more spread out.



Video 1. Preparation of hanging droplets on Petri dish

6. Add 10 ml of water or PBS to the bottom half of the Petri dish to prevent desiccation.
7. In one smooth movement, turn the lid with the droplets upside down and place on top of the bottom part of the Petri dish containing water or PBS.
8. Place in 37 °C, 5% CO₂ incubator for 48 h (Day 3).
9. EB formation can be observed directly using a non-inverted microscope or using an inverted microscope by placing the lid with hanging droplets onto the empty bottom part of a Petri dish

followed by carefully inverting the Petri dish. EBs should form within 24 h. See Figure 2 for example of EBs of v6.5 cells 24 h after preparation of hanging droplets.

Note: Differentiation can be confirmed by measuring the downregulation of pluripotency markers such as Nanog, Pou5f1 and Sox2 by quantitative real time PCR or Western blot.

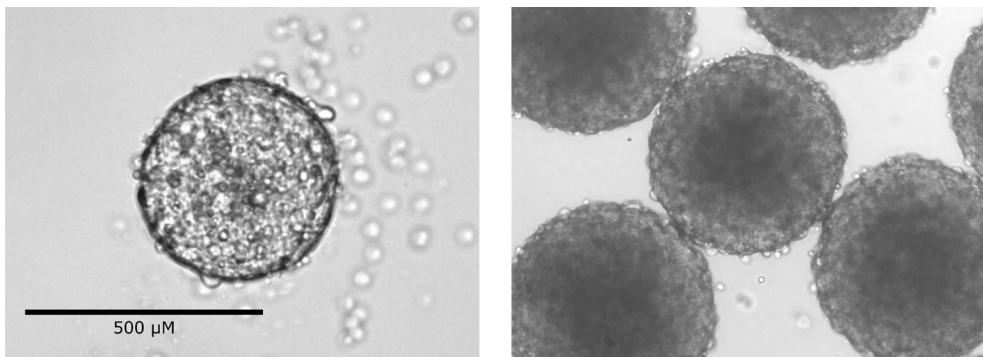


Figure 2. Typical morphology of embryoid body. v6.5 cells were cultured in hanging droplets in mESC medium without LIF for 24 h (left) and 48 h (right).

E. Differentiation of EBs using retinoic acid

1. After 48 h in droplets, the EBs are collected by adding PBS to the Petri dish lids and collecting the supernatant containing the EBs.

Note: It might require some pipetting up and down to dislodge all the EBs as they tend to stick to the plastic surface. Use serological pipettes as smaller pipettes are likely to damage the EBs.

2. The supernatant containing EBs can be collected in either 15 or 50 ml tubes depending on the volume used.

3. Leave the tubes upright for 5 min or more to allow the EBs to settle on the bottom of the tubes. Remove the supernatant and resuspend the EBs in 10 ml mESC medium without LIF.

4. Add the 10 ml EB suspension to a 90 mm Petri dish (not culture treated and not gelatin-coated) and grow for another 48 h at 37 °C and 5% CO₂ (day 5).

Note: The resuspension volume and size of the plate can be adjusted to the number of EBs required, we aim for approximately 100 EBs/90 mm dish.

5. After 48 h of incubation, replace the medium with 10 ml of fresh mESC medium without LIF using the method described in Steps E2 and E3 and add Retinoic acid to a final concentration of 250 nM.

Note: The concentration of Retinoic acid is likely to be cell line dependent.

6. Continue to culture for another 7 days (Day 12) whilst replacing the medium with fresh mESC medium without LIF supplemented with 250 nM retinoic acid every 2 days. See Figure 3 for example of EB 7 days after RA treatment.

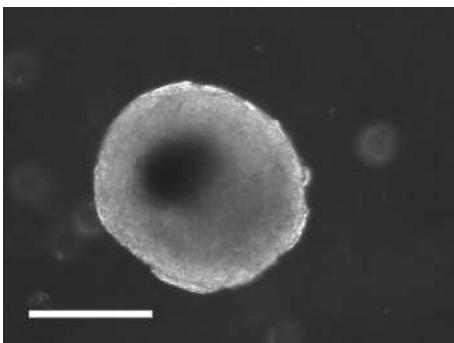


Figure 3. Embryoid bodies after 7 days of retinoic acid treatment. Scale bar = 500 µm.

7. After these 7 days (on Day 12), the EBs can be collected as described in Steps D2 and D3 and seeded on tissue-cultured treated, gelatin-coated plates or flasks. At this point, EBs will attach and cells will start to migrate from the EBs within 24 h. See Figure 4 for example of EB 24 h after attachment to gelatin-coated plate and Figure 5 for EBs 7 days after attachment, showing considerable migration and growth of cells of neuronal lineage.

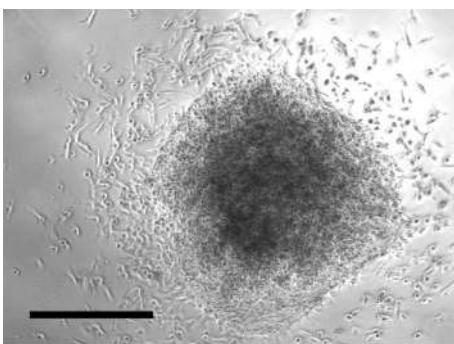


Figure 4. Embryoid bodies 24 h after plating on gelatin-coated plates showing migration of differentiated cells from embryoid body. Scale bar = 500 µm.

8. At this point, the cells are differentiated into cells of neuronal lineage and can be directly used for further experiments or frozen down.

Note: Differentiation into cell of neuronal lineage can be verified through measuring gene expression of genes such as Neurog2, Gata4, Gata6, Nestin, Sox1 and TuJ1 by quantitative real time PCR or Western blot (Ying et al., 2003; Witteveldt et al., 2019). To ensure a homogeneously differentiated population, lineage selection by cell-sorting based on fluorophore-expression driven by lineage-specific promoters or immune-isolation by lineage-specific extracellular markers (Jüngling et al., 2003; Ladewig et al., 2008) might be necessary. When multiple replicates are required we suggest to differentiate individual replicates.

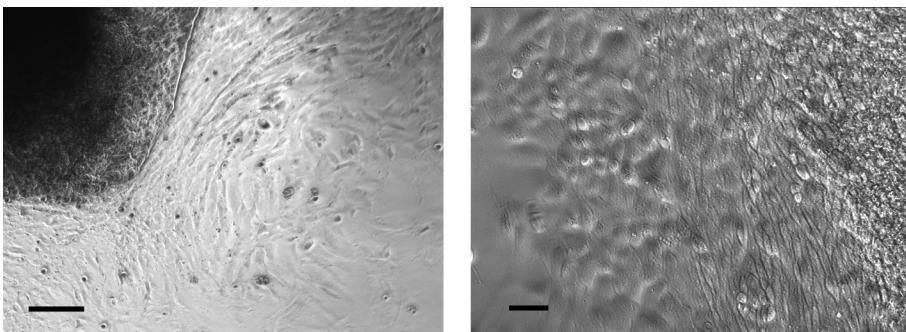


Figure 5. Embryoid bodies 7 days after plating on gelatin coated plates showing migration and growth of differentiated cells of neuronal lineage with clearly changed morphology. Scale bar = 20 μm .

Recipes

1. mESC medium with LIF
415 ml DMEM
5 ml Penicillin/Streptomycin
5 ml MEM Non-Essential amino acids
400 μl 2-Mercaptoethanol (40 μM final concentration)
75 ml Fetal bovine serum (15% final concentration)
50 μl LIF (final concentration of 1000 U/ml)
2. mESC medium without LIF
415 ml DMEM
5 ml Penicillin/Streptomycin
5 ml MEM Non-Essential amino acids
400 μl 2-Mercaptoethanol (40 μM final concentration)
75 ml Fetal bovine serum (15% final concentration)
3. 0.05% Trypsin
10 ml of 0.25% Trypsin (stock)
40 ml of PBS

Acknowledgments

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Competing interests

The authors declare no conflict of interests.

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***In vitro* Differentiation of Human iPSC-derived Retinal Pigment Epithelium Cells (iPSC-RPE)**

Agnieszka D'Antonio-Chronowska^{1,*}, Matteo D'Antonio¹ and Kelly A. Frazer^{1,2}

¹Department of Pediatrics, University of California, San Diego, La Jolla, USA; ²Institute for Genomic Medicine, University of California, San Diego, La Jolla, USA

*For correspondence: adantoniochronowska@health.ucsd.edu

[Abstract] Induced Pluripotent Stem Cells (iPSCs) serve as an excellent model system for studying the molecular underpinnings of tissue development. Human iPSC-derived retinal pigment epithelium (iPSC-RPE) cells have fetal-like molecular profiles. Hence, biobanks like iPSCORE, which contain iPSCs generated from hundreds of individuals, are an invaluable resource for examining how common genetic variants exert their effects during RPE development resulting in individuals having different propensities to develop Age-related Macular Degeneration (AMD) as adults. Here, we present an optimized, cost-effective and highly reproducible protocol for derivation of human iPSC-RPE cells using small molecules under serum-free condition and for their quality control using flow cytometry and immunofluorescence. While most previous protocols have required laborious manual selection to enrich for iPSC-RPE cells, our protocol uses whole culture passaging and yields a large number of iPSC-RPE cells with high purity (88-98.1% ZO-1 and MiTF double positive cells). The simplicity and robustness of this protocol would enable its adaption for high-throughput applications involving the generation of iPSC-RPE samples from hundreds of individuals.

Keywords: Human induced pluripotent stem (hiPSC), Retinal pigment epithelium (RPE), Human induced pluripotent stem cell-derived retinal pigment epithelium (hiPSC-RPE), Age-related macular degeneration (AMD), Differentiation, Genetic studies, Small molecules, Genetic variant

[Background] Age-related macular degeneration (AMD) is a leading cause of vision loss in developed countries affecting 11 million individuals in the United States and about 170 million worldwide (Pennington and DeAngelis, 2016). Moreover, considering age as a main factor, in our current aging society, the incidence of AMD is estimated to increase to 198 million in 2020 and 288 million by 2040 and to 22 million in the United States alone by 2050 (Wong *et al.*, 2014, Pennington and DeAngelis, 2016). Current therapeutic strategies, although effective, are expensive and limited to delaying the speed of disease progression, and AMD still eventually leads to a complete loss of vision (Al-Zamil and Yassin, 2017; Mitchell *et al.*, 2018). At the time of preparation of this article, there are seven active clinical trials aimed to evaluate the effectiveness and optimize the conditions of the transplantation of the human iPSC-RPEs or human embryonic stem cell-derived retinal pigment epithelium (ESC-RPE) cells (NIH-ClinicalTrials.gov). Thus, the development of a robust and cost-effective method for generating large amounts of high quality iPSC-RPEs is imperative for the advancement of future therapeutic treatments of AMD and potentially other eye diseases in humans as well as domestic animals (Sparrow *et al.*, 2010).

We have previously demonstrated the utility of employing iPSC-RPE cells to identify and study genetic variants playing a role in the development of AMD (Smith *et al.*, 2019). In this study, we derived iPSC-RPE cells from six individuals (3 European Americans, 2 East Asian Americans, and 1 African American) and showed that they have morphological and molecular characteristics similar to those of naïve RPE cells. We showed that iPSC-RPE gene expression profiles are highly similar to that of human fetal RPE, and that their ATAC-seq (Assay for Transposase-Accessible Chromatin using sequencing) peaks are enriched for relevant transcription factor motifs. We performed fine mapping of AMD risk loci integrating the molecular data from iPSC-RPE cells that resulted in the prioritization of four variants, including a potential regulatory SNP (rs943080) near VEGFA and a coding SNP (rs34882957, pP167S) in the C9 gene (Smith *et al.*, 2019). Our findings illustrate that iPSC-RPE cells are an excellent model system to study the molecular functions of genetic variation associated with AMD.

The initial protocols for deriving iPSC-RPE cells developed over a decade ago involved spontaneous differentiation and were highly inefficient requiring the manual separation of pigmented iPSC-RPE patches from non-differentiated cells (Klimanskaya *et al.*, 2004; Vugle *et al.*, 2008). Subsequently, induced directed differentiation protocols resulted in higher yield and quality of iPSC-RPE cells but required numerous animal-derived components and thus had relatively low reproducibility or were very time consuming (Aoki *et al.*, 2006; Osakada *et al.*, 2008; Buchholz *et al.*, 2009; Idelson *et al.*, 2009; Reichman *et al.*, 2014; Hazim *et al.*, 2017). The introduction of small molecules into iPSC-RPE differentiation protocols greatly simplified the procedure and resulted in higher reproducibility (Osakada *et al.*, 2009; Maruotti *et al.*, 2013; Maruotti *et al.*, 2015). Previous studies have optimized differentiation protocols to derive iPSC-RPEs from a limited number of iPSC or ESC lines and in most cases utilized small format culture vessels. Here, we present an optimized protocol for deriving iPSC-RPEs from multiple human iPSC lines in large sized culture flasks. It is similar to the protocol of Maruotti *et al.* (2015), but initiates differentiation when iPSCs reach 80% confluence, which we found to be optimal for all tested iPSCORE lines (Panopoulos *et al.*, 2017). Additionally, we modified the length of time of exposure to small molecules at two steps, which resulted in further increase of yield and purity of iPSC-RPEs (88-98.1% ZO-1 and MiTF double positive cells). Our optimized protocol allowed us to derive iPSC-RPEs from six iPSC lines generated from ethnically diverse individuals under identical culturing conditions without the requirement of any individualized optimization steps.

Materials and Reagents

A. iPSC Cell Culture

1. 6-well plates (Corning, catalog number: 3506)
2. Syringe filter 0.2 µm (VWR, catalog number: 28145-501)
3. Soft-Ject® 3-Part Disposable Syringe, Air-Tite-3 ml (VWR, catalog number: 89215-234)
4. 5 ml Borosilicate serological pipettes (Fisher Scientific, catalog number: 1367827E)
5. 5 ml Serological pipettes (Bio Pioneer, catalog number: GEX0050-S01)
6. 10 ml Serological pipettes (Bio Pioneer, catalog number: GEX0100-S01)

7. P20 pipette tips sterile with filter
8. P1000 pipette tips sterile with filter
9. 15 ml conical tubes (Bio Pioneer, catalog number: CNT-15R)
10. iPSC cells
11. 70% ethanol
12. UltraPure™ DNase/RNase-Free Distilled Water (Thermo Fisher Scientific, catalog number: 10977023)
13. Corning® MatriGel® Growth Factor Reduced (GFR) Basement Membrane Matrix (Corning, catalog number: 354230)
14. mTeSR™1 (Stem Cell Technologies, catalog number: 85850)
15. DMEM/F-12 medium (Thermo Fisher Scientific, catalog number: 11330057)
16. Dispase II (Thermo Fisher Scientific, catalog number: 17105041)
17. Matrigel solution (Matrigel) (see Recipes: Table 1)
18. 10 mM ROCK inhibitor, Y-27632 dihydrochloride solution (ROCK Inhibitor) (see Recipes: Table 2)
19. 10x Dispase (see Recipes: Table 3)
20. mTeSR™1 complete medium (mTeSR) (see Recipes: Table 4)

B. Monolayer plating

1. 100 mm tissue culture dishes (Corning, catalog number: 430167)
2. Automated cell counter slides (Bio-Rad Laboratories, catalog number: 1450019) or a hemocytometer (Hausser Scientific, catalog number: 1483) or equivalent.
3. 5 ml Serological pipettes (Bio Pioneer, catalog number: GEX0050-S01)
4. 10 ml Serological pipettes (Bio Pioneer, catalog number: GEX0100-S01)
5. P20 pipette tips sterile with filter
6. P200 pipette tips sterile with filter
7. P1000 pipette tips sterile with filter
8. 15 ml conical tubes (Bio Pioneer, catalog number: CNT-15R)
9. 50 ml conical tubes (Bio Pioneer catalog number: CNT-50R)
10. 70% ethanol
11. Corning® MatriGel® Growth Factor Reduced (GFR) Basement Membrane Matrix (Matrigel) (Corning, catalog number: 354230)
12. mTeSR™1 (Stem Cell Technologies, catalog number: 85850)
13. DMEM/F-12 medium (Thermo Fisher Scientific, catalog number: 11330-057)
14. Accutase (Innovative Cell Technologies, Inc., catalog number: AT 104)
15. Trypan Blue Solution, 0.4% (Thermo Fisher Scientific, catalog number: 15250061)
16. ROCK inhibitor, Y-27632 dihydrochloride (Selleck hem, catalog number: S1049)
17. iPSC cell culture
18. Matrigel solution (see Recipes: Table 1)

19. 10 mM ROCK inhibitor, Y-27632 dihydrochloride solution (see Recipes: Table 2)
20. mTeSR™1 complete medium (see Recipes: Table 4)

C. iPSC-RPE differentiation

1. 100 mm tissue culture dishes (Corning, catalog number: 430167)
2. (Optional) T150 tissue culture flasks, vented (Sigma, catalog number: Z707929)
Note: At the time of preparation of this manuscript Z707929 was no longer available. The same flasks are available under the catalog number Z707511-36EA (Sigma, catalog number Z707511-36EA).
3. 70 µm strainers (Fisher Scientific, catalog number: 431751)
4. Automated cell counter slides (Bio-Rad Laboratories, catalog number: 1450019) or a hemocytometer (Hausser Scientific, catalog number: 1483) or equivalent
5. 10 ml Serological pipettes (Bio Pioneer, catalog number: GEX0100-S01)
6. 25 ml Serological pipettes (Bio Pioneer catalog number: GEX250-S01)
7. 50 ml Serological pipettes (Bio Pioneer, catalog number: GEX500-S01)
8. P20 pipette tips sterile with filter
9. P200 pipette tips sterile with filter
10. P1000 pipette tips sterile with filter
11. Cell scraper (VWR International, catalog number: 179707)
12. 15 ml conical tubes (Bio Pioneer, catalog number: CNT-15R)
13. 50 ml conical tubes (Bio Pioneer, catalog number: CNT-50R)
14. Nalgene Cryogenic vials (Thermo Fisher Scientific, catalog number: 5000-1020)
15. iPSCs monolayer
16. 70% ethanol
17. Corning® Matrigel® Growth Factor Reduced (GFR) Basement Membrane Matrix (Matrigel) (Corning, catalog number: 354230)
18. DMEM/F-12 medium (Thermo Fisher Scientific, catalog number: 11330057)
19. DMEM medium (Thermo Fisher Scientific, catalog number: 11965092)
20. Ham's F12 Nutrient Mix (Thermo Fisher Scientific, catalog number: 11765054)
21. 1x Dulbecco's phosphate buffered saline (DPBS) without calcium and magnesium (Thermo Fisher Scientific, catalog number: 14190250)
22. B27 Supplement (50x), serum free (Thermo Fisher Scientific, catalog number: 17504044)
23. KnockOut™ Serum Replacement (KOSR) (Thermo Fisher Scientific, catalog number: 10828028)
24. L-Glutamine 200 mM (Thermo Fisher Scientific, catalog number: 25030081)
25. MEM Non-Essential Amino Acids Solution 100x (Thermo Fisher Scientific, catalog number: 11140050)
26. Penicillin-Streptomycin (10,000 U/ml) (Thermo Fisher Scientific, catalog number: 15140122)
27. β-Mercaptoethanol (Thermo Fisher Scientific, catalog number: 21985023)
28. Accutase (Innovative Cell Technologies, Inc., catalog number: AT 104)

29. Nicotinamide (Sigma, catalog number: N3376)
30. Chetomin (Sigma, catalog number: C9623)
31. Trypan Blue Solution, 0.4% (Thermo Fisher Scientific, catalog number: 15250061)
32. Dimethyl Sulfoxide (DMSO) (Sigma, catalog number: D2650-100ML)
33. Liquid nitrogen
34. RPE DM medium (see Recipes: Table 5)
35. RPE medium (see Recipes: Table 6)
36. 2x iPSC-RPE freezing medium (see Recipes: Table 7)
37. 1 mM Chetomin solution (see Recipes: Table 8)
38. 1 M Nicotinamide (100x) solution (see Recipes: Table 9)

D. Flow cytometry

1. 96-well round bottom assay plates (Genesee Scientific, catalog number: 25-224)
2. Corning™ Falcon™ Test Tube with Cell Strainer Snap Cap (Fisher Scientific, catalog number: 352235)
3. Corning™ Costar™ Sterile Disposable Reagent Reservoirs (Fisher Scientific, catalog number: 4870)
4. 5 ml Serological pipettes (Bio Pioneer, catalog number: GEX0050-S01)
5. 10 ml Serological pipettes (Bio Pioneer, catalog number: GEX0100-S01)
6. P20 pipette tips sterile with filter
7. P200 pipette tips sterile with filter
8. P1000 pipette tips sterile with filter
9. P20 pipette tips without filter
10. (Optional) P200 pipette tips without filter
11. Fixation/Permeabilization Solution Kit with BD GolgiStop™ (BD Biosciences, catalog number: 554715)
12. 1x Dulbecco's phosphate buffered saline (DPBS) without calcium and magnesium (Thermo Fisher Scientific, catalog number: 14190250)
13. Bovine Serum Albumin (BSA) (Sigma, catalog number: A2153-100G)
14. (Optional) NaN₃ (Sigma, catalog number: S2002-5G)
15. 37% Formaldehyde (Sigma, catalog number: F-1635-500ML)
16. Rabbit polyclonal anti-ZO-1 antibody (abcam, catalog number: ab59720)
Note: During preparation of this manuscript the antibody ab59720 was no longer available. A potential replacement: abcam, catalog number: ab221547.
17. Mouse monoclonal anti-MiTf antibody (abcam, catalog number: ab12039)
18. Recombinant Rabbit IgG, monoclonal [EPR25A]–Isotype Control (abcam, catalog number: ab172730)
19. Mouse IgG1, kappa monoclonal [15-6E10A7]–Isotype Control (abcam, catalog number: ab170190)

20. Donkey-anti-Rabbit Alexa Fluor™ 647 conjugated antibody (abcam, catalog number: ab150075)
21. Goat-anti-Mouse Alexa Fluor™ 488 conjugated antibody (Thermo Scientific, catalog number: A-11001)
22. FACS Buffer (see Recipes: Table 10)
23. FACS-FIX Buffer (see Recipes: Table 11)

Note: For antibody working concentration, see Recipes: Table 15.

E. Immunofluorescence

1. Millicell EZ SLIDE 8-well glass slides (Millipore, catalog number: PEZGS0816)
2. Cover glass slides (Fisherbrand, catalog number: 12-545-F)
3. 5 ml Serological pipettes (Bio Pioneer, catalog number: GEX0050-S01)
4. 10 ml Serological pipettes (Bio Pioneer, catalog number: GEX0100-S01)
5. P20 pipette tips sterile with filter
6. P200 pipette tips sterile with filter
7. 1x Dulbecco's phosphate buffered saline (DPBS) without calcium and magnesium (Thermo Fisher Scientific, catalog number: 14190250)
8. Bovine Serum Albumin (BSA) (Sigma, catalog number: A2153-100G)
9. Paraformaldehyde (PFA)
10. Tween® 20 (Sigma, catalog number: P9416-100ML)
11. Triton X-100 (Manufacturer, catalog number: X-100-500ML)
12. Corning® Matrigel® Growth Factor Reduced (GFR) Basement Membrane Matrix (Matrigel) (Corning, catalog number: 354230)
13. Rabbit polyclonal anti-ZO-1 antibody (abcam, catalog number: ab59720)
14. Mouse monoclonal anti-MiTf antibody (abcam, catalog number: ab12039)
15. Mouse monoclonal anti-Bestrophin 1 antibody (Novus Biologicals, catalog number: NB300-164SS)
16. Recombinant Rabbit IgG, monoclonal [EPR25A]-Isotype Control (abcam, catalog number: ab172730)
17. Mouse IgG1, kappa monoclonal [15-6E10A7]-Isotype Control (abcam, catalog number: ab170190)
18. Donkey-anti-Rabbit Alexa Fluor™ 647 conjugated antibody (abcam, catalog number: ab150075)
19. Goat-anti-Mouse Alexa Fluor™ 488 conjugated antibody (Thermo Scientific, catalog number: A-11001)
20. ProLong Gold Antifade Reagent with DAPI (Cell Signaling Technologies, catalog number: 8961)
21. IF Wash Buffer (see Recipes: Table 12)
22. IF Perm Buffer (see Recipes: Table 13)
23. IF Staining Buffer (see Recipes: Table 14)

Note: For antibody working concentration, see Recipes: Table 15.

Equipment

A. iPSC Cell Culture

1. Biosafety cabinet (Labconco, model: Logic+)
2. Incubator with humidity and gas control set to maintain 37 °C and 95% humidity in an atmosphere of 5% CO₂ in air (Panasonic, model: MCO-170AICUVH-PA)
3. Water bath (Thermo Scientific, model: Precision)
4. Tissue culture centrifuge with rotors for 15 ml conical tubes and 50 ml conical tubes (Thermo Scientific, model: Legend RT+)
5. Phase contrast inverted microscope (objectives: x4, x10, x20) (Olympus, model: CKX41SF)
6. (Optional) Phase contrast inverted microscope with camera (objectives: x4, x10, x20) (Thermo Scientific, model: EVOS XL Core)
7. Microscope Object marker (Nikon, model: MBW10020)
8. Pipette aid
9. P20 Micropipette
10. Freezer -20 °C
11. Refrigerator 2-8 °C

B. Monolayer plating

1. Biosafety cabinet (Labconco, model: Logic+)
2. Incubator with humidity and gas control set to maintain 37 °C and 95% humidity in an atmosphere of 5% CO₂ in air (Panasonic, model: MCO-170AICUVH-PA)
3. Tissue culture centrifuge with rotors for 15 ml conical tubes and 50 ml conical tubes (Thermo Scientific, model: Legend RT+)
4. Phase contrast inverted microscope (objectives: x4, x10, x20) (Olympus, model: CKX41SF)
5. Phase contrast inverted microscope with camera (objectives: x4, x10, x20) (Thermo Scientific, model: EVOS XL Core)—Optional
6. Pipette aid
7. P20 Micropipette
8. P200 Micropipette
9. P1000 Micropipette
10. Automated cell counter (Bio-Rad, model: TC20) or a hemocytometer (Hausser Scientific, catalog number: 1483) or equivalent
11. Freezer -20 °C
12. Refrigerator 2-8 °C

C. iPSC-RPE differentiation and cryopreservation

1. Biosafety cabinet (Labconco, model: Logic+)
2. Incubator with humidity and gas control set to maintain 37 °C and 95% humidity in an atmosphere

of 5% CO₂ in air (Panasonic, model: MCO-170AICUVH-PA)

3. Tissue culture centrifuge with rotors for 15 ml conical tubes and 50 ml conical tubes (Thermo Scientific, model: Legend RT+)
4. Phase contrast inverted microscope (objectives: x4, x10, x20) (Olympus, model: CKX41SF)
5. (Optional) Phase contrast inverted microscope with camera (objectives: x4, x10, x20) (Thermo Scientific, model: EVOS XL Core)
6. Pipette aid
7. P20 Micropipette
8. P200 Micropipette
9. P1000 Micropipette
10. Automated cell counter (Bio-Rad, model: TC20) or a hemocytometer (Hausser Scientific, catalog number: 1483) or equivalent.
11. Mr. Frosty freezing container (Corning, model: CoolCell® FTS30)
12. Refrigerator 2-8 °C
13. Freezer -20 °C
14. Freezer -80 °C
15. Liquid nitrogen vapor tank

D. Flow cytometry

1. Pipette aid
2. P20 Micropipette
3. P200 Micropipette
4. P1000 Micropipette
5. P200 Multichannel micropipette
6. Refrigerator 2-8 °C
7. Freezer -20 °C
8. Flow cytometer (BD Biosciences, model: FACSCanto II) or equivalent

E. Immunofluorescence

1. Pipette aid
2. P20 Micropipette
3. P200 Micropipette
4. P1000 Micropipette
5. Refrigerator 2-8 °C
6. Freezer -20 °C
7. Confocal laser scanning fluorescence microscope (Olympus, Fluoview1000)

Software

1. FlowJo (Version 10) (FlowJo, LLC, <https://www.flowjo.com/>)
2. FlowView ASW V03.01.03.03 or V4.2a (Olympus Life Science, <https://www.olympus-lifescience.com/en/support/downloads/>)

Procedure

A. iPSC cell culture

1. Thaw iPSC cells
 - a. Prepare 12 ml of mTeSR containing 10 µM ROCK Inhibitor.
 - b. Transfer 9 ml of mTeSR containing 10 µM ROCK Inhibitor into a sterile conical tube labeled with the name of the line.
 - c. Remove vial of cryopreserved cells from liquid nitrogen tank. Keep vial on dry ice.
 - d. Place and shake gently in a 37 °C water bath until a pea-sized ice crystal remains (around 2 min).
 - e. Wipe off excess water from the vial, spray with 70% ethanol before placing in the hood.
 - f. Remove thawed cells from the vial and add gently into 9 ml mTeSR containing 10 µM ROCK Inhibitor in a conical tube. Wash the vial with 1-2 ml of mTeSR containing 10 µM ROCK Inhibitor. Collect all cells in the same conical tube.
 - g. Centrifuge cells for 5 min at 53 x g (500 RPM in a Sorvall 75006445 rotor with 75006441 K buckets) at room temperature.
 - h. Aspirate supernatant, and gently resuspend cell pellet in 2 ml of mTeSR containing 10 µM ROCK Inhibitor (1 cryovial is thawed into 1 well of 6-well plate).
 - i. Label a Matrigel plate with name of line, clone and passage number. Aspirate DMEM/F-12 from Matrigel-coated plate. Add +1 to the passage number after thawing.
Note: Do not add +1 to the passage number if the passage number was increased during cryopreservation of iPSCs.
 - j. Plate cells resuspended in 2 ml into one well of a Matrigel-coated 6-well plate (final volume 2 ml/well).
 - k. 24 h after plating, observe cells. Wash cells gently with DMEM/F-12 (2 ml/well) to remove cell debris and feed using fresh mTeSR medium without ROCK Inhibitor (2 ml/well).
 - l. Daily, observe the iPSCs, remove the differentiated cells, and change the medium with fresh mTeSR (2 ml/well).
Note: It is critical to maintain iPSC culture differentiation free.
 - m. Cells should reach 80-90% of confluence and be ready for passage in about 5 days.
2. iPSC passaging using Dispase
 - a. Prepare 1x (2 mg/ml) Dispase solution by adding 9 ml DMEM/F-12 to 1 ml of 10x Dispase (20 mg/ml).

- b. Allow 1x Dispase solution to come to room temperature.
Note: 1x Dispase solution can be stored at 4 °C for maximum 2 weeks.
- c. Mark any areas of differentiation on the well to be split using the Microscope Object marker.
- d. Aspirate spent media. Aspirate marked areas of differentiation, if any, by gently tapping a Pasteur pipette within the marked circle. Wash with 2 ml of DMEM/F-12 per well.
- e. Add 1 ml of 1x Dispase in each well to be split. Incubate at 37 °C for 5 min.
- f. Check morphology of colonies after 5 min.
When edges of the colonies are slightly curled up, cells are ready to be passaged. If edges of colonies are not curled up, incubate cells at 37 °C for another 1-2 min. Do not incubate with Dispase for longer than 8 min.
- g. Aspirate Dispase from all wells.
- h. Rinse the wells gently 3 times with DMEM/F12 (2 ml/well).
- i. Add 1 ml of mTeSR media to each well to be passaged.
- j. Use a glass serological pipette to detach colonies. Hold the pipette at a 90° angle to the surface of the plate. Scrape across the surface of the 6-well plate in the motion outlined in Figure 1 (start from top left side of the well and zig-zag tightly down to bottom-right side, then turn plate clockwise or counterclockwise and scrape again). Scrape until at least 90% of the colonies are detached from the well.

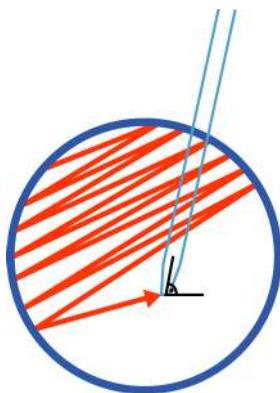


Figure 1. Pattern of movement of a glass serological pipette during the iPSCs passaging with Dispase. After scraping the well in one direction, turn the plate clockwise or counterclockwise by 90° and scrape remaining iPSC colonies again. About 90% cells should be detached from the well. Please refer to the section on iPSC passaging using Dispase for details.

- k. Wash plate with the volume of mTeSR required to bring cells up to the final volume needed to seed a new Matrigel-coated vessel. Calculate the final volume considering 2 ml per each well to be seeded with passaged cells. For example, if cells are to be passaged 1 to 3 the final volume will be 6 ml, therefore the volume of mTeSR used to wash the plate is 5 ml.
- l. Seed cells on a new Matrigel plate plating 1 ml per well and then add 1 ml more. Plate cells dropwise across the entire surface of the well to ensure uniform plating.

- m. Observe seeded cells under microscope to ensure even plating.
- n. Place in a 37 °C incubator. Shake the plate in T-shape to homogenously distribute the colonies pieces in the well.
- o. Twenty-four hours after plating gently, wash cells with DMEM/F-12 before adding fresh mTeSR medium.

Note: For a healthy and efficient iPSC culture, it is critical to plate cells uniformly. Plate cells uniformly across the entire surface of the well and, when plating multiple wells, plate cells uniformly across all wells.

B. Monolayer plating

Note: After thawing an iPSC line, passage cells with Dispase at least once before plating monolayer.

1. Remove 6-well plates from the incubator. When iPSC cells are at around 80% confluence (cells are ready for a passage), iPSC cells are ready for Monolayer. Mark all differentiated cells, which need to be removed.
2. Aspirate the spent medium. Remove all marked differentiated cells and wash cells with DMEM/F-12 (2 ml/well).
3. Aspirate DMEM/F-12 and add 1 ml of room temperature Accutase to well of a 6-well plate. Incubate cell for 8 min at 37 °C.
4. After 8 min of incubation, add 1 ml per well of mTeSR containing 5 µM ROCK inhibitor and re-suspend cells as single cells without scraping plate surface, using a P1000 pipette. Pipette cells 10-12 times, turn the dish by 180° (upside down) and pipette 5 more times. Collect cells from all wells in a 50 ml conical tube. You should not see any cell clumps.
5. Wash all wells twice with 5 ml of mTeSR containing 5 µM ROCK Inhibitor. Collect all cells in the same conical tube.
6. Centrifuge the cells at 53 x g (500 RPM in a Sorvall 75006445 rotor with 75006441 K buckets) for 7 min at room temperature. Aspirate the supernatant and resuspend cells in 10 ml of mTeSR containing ROCK Inhibitor.
7. Mix the pooled cell suspension by inverting 20 times or more if necessary. Perform the live cell count using 0.4% Trypan Blue Solution.

Note: iPSC cell viability should be not lower than 80%.

8. Prepare required number of cells. Optimal cell number will vary depending on the scale of differentiation. iPSC-RPE differentiation protocol requires 2.5×10^4 live cells per cm^2 (2×10^6 per one 100 mm dish). For one 100 mm dish, prepare in a 15 ml conical tube 11 ml of cell suspension containing 2.2×10^6 cells. Mix cell suspension very well by inverting the tube 20 times.
9. Add 10 ml of cell suspension per 100 mm dish dropwise using a 10 ml pipette.

Note: It is critical to plate cells uniformly on the entire surface of the plate. To help distribute the cells uniformly plate one dish at the time and shake the newly plated dish in a cross shape (T-shape).

10. Place plates in the incubator without stacking the plates. Incubate the cells until next morning, at 37 °C, 5% CO₂.
11. Next day change medium for fresh mTeSR without ROCK inhibitor (10 ml/100 mm dish). Monolayer for iPSC-RPE cell differentiation requires culturing cells for about 4-5 days until the monolayer reaches 80% confluence. Change medium with fresh mTeSR daily.

C. iPSC-RPE differentiation

Refer to Figure 2 for a schematic representation of the differentiation protocol.

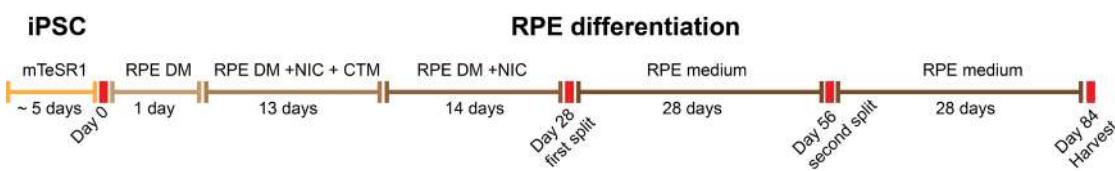


Figure 2. Schematic representation of the iPSC-RPE differentiation protocol. Nicotinamide (NIC), Chetomin (CTM). iPSC monolayer is cultured until the cells reach 80% confluence. iPSC-RPE differentiation is initiated and driven using small molecules (NIC and CTM). iPSC-RPEs continue to differentiate and expand for a total of 12 weeks. Whole culture passages promote expansion of iPSC-RPE cells. Adapted from Smith *et al.*, 2019.

1. Day 0 (D0)—When iPSC monolayer reaches about 80% confluence initiate the iPSC-RPE differentiation by replacing mTeSR medium with RPE DM medium (see Recipes: Table 5) (24 ml/100 mm dish).
2. D1–24 h after initiation of the differentiation replace spent RPE DM medium with fresh RPE DM medium supplemented with 10 mM Nicotinamide (see Recipes: Table 9) and 50 nM Chetomin (see Recipes: Table 8) (24 ml/100 mm dish).
3. Daily change spent medium with fresh RPE DM medium supplemented with 10 mM Nicotinamide (NIC) and 50 nM Chetomin (CTM) (24 ml/10 cm dish).
Note: Due to very high cell number, high proliferative rate and metabolic activity of iPSC-RPE cells to obtain a healthy and robust iPSC culture it is strongly recommended to maintain the schedule of 24 h media change throughout the entire differentiation.
4. On D14 change spent medium with fresh RPE DM medium supplemented with 10 mM Nicotinamide (24 ml/100 mm dish).
5. Daily change spent medium with fresh RPE DM medium supplemented with 10 mM Nicotinamide (24 ml/100 mm dish).
Note: First cells start to acquire melanin pigmentation and the characteristic polygonal shape after 2-3 weeks.
6. On D28 passage the cells—first passage.
 - a. (Optional) Take images of the cells—(Figure 3-D28-left panel).

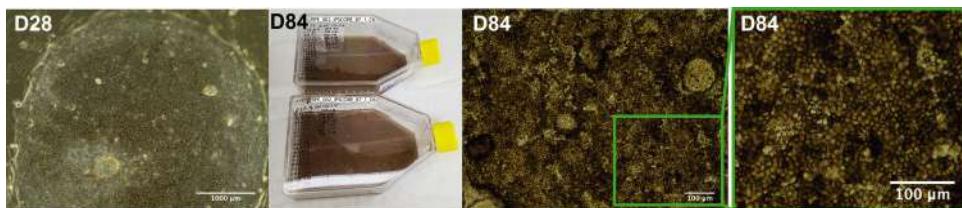


Figure 3. Images of the iPSC-RPE cells. Bright-field image of iPSC-RPE at Day 28 (D28) of the differentiation iPSC-RPE cells appear as small clusters of polygonal pigmented cells which expand over time (left); T150 flasks containing iPSC-RPE cells (iPSCORE 87_1) at Day 84 (D84) (middle left); Bright-field image of iPSC-RPE sample (iPSCORE_42_1) at Day 84 illustrating a highly organized monolayer with strong melanin pigmentation (middle right) and characteristic polygonal shape (right). Adapted from Smith *et al.*, 2019.

- b. Aspirate spent medium and wash cells with PBS (10 ml/100 mm dish)
- c. Aspirate PBS and add 5 ml of room temperature Accutase to a 100 mm dish. Incubate cells for 12 min at 37 °C.
- d. After 12 min of incubation add 5 ml per dish of RPE DM medium, re-suspend cells in Accutase as single cells without scraping the surface of the plate, using a P1000 pipette. If cells are difficult to remove from a dish, use a cell scraper.
- e. Collect cells in a 50 ml conical tube.
- f. Wash plate three additional times, each with 5 ml of RPE DM medium. Collect all cells in the same conical tube.
- g. Centrifuge cells for 8 min at 136 \times g (800 RPE in a Sorvall 75006445 rotor with 75006441 K buckets) at room temperature.
- h. After centrifugation, aspirate the supernatant and resuspend the cells in 10 ml of RPE medium (see Recipes: Table 6).
- i. Gently pass all cells through a 70 μm strainer. Add medium for a total of 20 ml of final volume passing it through the strainer.
- j. Plate cells on two fresh 100 mm dishes which were coated overnight with Matrigel.
- k. Using a 10 ml pipette add dropwise 10 ml of cell suspension per 100 mm dish.

Note: It is critical to plate cells uniformly on the entire surface of the plates. To help distribute the cells uniformly plate one dish at the time and shake the newly plated dish in a cross shape (T-shape).

Optional: Instead of using two 100 mm dishes plate cells on one T150 flask.

- I. Place plates in the incubator. Incubate the cells until next day, at 37 °C, 5% CO₂ without stacking the plates.
7. Twenty-four hours after split, change medium for fresh RPE medium (10 ml/100 mm dish). If using T150 flask use 25 ml/T150 flask.
8. Change medium daily for fresh RPE medium (10 ml/100 mm dish) for 4 weeks. If using T150 flask use 25 ml/T150 flask.

9. On D56 passage the cells—second passage.

Perform the passage following Steps C6a-C6g.

Note: If cells were plated on a T150 flask, incubate cells with 10 ml of Accutase per flask and use a cell scraper to recover all the cells. To collect all the cells, wash flask three times, each time with 10 ml of medium.

- a. After centrifugation, aspirate the supernatant and resuspend the cells in 20 ml of RPE medium.
- b. Gently pass all cells through a 70 µm strainer. Add medium for a total of 50 ml of final volume passing at least 20 ml of it through the strainer.
- c. Plate cells onto two fresh T150 flasks which were coated overnight with Matrigel.
- d. Add 25 ml of cell suspension per each T150 flask dropwise using a 10 ml pipette.

Note: It is critical to plate cells uniformly on the entire surface of the plates which were coated overnight with Matrigel. To help distribute the cells uniformly plate one flask at the time and shake the newly plated flasks in a cross shape (T-shape).

- e. Place flasks in the incubator without stacking. Incubate the cells until next morning, at 37 °C, 5% CO₂.

10. Twenty-four hours after split, change medium with fresh RPE medium (25 ml/T150 flask).

11. Change medium daily with fresh RPE medium (25 ml/T150 flask) for 4 weeks.

12. On D84 collect all cells.

- a. (Optional) Take images of the cells. Refer to Figure 3 for an example of expected iPSC-RPE yield, pigmentation (Figure 3—panel middle left and middle right) and cell morphology (Figure 3—panel middle right and right).
- b. Aspirate spent medium and wash cells with PBS (20 ml/T150 flask).
- c. Aspirate PBS and add 10 ml of room temperature Accutase to each flask. Incubate for 12 min at 37 °C.
- d. After 12 min of incubation, add 10 ml per well of RPE medium re-suspend cells. Use cell scraper to remove all cells.
- e. Collect cells in a 50 ml conical tube.
- f. Wash plate three additional times with 10 ml of RPE medium. Collect all cells in the same conical tube.
- g. Centrifuge cells for 8 min at 136 x g (800 RPE in a Sorvall 75006445 rotor with 75006441 K buckets) at room temperature.
- h. After centrifugation, aspirate the supernatant and resuspend the cells in 20 ml of RPE medium.
- i. Gently pass all cells through a 70 µm strainer. Add medium for a total of 40 ml of final volume passing at least 20 ml of it through the strainer.
- j. Mix the cell suspension by inverting 20-30 times. Perform the live cell count using 0.4% Trypan Blue Solution.

Notes:

- a. When performing the live cell count of iPSC-RPE using automated cell counter, cell viability may be inaccurately scored as high due to the high melanin concentration in the cells. Depending on the experimental needs, iPSC-RPE cells can be cryopreserved for future experiments, fixed for flow cytometry analysis and/or plated for immunofluorescence analysis.
- b. Cells start to acquire melanin pigmentation and the characteristic polygonal shape after 2-3 weeks in culture and about 1 week after each passage. The first signs of the cells starting to acquire the pigmentation is slightly grayish (“dirty”) hue of the color of the medium. When cells are strongly pigmented the medium also acquires dark color. Cell suspension of concentrated iPSC-RPE at D84 appears black, similarly to the pelleted iPSC-RPE-cells which are also black (Figure 4).



Figure 4. Images of the iPSC-RPE cells at D84. iPSC-RPE cells (iPSCORE_29_1) collected prior to the centrifugation (left) and after centrifugation (right).

D. Cryopreservation of iPSC-RPE

1. Prepare 2x iPSC-RPE freezing medium by preparing 20% DMSO solution in FBS. Prepare 0.25 ml of 2x iPSC-RPE freezing medium per each cryovial intended to be cryopreserved. Freeze cells at a final density of $1.2 \times 10^7/\text{ml}$ (depending on the downstream experiments the volume and the concentration of the cryopreserved iPSC-RPE cells in a single cryovial can be modified). *Optional: If a serum free conditions are required, prepare the 2x iPSC-RPE freezing medium using KOSR instead of FBS.*
2. Prepare and print the labels for cryovials. Prepare $n + 2$ number of labels ($n =$ number of cryovials to be cryopreserved). Prepare and affix the labels on all cryovials to be frozen, use one label for the Mr. Frosty and one label for record keeping (i.e., lab book).
3. After the live cell count (Step C12j), determine how many cells should be cryopreserved and transfer desired number of cells into a new 15 ml or 50 ml conical tube.
4. Centrifuge cells for 5-8 min at $136 \times g$ (800 RPM in a Sorvall 75006445 rotor with 75006441 K buckets) at room temperature (adjust the time of centrifugation depending on the volume of cells).
5. After centrifugation, aspirate the supernatant and resuspend the cells in 0.25 ml of FBS (or KOSR) per each cryovial to be frozen at the concentration of $2.4 \times 10^7/\text{ml}$ (i.e., for 10 cryopreserved vials resuspend 6×10^7 cells in 2.5 ml of FBS or KOSR).
6. Open all pre-labeled cryovials and add 0.25 ml of cell suspension to each cryovial.

7. Add 0.25 ml of 2x iPSC-RPE freezing medium to each cryovial containing the iPSC-RPE cell suspension.
8. Close all cryovials and gently invert them 5-6 times to mix cell suspension and 2x iPSC-RPE freezing medium. Transfer cryovials to Mr. Frosty freezing container.
9. Immediately transfer Mr. Frosty into a -80 °C freezer. When freezing large number of cryovials (*i.e.*, multiple Mr. Frosties) prepare individual batches, with each batch containing only the number of cryovials that will fit into one Mr. Frosty.
10. After 24-48 h, transfer the cells into a liquid nitrogen vapor tank. Update accordingly the records (*i.e.*, box maps).

Here, we provide detailed protocols for flow cytometry (FC) and immunofluorescence (IF) which can be applied to perform quantitative (FC) and qualitative (FC and IF) quality control of derived iPSC-RPE cells.

E. Flow cytometry

1. After the live cell count (Step C12j), determine how many cells should be fixed for flow cytometry analysis and transfer desired number of cells into a 15 ml conical tube. Use at least $2\text{-}5 \times 10^6$ cells.
2. Centrifuge cells for 5 min at $136 \times g$ (800 RPM in a Sorvall 75006445 rotor with 75006441 K buckets) at room temperature.
3. After centrifugation, aspirate the supernatant and resuspend the cells in 10 ml of PBS.
Optional: If the volume of cell suspension used for flow cytometry is smaller than 0.5 ml then add directly to the cells 14 ml of PBS and centrifuge mix of cells and PBS for 8 min at $136 \times g$ (800 rpm in a Sorvall 75006445 rotor with 75006441 K buckets) at room temperature.
4. Fix and permeabilize iPSC-RPE cells using the Fixation/Permeabilization Solution Kit with BD GolgiStop™ following manufacturer recommendations. After the last centrifugation, aspirate the supernatant and resuspend the cells in the 1x BD Perm/Wash™ Buffer at the concentration of $1 \times 10^7/\text{ml}$. For each flow cytometry staining use 2.5×10^5 cells.
Note: Staining of 2.5×10^5 cells allows for an efficient cells and reagent usage, however it is also possible to use 1×10^6 cells per staining maintaining the same antibodies dilution ratios.
5. Transfer 25 μl of fixed and permeabilized cells into 5 wells of a 96-well round bottom assay plate. In order to limit usage of the antibodies and cells when staining multiple lines, mix equal number of cells from each line and transfer 25 μl of the cell mix into four control wells [Recombinant Rabbit IgG, monoclonal class control antibody (Rb-IgG), Mouse IgG1, kappa monoclonal (M-IgG1), anti ZO-1 antibody (ZO1) and anti MiTF antibody (MiTF)].
6. Following Table 15 add appropriate concentrations of the antibodies in each well. Using a multichannel pipette set for 20 μl mix cells and antibodies gently by pipetting up and down 20 times.
7. Incubate cells with primary antibodies for 1 h at room temperature.
8. After 1 h, add 150 μl of FACS buffer (see Recipes: Table 10).

9. Centrifuge plate at $863 \times g$ (2,000 RPM in a Sorvall 75006445 rotor with 75006441 K buckets) for 8-10 s counting from when the speed reaches $863 \times g$ (2,000 rpm in a Sorvall 75006445 rotor with 75006441 K buckets) at room temperature.
In detail:
 - a. Set the centrifuge for $863 \times g$ (2,000 RPM), 1 min, room temperature); start the centrifuge and wait until the speed reaches $863 \times g$ (2,000 rpm).
 - b. Count to 8-10 s and stop the centrifuge.) The pellet after the centrifugation should be clearly visible especially when using 1×10^6 cells.
10. After centrifugation, gently aspirate the supernatant, being very careful not to aspirate any cells. If using vacuum to aspirate cells, use a P20 tip without a filter (or a P200 + P20 tips without filters). Leave about 20 μl of liquid in each well to avoid aspirating the cells.
11. Using a multichannel pipette add 200 μl of FACS buffer and mix cells gently 5-6 times.
12. Centrifuge plate like in Step E9.
13. Repeat Steps E10-E12 to wash the cells one more time.
14. Resuspend cells in 50 μl of 1x BD Perm/Wash™ Buffer.
15. Following Table 15 add appropriate concentrations of the antibodies in each well. Using a multichannel pipette set for 40 μl mix cells and antibodies gently by pipetting up and down 20 times.
16. Incubate cells with secondary antibodies for 45 min at room temperature in darkness.
17. After 45 min repeat Steps E8-E13.
18. After the last centrifugation aspirate the supernatant and resuspend the cells in 200 μl of FACS-FIX Buffer (see Recipes: Table 11). Using a multichannel pipette resuspend the cells by pipetting 5-6 times.
19. Using a P1000 pipette transfer each sample, one at a time, into a Corning™ Falcon™ Test Tubes with Cell Strainer Snap Cap passing the cells through the strainer in a cap.
20. With an additional 250 μl wash each well and transfer to the appropriate tube passing the cells through the strainer in a cap. Depending on the number of cells used for staining dilute the cells to an appropriate concentration to avoid clogging the flow cytometer.
21. Place all the tubes in an appropriate rack and wrap them in an aluminum foil to protect from light.
22. Proceed with acquisition using a flow cytometer FACS Canto II (or alternative flow cytometer).
23. Perform the flow cytometry analysis using FlowJo software V 10.4. Refer to the Figure 5 for an example of iPSC-RPE flow cytometry staining results.

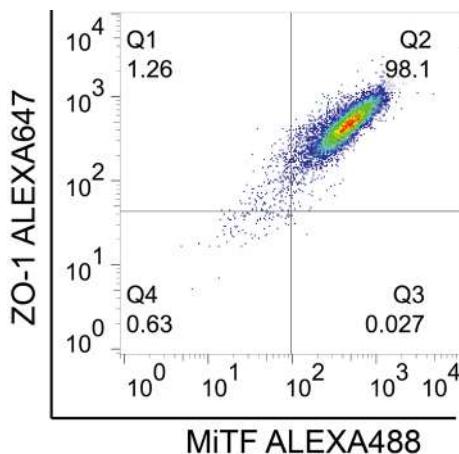


Figure 5. Flow-cytometry analysis of iPSC-RPE (iPSCORE_42_1) at Day 84 showing high co-staining of Zonula Occludens 1 (ZO-1) and Microphthalmia-associated Transcription Factor (MiTF). Adapted from Smith et al., 2019.

F. Immunofluorescence

1. Coat Millicell EZ SLIDE 8-well glass slides overnight with Matrigel.
2. Plate fresh or cryopreserved iPSC-RPE cells on the Matrigel coated Millicell EZ SLIDE 8-well glass slides. Plate at least 6 wells per line at the density of $1\text{-}1.5 \times 10^6/\text{cm}^2$.
3. Culture cells for 10 days until they reach full confluence, re-acquire polygonal shape and pigmentation.
4. Aspirate the medium and wash cells twice with PBS. Aspirate the PBS.
5. Fix cells with 4% PFA for 10 min at room temperature.
6. Remove the PFA solution and wash cells twice with freshly prepared IF Wash Buffer (see Recipes: Table 12). Aspirate the IF Wash Buffer.
7. Saturate and permeabilize the cells using IF Perm Buffer (see Recipes: Table 13). Incubate the cells for 20 min at room temperature.
8. In the last 5 min of the saturation and permeabilization prepare the primary antibody solutions in IF Staining Buffer (see Recipes: Table 14 and Table 15) for the appropriate concentrations of the antibodies. Store antibodies solutions on ice until use.
9. After saturation and permeabilization aspirate all the buffer and add antibodies solutions to the appropriate wells.
10. Incubate cells with the antibodies solution overnight at 4 °C.
11. Next day (morning) prepare the secondary antibody solutions in IF staining Buffer. Refer to the Table 15 for the appropriate concentrations of the antibodies. Keep antibodies solutions on ice until use, protected from light.
12. Aspirate the primary antibodies solutions and wash cells three times with PBS. After last wash aspirate all PBS.
13. Immediately add the secondary antibodies solutions to the appropriate wells. Incubate cells for 1 h at room temperature in darkness.

14. Aspirate the secondary antibodies solutions and wash cells three times with PBS. After last wash aspirate all PBS.
15. Detach the walls of the Millicell EZ SLIDE 8-well glass slides.
16. Add ProLong Gold Antifade Reagent with DAPI following manufacturer's recommendations and gently mount the cover glass slide avoiding bubbles. Use a pencil rubber to gently remove any bubbles. Store the slide(s) at room temperature for several hours (best until next day) in darkness to allow proper mounting.
17. Acquire images using an appropriate immunofluorescence microscope (best is a confocal laser scanning fluorescence microscope). Refer to Figure 6 for an example of iPSC-RPE immunofluorescence staining.

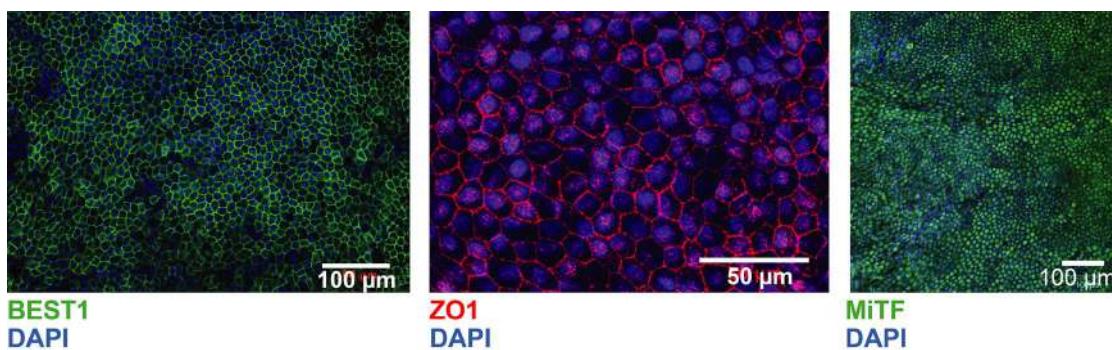


Figure 6. Immunofluorescence analysis of Bestrofin 1 (BEST1) (iPSCORE_29_1), ZO-1 (iPSCORE_29_1), and MiTF (iPSCORE_42_1). ZO-1 appears as sharp cell membrane staining; BEST1 membrane staining appears “fuzzier” compared to ZO1 staining; MiTF nuclear staining. Adapted from Smith *et al.*, 2019.

Recipes

A. Cell culture reagents and media preparation

Table 1. Preparation of Matrigel solution

Reagent	Matrigel solution (referred to as Matrigel)
Components and preparation	1 mg Corning® Matrigel® Growth Factor Reduced (GFR) Basement Membrane Matrix 24 ml of ice cold DMEM/F-12 medium Mix well by pipetting and inverting
Method of sterilization	None
Notes	<ul style="list-style-type: none">All plasticware used for preparation of Matrigel should be cold. Best if stored in a -20 °C freezer and removed immediately prior to usePrepare the Matrigel solution on iceVolumes of Matrigel solution used for coating:2 ml–1 well of a 6-well plate10 ml–100 mm tissue culture dish20 ml–T150 tissue culture flaskWhen using other vessel adjust the volume of Matrigel to 210 µl/cm²
Storage	<ul style="list-style-type: none">store aliquots of Matrigel at -80 °Cstore coated plates in an incubator with humidity and gas control set to maintain 37 °C and 95% humidityplates coated with Matrigel should be used within two weeks; best if prepared a day before use

Table 2. Preparation of 1.10 mM ROCK inhibitor, Y-27632 dihydrochloride

Reagent	10 mM ROCK inhibitor, Y-27632 dihydrochloride (referred to as ROCK Inhibitor)
Components and preparation	5 mg ROCK inhibitor, Y-27632 dihydrochloride 1,561 µl UltraPure™ DNase/RNase-Free Distilled Water Mix well by pipetting
Method of sterilization	Sterile filter with a 0.2 µm filter
Notes	<ul style="list-style-type: none">Prepare 50-100 µl aliquotsProtect from lightWe recommend 10 µM ROCK Inhibitor for thawing and 5 or 10 µM for single cell iPSC passages
Storage	1 month at -20 °C

Table 3. Preparation of 10x Dispase Solution

Reagent	10x Dispase
Components and preparation	200 mg Dispase 10 ml DMEM/F-12 medium Mix well by pipetting and inverting
Method of sterilization	Sterile filter with a 0.2 µm filter
Notes	<ul style="list-style-type: none">• Prepare 1 ml aliquots in a 15 ml conical tubes• To prepare 1x Dispase add 9 ml of DMEM/F-12
Storage	10x: one month at -20 °C 1x: two weeks at 4 °C

Table 4. Preparation of mTeSR™1 complete medium

Medium	mTeSR™1 complete medium (referred to as mTeSR)
Components and preparation	100 ml of 5x Supplement 400 ml of Basal medium Mix well by inverting
Method of sterilization	none
Notes	Prepare aliquots if needed
Storage	4 °C

Table 5. Preparation of RPE DM medium (following Maruotti et al., 2015)

Medium	RPE DM medium (following Maruotti et al. [2015])
Components and preparation	425 ml DMEM/F-12 medium 75 ml KnockOut™ Serum Replacement (KOSR) 5 ml L-Glutamine (200 mM) 5 ml MEM Non-Essential Amino Acids Solution 100x 5 ml Penicillin-Streptomycin (10,000 U/ml) 3.5 µl β-Mercaptoethanol Mix well by inverting
Method of sterilization	None or sterile filter with a 0.2 µm filter if needed
Notes	Prepare aliquots if needed
Storage	4 °C

Table 6. Preparation of RPE medium (following Maruotti et al., 2015)

Medium	RPE medium (following Maruotti et al.[2015])
Components and preparation	350 ml DMEM medium 150 ml Ham's F12 Nutrient Mixture 10 ml B27 Supplement (50x) 5 ml Penicillin-Streptomycin (10,000 U/ml) Mix well by inverting
Method of sterilization	None or sterile filter with a 0.2 µm filter if needed
Notes	Prepare aliquots if needed
Storage	4 °C

Table 7. Preparation of 2x iPSC-RPE freezing medium (for 5 ml)

Medium	2x iPSC-RPE freezing medium (for 5 ml)
Components and preparation	4 ml FBS or KnockOut™ Serum Replacement (KOSR) 1 ml DMSO Mix well by inverting or pipetting
Method of sterilization	None
Notes	Prepare fresh
Storage	Do not store

Table 8. Preparation of 1 mM Chetomin solution

Reagent	1 mM Chetomin
Components and preparation	1 mg Chetomin 1.4 ml DMSO Mix well by pipetting
Method of sterilization	none
Notes	<ul style="list-style-type: none">• Prepare 10 µl aliquots.• Protect from light.• It is possible to freeze thaw aliquots 2-3 times without affecting iPSC-RPE differentiation efficiency (Maruotti et al., 2015)
Storage	-80 °C: several months

Table 9. Preparation of 1M Nicotinamide (100x) solution

Reagent	1 M Nicotinamide (100x)
Components and preparation	6.1 g Nicotinamide 50 ml UltraPure™ DNase/RNase-Free Distilled Water Mix well by pipetting and inverting
Method of sterilization	Sterile filter with a 0.2 µm filter
Notes	Prepare 1 ml aliquots.
Storage	4 °C: one month

B. Buffer preparation

Table 10. Preparation of FACS Buffer

Buffer	FACS Buffer
Components and preparation	500 ml PBS 1% BSA 0.05% NaN ₃ - Optional Mix well by inverting
Method of sterilization	Sterile filter with a 0.2 µm filter
Notes	Prepare aliquots if needed
Storage	4 °C

Table 11. Preparation of FACS-FIX Buffer

Buffer	FACS-FIX Buffer
Components and preparation	FACS Buffer 1% Formaldehyde Mix well by inverting
Method of sterilization	None
Notes	Prepare aliquots if needed
Storage	4 °C for several months

Table 12. Preparation of IF Wash Buffer

Buffer	IF Wash Buffer
Components and preparation	PBS 0.1% Tween® 20 Mix well by inverting
Method of sterilization	None
Notes	Prepare fresh
Storage	Do not store

Table 13. Preparation of IF Perm Buffer

Buffer	IF Perm Buffer
Components and preparation	PBS 1% BSA 0.1% Triton X-100 Mix well by inverting
Method of sterilization	None
Notes	Prepare fresh
Storage	Do not store

Table 14. Preparation of IF Staining Buffer

Buffer	IF Staining Buffer
Components and preparation	PBS 1% BSA Mix well by inverting
Method of sterilization	None
Notes	<ul style="list-style-type: none">• Prepare fresh• Add antibodies few minutes before adding to the fixed and permeabilized cells
Storage	Do not store. Keep on ice and protected from light when necessary prior to use

C. Antibodies

Table 15. Antibodies concentrations

Antibody	Catalog number	Concentration
ZO-1	ab59720	1:10–flow cytometry 1:250–immunofluorescence
MiTf	ab12039	1:10–flow cytometry 1:100–immunofluorescence
BEST1	NB300-164SS	1:150–immunofluorescence
Goat-anti-Mouse Alexa Fluor™ 488	A-11001	1:200–flow cytometry 1:250–immunofluorescence
Donkey-anti-Rabbit Alexa Fluor™ 647	ab150075	1:200–flow cytometry 1:250–immunofluorescence

Acknowledgments

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Competing interests

Authors declare no competing interests.

Ethics

iPSC lines generated from individuals of different ethnicities (3 European Americans, 2 East Asian Americans, and 1 African American) were obtained from iPSCORE (Panopoulos *et al.*, 2017).

Donors were all females ranging from 21 to 62 years of age at the time of donation. The recruitment of these individuals was approved by the Institutional Review Boards of the University of California, San Diego, and The Salk Institute (project no. 110776ZF).

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Retina Injury and Retina Tissue Preparation to Study Regeneration in Zebrafish

Poonam Sharma and Rajesh Ramachandran*

Department of Biological Sciences, Indian Institute of Science Education and Research, Mohali, India

*For correspondence: rajeshra@iisermohali.ac.in

[Abstract] Unlike mammals, primitive vertebrates have immense capability to regenerate almost all of their organs including the central nervous system. Among primitive organisms, zebrafish have been extensively used as a model system for regeneration studies. The retina is a part of the central nervous system and mammals lack the potential to repair any damage caused to it. Zebrafish have been used for retina regeneration studies because of ease in handling and maintenance. In zebrafish, Muller glia cells respond to damage and enter the regenerative cascade to maintain the retinal homeostasis. Zebrafish retinal damage can be induced by light, chemical or mechanical methods. Here we are describing the mechanical method of retinal injury, which ensures uniform damage to all retinal layers. Alongside this, we have also described *in vivo* manipulation strategies for the regeneration associated genes and preparation of retinal tissue for immunohistochemical analysis.

Keywords: Zebrafish, Retina, Electroporation, Intravitreal, Transfection, Eye

[Background] Retina is the sensory part of the eye and any physical or physiological damage leads to impairment of vision. In the course of evolution higher vertebrates have lost the regenerative potential while primitive vertebrates have enormous capability to regenerate their lost vision. Studying the regenerative events in primitive organisms such as zebrafish can be a hope for mammalian regeneration studies. Zebrafish have been used for retina regeneration studies with various injury paradigms being used. The photobleaching method damages the photoreceptor cells, while chemical methods damage the ganglion cell layer. Mechanical injury by the stab wound method maintains a uniform injury to all retinal layers. Various approaches including transgenic approaches have been used to find the relevance of genetic events during retina regeneration. Here we are describing the *in vivo* method for mRNA transfection which allows manipulation of gene expression levels above endogenous levels.

Materials and Reagents

1. 30G needle (BD Microlance, 30G ½", 0.3 x 13 mm, catalog number: 304000)
2. Centrifuge tubes (Tarsons, catalog number: 500010X)
3. Paper towels (Scott SCOTTFOLD Towels, 31.4 cm x 19.9 cm, catalog number: 01960)
4. Hamilton syringe (Hamilton Company, 10 µl, catalog number: MICROLITER™ #701)
5. Staining box (Custom made by fixing two plastic pipettes parallel to each other at the bottom of a rectangular plastic box)
6. Glass slides (Superfrost Plus Microscope Slides) (Fisher Scientific, catalog number: 12-550-15)

7. pCS2+ plasmid (David Turner, University of Michigan, Ann Arbor)
8. Ethyl 3-aminobenzoate, methane sulfonic acid salt, Tricaine methanesulfonate (Acros, catalog number: 118000500)
9. 2x HBSS (Diluted from 10x solution, see Recipes)
10. mMESSAGE mMACHINE SP6 kit (Thermo Fisher, catalog number: AM1340)
11. Lipofectamine messenger max reagent (Invitrogen, catalog number: LMRNA001)
12. DABCO (1,4 diazabicyclo [2.2.2] octane, Sigma-Aldrich, catalog number: D27802)
13. Na₂HPO₄ (Sodium phosphate dibasic, Sigma-Aldrich, catalog number: 255793)
14. NaH₂PO₄ (Sodium phosphate monobasic, Sigma-Aldrich, catalog number: 33198)
15. KCl (Potassium chloride, Himedia, catalog number: MB043)
16. HEPES (Sigma-Aldrich, catalog number: H3375)
17. Glucose (Sigma-Aldrich, catalog number: G8270)
18. Tissue-Plus O.C.T. compound (Fisher Health care, catalog number: 4585)
19. BSA (Bovine serum albumin fraction-V, Himedia, catalog number: GRM105)
20. Triton X-100 (Sigma-Aldrich, catalog number: T8787)
21. BrdU (5-Bromo-2'-deoxyuridine, Sigma-Aldrich, catalog number: B5002)
22. PFA (Paraformaldehyde, Sigma-Aldrich, catalog number: P6148)
23. PVA (Polyvinyl alcohol, Sigma-Aldrich, catalog number: P8136)
24. Glycerol (Sigma-Aldrich, catalog number: G7757)
25. Tris Base (Trizma base, Sigma-Aldrich, catalog number: T1503)
26. HCl (Hydrochloric acid, Sigma-Aldrich, catalog number: 320331)
27. Sucrose (Sigma-Aldrich, catalog number: S0389)
28. Tris-HCl (1 M, pH 7.5, 100 ml) (see Recipes)
29. Tricaine methanesulfonate solution (100 ml) (see Recipes)
30. Phosphate buffer (PB, 1 M, pH 7.4, 100 ml) (see Recipes)
31. Phosphate buffered saline (PBS, 10x, pH 7.4, 1 L) (see Recipes)
32. HBSS (Hanks balanced salt solution, 10x, pH 7.14, 100 ml) (see Recipes)
33. 4% PFA (50 ml) (see Recipes)
34. DABCO (2.5%, 50 ml) (see Recipes)
35. Sucrose solutions (50 ml) (see Recipes)

Equipment

1. Forceps (McPherson Suture Tying Forceps Straight-With Tying Platform 10cm (4") 5 mm size, jaw length, Surtex Instruments, catalog number: FR-780-10)
2. Stereomicroscope (Carl Zeiss™ Stemi™ DV4 Series Stereomicroscopes with LED Illumination)
3. Electroporator (Electro Square Porator, BTX Harvard Apparatus, model: ECM 830)
4. Electrodes (Platinum Tweezertrode, 5 mm Diameter with 45-0204 cables, BTX, catalog number: 45-0489)

5. Cryostat (Leica, model: CM3050 S)
6. Rotospin Rotary Mixer (Tarsons)
7. Shaking water bath (Stuart, model: SBS40)
8. Centrifuge (Labocene, catalog number: ScanSpeed 1736R)
9. Nikon Ni-E fluorescence microscope and Nikon A1 confocal imaging system (Nikon A1-SHS, catalog number: 10225)

Procedure

- A. Retina Injury (Kaur *et al.*, 2018; Mitra *et al.*, 2018; Mitra *et al.*, 2019; Sharma *et al.*, 2019)
1. Prepare 50 ml of 1x tricaine methane sulfonate solution in the fish water.
 2. Anesthetize the fish by putting in tricaine solution till the gill's movement slows down.
 3. Keep the anesthetized fish on a wet paper towel bedding with the right side facing upward.
 4. Focus the zebrafish eye under a stereomicroscope and tilt the dorsal side of eyeball gently with forceps.
 5. Poke one edge of the ventral side of the eye with 30 G needle by stab wound injury and then injure the other edge of the eye.
 6. Repeat the injuries on the dorsal side of the eye by tilting it gently from the ventral side so that a total of four injuries are made.
 7. Let the fish revive in the system water (see Video 1 for details).



Video 1. Retinal injury in zebrafish

- B. *In vivo* mRNA transfection (Mitra *et al.*, 2018; Sharma *et al.*, 2019)

In vivo overexpression of genes is achieved by mRNA transfection in the retina. It involves the following steps (see Video 2 for details):



Video 2. Intravitreal injection and morpholino electroporation

1. Preparation of transfection reagent

- a. Clone GFP or gene of interest into pCS2+ plasmid. Linearize the vector containing insert from the 3' end. Prepare mRNA with a sp6 message machine *in vitro* transcription kit. Precipitate mRNA and dissolve in nuclease-free water to make a stock of 2,000 ng/ μ l.
- b. Prepare transfection mixture (8 μ l) with lipofectamine 2,000 transfection reagent as follows:
 - i. Mix 2 μ l each of mRNA (2,000 ng/ μ l) and 2x HBSS solution at room temperature.
 - ii. Mix 2 μ l of lipofectamine with an equal volume of 2x HBSS solution at a ratio of 1:1 and keep the solution at room temperature.
 - iii. Let both the solutions stand at room temperature for five minutes.
 - iv. Mix the solutions (i) and (ii), dropwise at a ratio of 1:1 and keep the mixture the room temperature for 30 min. Use the final transfection mixture containing 500 ng/ μ l of mRNA for intravitreal injection.

Note: This transfection mixture is for intravitreal injections in 4-5 zebrafish and only a single concentration of mRNA has been used. Depending upon the experimental need, these volumes can be scaled up or down, without any loss in transfection efficiency.

2. *In vivo* overexpression by intravitreal injection

- a. Anesthetize the fish and injure the right eye by making four stab wounds with a 30 G needle.
- b. From the fourth poke inject 1 μ l of transfection mixture using Hamilton syringe.
- c. Electroporate the fish by placing a negative electrode on the right eye and positive electrode on the other side.
- d. Electroporate with five pulses at 70 V of 50 milliseconds duration with a gap period of 950 milliseconds between the pulses.
- e. Harvest the eyes on 4 days post-injury as described in sections E and F.
- f. In gene overexpression retina check the proliferation in terms of BrdU positive cells in comparison to GFP transfected retinae (see Procedures E, F and G).
- g. Check the transfection by immunostaining for GFP and subsequent imaging which shows GFP in the whole retina. Two representative images to show GFP expression at the injury site and away from the injury site are appended here for reference (Figure 1).

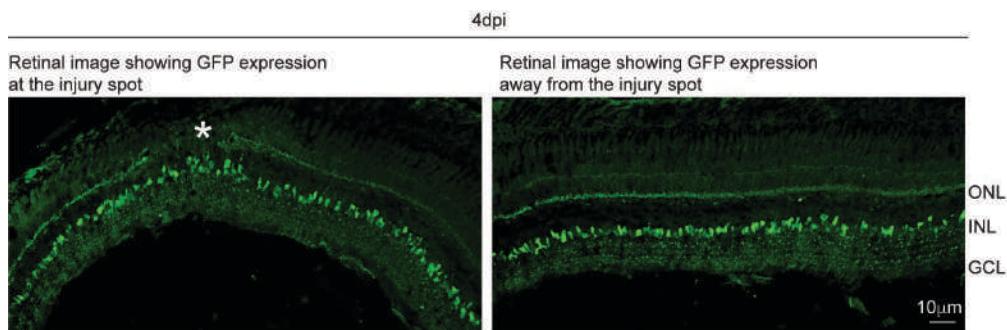


Figure 1. Showing GFP expression in zebrafish retina after GFP mRNA transfection.

Asterisk marks the injury spot. ONL—outer nuclear layer; OPL—outer plexiform layer; INL—inner nuclear layer; IPL—inner plexiform layer; GCL—ganglion cell layer.

C. Drug delivery and Morpholino electroporation (Kaur *et al.*, 2018; Mitra *et al.*, 2018; Mitra *et al.*, 2019; Sharma *et al.*, 2019)

To study the localized effect of drugs, proteins, and gene knockdown approaches, intravitreal injections are made.

1. Preparation of solution for injection:

- Dissolve the lyophilized morpholino (300 ng) in 300 μl of autoclaved Milli-Q water to make a stock of 1 mM. Use neat for 1 mM or dilute with Milli-Q water to make stocks of 0.5 or 0.25 mM for injection.
- Dissolve the protein in the recommended solvent to make a stock solution and dilute further in 1x PBS or recommended diluent.
- Dissolve the drugs in 1 ml DMSO (Dimethyl sulfoxide) or recommended solvent to make a stock solution of final concentration depending upon the molecular mass and weight of the pharmacological inhibitor. Make the required working concentrations from the main stock by diluting with Milli-Q water.

2. Injection of the solution:

- Injure one retina by stab wound injury as described above.
- From the fourth poke inject around 1 μl of the reagent with Hamilton syringe.
- In the case of morpholino delivery, electroporate the morpholino to make their entry in the retina.
- Place the positive electrode on the eye injected with morpholino and the negative electrode on the other eye.
- Electroporate at the conditions given above.
- Harvest the eyes on 4 days post retinal injury (dpi) as described below.

D. BrdU labeling

BrdU pulse labeling is done for 3 h before harvesting the eye on 4 dpi. It labels actively proliferating cells by incorporating thymidine analog (BrdU) in the replicating DNA.

1. Make 5 mM solution of BrdU by dissolving 15.35 mg of BrdU powder in 10ml of autoclaved Milli-Q water. Make 1 ml aliquots and store at -20 °C for later use.
2. Bring the BrdU solution at room temperature before use and fill in the insulin syringe.
3. Anesthetize the fish and hold on a wet paper towel under the microscope.
4. Insert the insulin needle gently in the midway between pelvic fins and making needle parallel to the fish body taking care not to damage internal organs.
5. Inject around one unit (15-20 µl) of BrdU solution and leave the fish for 3 h in the system water.

E. Harvesting and tissue preparation

1. Harvesting of eye (see Video 3 for details)
 - a. Euthanize the fish by tricaine overdosing (prolonged immersion in 1x tricaine solution).
 - b. Gently pull out the eye out of the socket with forceps.
 - c. Keep the eye in chilled fixative in the Petri plate and focus under a stereomicroscope.
 - d. Hold the eye with forceps and pierce the cornea with a needle. With the help of forceps tear the cornea and gently push the eye allowing the lens come out.



Video 3. Zebrafish euthanasia and harvesting of the eye

2. Tissue fixation
 - a. Fix the eye overnight at 4 °C in 4% PFA with continuous rotation at 12 rpm on rotor spin.
 - b. Remove the PFA and dehydrate the tissue in the sucrose gradient for 45 min each at room temperature with continuous rotation. Add the sucrose solutions as follows:
 - 5% sucrose, 1 volume
 - 5%:20% sucrose, 2:1 volume
 - 5%:20% sucrose, 1:1 volume
 - 5%:20% sucrose, 1:2 volume
 - 20% sucrose, 1 volume
 - c. Finally, replace 20% sucrose with 500 µl of fresh sucrose solution and add an equal volume of OCT into it. Mix by rotation at room temperature for 30 min.
 - d. Make cubic molds of aluminum foil by wrapping on cubits.

- e. Fill the mold till half with OCT, put a labeled flag at the top and place the eye dorsoventrally parallel to it.
- f. Freeze the positioned eye immediately by keeping at -80 °C.

F. Cryo-sectioning and Immunostaining

1. Cryo-sectioning
 - a. Turn on the cryostat and keep eye blocks inside the chamber for 10 min.
 - b. Fix the tissue on specimen disc with the help of OCT.
 - c. Fix the specimen disc on the specimen head and label the glass slides.
 - d. Remove the excess of OCT till retinal tissue starts appearing.
 - e. Take 8-10 µm thin serial sections on glass slides.
 - f. Allow the slides dry overnight at room temperature.
2. Immunostaining
 - a. Take one set of slides and lay them horizontally on the immunostaining rack.
 - b. Give three washes with 1x PBS for 10 min each to remove OCT.
 - c. Block the tissues with 5% BSA in PBST (1x PBS with 0.01% Triton X-100) for 2-3 h at room temperature.
 - d. Remove the blocking solution and overlay the slides with the primary antibody at 4 °C overnight.
 - e. Next day collect the primary antibody and wash the slides three times with PBST at room temperature for 10 min each.
 - f. Overlay the slides with fluorescently labeled secondary antibody for 2-3 h at room temperature.
 - g. Collect the secondary antibody and wash the slides thrice with PBST.
 - h. Stop the reaction by washing with autoclaved water.
 - i. Let the slides dry vertically in the dark for 10-15 min.
 - j. Mount the slides with 60 µl DABCO and coverslip them.
 - k. Let the slides dry overnight at room temperature in a dark chamber.

G. Image acquisition

Examine the slides under a fluorescence microscope and take images with a confocal imaging system with a Nikon Ni-E fluorescence microscope equipped with fluorescence optics and Nikon A1 confocal imaging system.

Data analysis

Count the BrdU labeled cells under a fluorescence microscope by observation of their fluorescence in retinal sections. In both experimental and treatment sets count the cells at the main injury spot and avoid the spots away from the main injury. The injury spot can be demarcated by discontinuous

outer nuclear layer as shown below (Figure 2). Take a minimum of three eyes in each experimental setup. Count the labeled cells in all the main injury spots present in the three retinal sections. Exclude the outliers (extremely low and extremely high values) and calculate the average and standard deviation by analyzing the data in an Excel sheet.

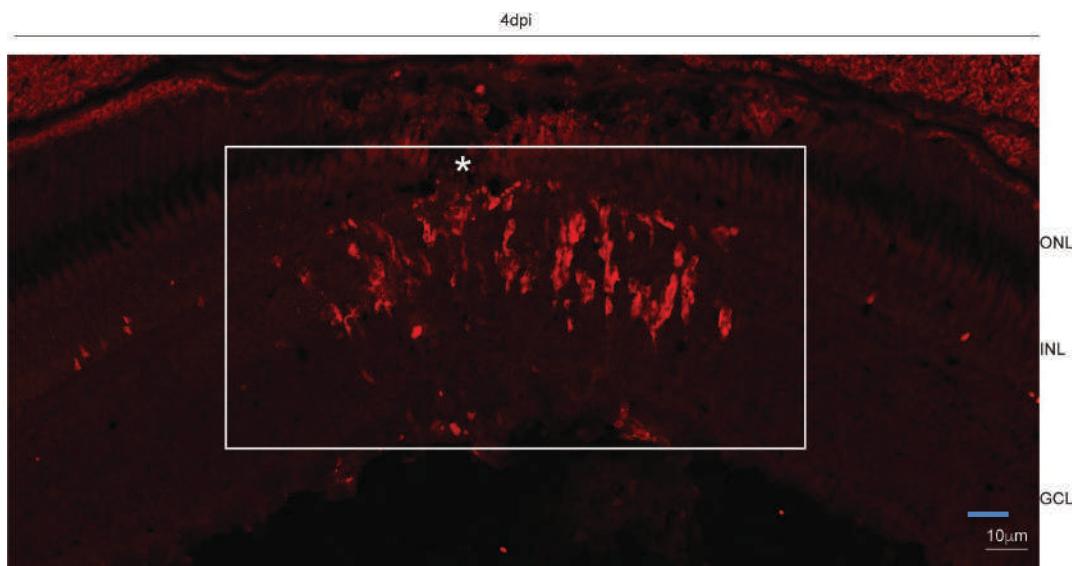


Figure 2. Showing injury spot (demarcated by an asterisk) with BrdU positive cells labeled in red. Square around the injury spot marking the area for cell count. Scale bar—10 μ m; ONL—outer nuclear layer; INL—inner nuclear layer; GCL—ganglion cell layer.

Recipes

1. Tris-HCl (1 M, pH 7.5, 100 ml)
 - a. Make 1 M stock of Tris-HCl by dissolving 12.1 g of Tris base in 80 ml of Milli-Q water
 - b. Adjust the pH to 7.5 with concentrated HCl solution and make the final volume to 100 ml with Milli-Q water
2. Tricaine methane sulfonate solution (100 ml)
 - a. Measure 2 g of Ethyl 3-aminobenzoate, methane sulfonic acid salt and dissolve it in 100 ml of 0.1 M Tris-HCl (pH 7.5) solution (Dilute the stock solution to 0.1 M by taking 10 ml of 1 M solution in 90 ml of Milli-Q water)
 - b. Keep the solution at 4 °C and dilute it (1:100) in fish system water before use
3. Phosphate buffer (PB, 1 M, pH 7.4, 100 ml)
 - a. Weigh the following components:

Na ₂ HPO ₄	10.9877 g
NaH ₂ PO ₄	2.711548 g
 - b. First, dissolve Na₂HPO₄ in 80 ml of Milli-Q H₂O, then add NaH₂PO₄
 - c. Adjust the pH to 7.4 and make up the volume to 100 ml with Milli-Q H₂O

- d. Autoclave the solution and dilute to a working stock of 0.1 M with autoclaved Milli-Q water
4. Phosphate buffered saline (PBS, 10x, pH 7.4, 1 L)
 - a. Measure the following components:

NaCl	75.97 g
Na ₂ HPO ₄	9.937 g
NaH ₂ PO ₄	3.59 g
 - b. Dissolve all the components in 800 ml of Milli-Q water by vigorous shaking or with magnetic beads
 - c. Once all the components have dissolved adjust the pH to 7.4 and make up the final volume to 1,000 ml
 - d. Autoclave the solution and use it as a 1x solution diluted with Milli-Q water
5. HBSS (Hanks balanced salt solution, 10x, pH 7.14, 100 ml)

NaCl	8 g
KCl	0.354 g
Glucose	1.351 g
HEPES	5.466 g
Na ₂ HPO ₄	99.35 mg

 - a. Measure these components and dissolve in 80 ml of Milli-Q water
 - b. Adjust the pH to 7.14 and make up the final volume to 100 ml
 - c. Prepare 2x solution from 10x stock and store in 1 ml aliquots at -20 °C
6. 4% PFA (50 ml)
 - a. Measure 2 g of PFA in a 50 ml dry centrifuge tube and add 5 ml of 1 M PB into it
 - b. Make the volume to 45 ml with autoclaved Milli-Q water and dissolve PFA by heating at 65 °C in a shaking water bath
 - c. Take out the centrifuge tube in-between to mix settled powder by inverting vigorously. It takes around 15-30 min to dissolve completely
 - d. After dissolving properly, adjust the volume to 50 ml with autoclaved Milli-Q water and keep immediately on ice. For long term storage, make small aliquots and store at -20 °C
7. DABCO (2.5%, 50 ml)
 - a. Measure 12 g of glycerol in a 50 ml centrifuge tube
 - b. Measure 4.8 g of PVA and add it to glycerol. Mix well by gently inverting the tube till homogenous solution
 - c. Add 12 ml of autoclaved Milli-Q water and mix overnight on rotor. Then spin at room temperature
 - d. Add 24 ml of 0.2 M Tris-HCl, pH 8-8.5
 - i. Make 100 ml of 1 M Tris-HCl solution by dissolving 12.1 g of Tris base in 80 ml of Milli-Q water
 - ii. Adjust the pH to 8.2 with a concentrated HCl solution and make up the final volume to 100 ml

- iii. To make 0.2 M Tris-HCl take 6 ml of 1 M stock in 24 ml of Milli-Q water
 - e. Heat the solution to 50 °C in a shaker water bath for 15-30 min and centrifuge at 5,000 x g for 15 min
 - f. Carefully remove the supernatant and add 1.25 g of DABCO into the supernatant
 - g. Centrifuge at 5,000 x g for 15 min
 - h. Aliquot the supernatant in 1 ml tubes and store the at -20 °C for later use
8. Sucrose solutions (50 ml)
 - a. 5% sucrose: Measure 2.5 g of sucrose and dissolve it in 40 ml of 0.1 M PB solution. Adjust the volume to 50 ml with 0.1 M PB
 - b. 20% sucrose: Measure 10 g of sucrose and dissolve it in 40 ml of 0.1 M PB solution. After dissolving it by rotation, adjust the volume to 50 ml with 0.1 M PB
 - c. Store the solutions at 4 °C for long term use

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Competing interests

The authors declare that they have no conflict of interest.

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***In vitro* Self-organized Mouse Small Intestinal Epithelial Monolayer Protocol**

Gizem Altay¹, Eduard Batlle^{2, 3, 4}, Vanesa Fernández-Majada^{1, # *} and Elena Martínez^{1, 5, 6, #, *}

¹Biomimetic Systems for Cell Engineering Laboratory, Institute for Bioengineering of Catalonia (IBEC), The Barcelona Institute of Science and Technology (BIST), Barcelona, Spain; ²Colorectal Cancer Laboratory, Institute for Research in Biomedicine (IRB Barcelona), The Barcelona Institute of Science and Technology (BIST), Baldiri Reixac 10-12, Barcelona 08028, Spain; ³Centro de Investigación Biomédica en Red de Cáncer (CIBERONC), Barcelona, Spain; ⁴ICREA, Passeig Lluís Companys 23, 08010 Barcelona, Spain; ⁵Centro de Investigación Biomédica en Red (CIBER), Madrid, Spain; ⁶Department of Electronics and Biomedical Engineering, University of Barcelona (UB), Barcelona, 08028 Spain

#Contributed equally to this work

*For correspondence: emartinez@ibecbarcelona.eu; vfernandez@ibecbarcelona.eu

[Abstract] Developing protocols to obtain intestinal epithelial monolayers that recapitulate *in vivo* physiology to overcome the limitations of the organoids' closed geometry has become of great interest during the last few years. Most of the developed culture models showed physiological-relevant cell composition but did not prove self-renewing capacities. Here, we show a simple method to obtain mouse small intestine-derived epithelial monolayers organized into proliferative crypt-like domains, containing stem cells, and differentiated villus-like regions, closely resembling the *in vivo* cell composition and distribution. In addition, we adapted our model to a tissue culture format compatible with functional studies and prove close to physiological barrier properties of our *in vitro* epithelial monolayers. Thus, we have set-up a protocol to generate physiologically relevant intestinal epithelial monolayers to be employed in assays where independent access to both luminal and basolateral compartments is needed, such as drug absorption, intracellular trafficking and microbiome-epithelium interaction assays.

Keywords: Mouse intestinal organoids, Adult intestinal stem cells, Matrigel, Intestinal epithelial monolayer, *In vitro* intestinal epithelial model, Tissue-like functionality, TEER

[Background] The development of epithelial organoid culture systems is a major scientific achievement that has been established as an important tool in many basic biology and clinical applications due to their similarity in cell composition and function to the *in vivo* organ. Specifically, intestinal epithelial organoids are spherical formations with budding structures containing stem, Paneth and proliferative cells, corresponding to intestinal crypts, and inter-budding areas of differentiated cells representing the intestinal villi (Sato *et al.*, 2009; Ootani *et al.*, 2009). One of the major drawbacks of the intestinal organoid system is their closed-spherical structure which hampers their use in standard functional assays in which direct access to the organoid lumen or apical site of the epithelium is required. In the last few years, several attempts to open-up the spherical organoids into 2D monolayers have been established (Moon *et al.*, 2014; VanDussen *et al.*, 2015; Ettayebi *et al.*, 2016; In *et al.*, 2016; Kozuka

et al., 2017). However, the reported epithelial monolayers were mainly composed of mature epithelial cells with poor characterization of the proliferation and self-renewing capacities. More recently, methods to obtain monolayers with both proliferative and differentiated regions have been published. Although showing a physiologically relevant cell composition and distribution, those monolayers did not expand enough to cover the full substrate area and did not allow access to the basolateral site preventing their use in functional assays (Wang *et al.*, 2017a; Liu *et al.*, 2018; Thorne *et al.*, 2018).

Here, we describe an experimental protocol in which we grow either *Lgr5-EGFP-ires-Cre ERT2* mouse organoid-derived crypts or single cells on a thin layer of Matrigel to obtain intestinal epithelial monolayers that self-organize in crypt foci, containing stem cells, Paneth and proliferative cells, and villus-like regions composed of differentiated cells, resembling the cell distribution found in the *in vivo* small intestine. Importantly, our results indicate that the capacity of the intestinal epithelial cells to first self-organize into crypt-like domains and then originate villus-like regions is regulated intrinsically and is not due to the preservation of pre-established crypt configuration. We successfully adapted this method to a Transwell inserts culture format, as previously described (Wang *et al.*, 2017b), which allow an independent access to the basolateral and apical compartments, and demonstrate that the basolateral administration of crypt medium supplemented with non-epithelial niche-derived biochemical factors boosted epithelial monolayer expansion to fully cover the tissue culture substrate without altering the crypt-villus-like cell distribution.

These features of our model enable to perform functional assays such as to record transepithelial electrical resistance (TEER) measurements to determine the monolayers' epithelial barrier properties. We found that TEER values were within the expected physiological range ($40\text{-}100 \Omega \text{ cm}^2$) for mouse small intestine, demonstrating an adequate maturation of *de novo* generated epithelium. Overall, we have developed a protocol to generate organoid-derived intestinal epithelial monolayers with *in vivo*-like structural and functional features in a culture format compatible with functional assays. We believe our technology meet the necessary features of the complex 3D organotypic cell culture systems and 2D formats for a close to physiological high-throughput testing.

Materials and Reagents

A. Materials

1. Petri dishes, 94 x 16 mm, sterile (Biolab, catalog number: 020005)
2. Glass coverslips 24 x 24 mm (Deltalab, catalog number: D102424)
3. Serological pipettes 5 ml, 10 ml, 25 ml (Nunc, catalog numbers: 056815, 056816, 056817)
4. Racked filter pipette tips 20 μl , 200 μl , 1,000 μl , sterile (Labclinics, catalog numbers: LAB20ULFNL, LAB200ULFNL, LAB1000ULFNL)
5. 70 μm pore cell strainer (Biologix Research Co., catalog number: 151070)
6. 24-well multiwell plates (Nunc, catalog number: 142485)
7. Falcon, 50 ml centrifuge tubes (Deltalab, Eurotubo, catalog number: DEL429927)
8. Falcon, 15 ml centrifuge tubes (Deltalab, Eurotubo, catalog number: DEL429946)

9. Ibidi 8-well μ -Slides (Ibidi GmbH, catalog number: 80826)
10. Transwell polycarbonate membrane cell culture inserts, 6.5 mm diameter, 0.4 μ m pore size (Corning, catalog number: CLS3413)
11. 23 G 1" needle (BD Microlance 3, catalog number: 300800)
12. Disposable 1 ml syringe (B-Braun, catalog number: 9166017V)
13. Disposable 10 ml syringe (Becton Dickinson, catalog number: 309110)
14. Cell culture flasks, filter cap 25 cm² (Nunc EasYFlasks, catalog number: 156367)
15. Syringe filter, 0.22 μ m pore size, sterile (Millipore, Millex, catalog number: SLGP033RS)

B. Reagents

1. Phosphate buffered saline (PBS) powder, pH 7.4 (Sigma-Aldrich, catalog number: P3813), store at RT
2. Ethylenediaminetetraacetic acid (EDTA) solution, pH 8.0, ~0.5 M (Sigma-Aldrich, catalog number: 03690), store at RT
3. Matrigel Basement membrane matrix, LDEV-free (Corning, catalog number: 354234), store at -20 °C
Note: Thaw O/N on ice and aliquot in 0.5-1 ml vials upon arrival, to minimize freeze and thaw cycles.
4. Advance Dulbecco's modified medium (DMEM)/F12 (Thermo Fisher Scientific, Gibco, catalog number: 12634010), store at 2-8 °C
5. GlutaMAX supplement, 100x (Thermo Fisher Scientific, Gibco, catalog number: 35050038), store at 2-8 °C
6. 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer solution, 1 M (Thermo Fisher Scientific, Gibco, catalog number: 15630056), store at 2-8 °C
7. Normocin (InvivoGen, catalog number: ant-nr-2), store at -20 °C
8. B-27 supplement, minus vitamin A, 50x (Thermo Fisher Scientific, Gibco, catalog number: 12587010), store at -20 °C aliquoted
9. N-2 supplement, 100x (Thermo Fisher Scientific, Gibco, catalog number: 17502048), store at -20 °C aliquoted
10. N-Acetyl-L-cysteine (Sigma-Aldrich, catalog number: A9165), store at -20 °C aliquoted
11. Recombinant murine Epidermal growth factor (EGF) (Thermo Fisher Scientific, Gibco, catalog number: PMG8043), store at -20 °C aliquoted
12. Recombinant human R-spondin 1 (R&D Systems, catalog number: 4645-RS-250), store at -20 °C aliquoted
13. Recombinant murine Noggin (Peprotech, catalog number: 250-38), store at -20 °C aliquoted
14. Y-27632 Dichloride, powder (Sigma-Aldrich, catalog number: Y0503, reconstitute at 1 mM in sterile Milli-Q and store at -20 °C aliquoted)
15. CHIR99021 (Stemgent, catalog number: 04-0004), store at -20 °C aliquoted
16. Valproic acid sodium salt (Sigma-Aldrich, catalog number: P4543, reconstitute at 1 M in sterile

Milli-Q and store at -20 aliquoted)

17. TrypLE Express enzyme, with phenol red, 1x (Thermo Fisher Scientific, Gibco, catalog number: 12605010), store at 2-8 °C
18. Fetal bovine serum (FBS), heat inactivated (Thermo Fisher Scientific, Gibco, catalog number: 10500064), store at -20 °C
19. DMEM, high glucose, GlutaMAX supplement, pyruvate (Thermo Fisher Scientific, Gibco, catalog number: 31966021), store at 2-8 °C
20. Penicillin-streptomycin, with 10,000 units penicillin and 10 mg streptomycin per ml in 0.9% NaCl (Sigma-Aldrich, catalog number: P0781), store at -20 °C
21. Minimum essential medium non-essential amino acid (MEM-NEA) solution, 100x (Thermo Fisher Scientific, Gibco, catalog number: 11140035), store at 2-8 °C
22. Collagenase (Sigma-Aldrich, catalog number: C0130), store at -20 °C
23. ACK Lysing Buffer (Gibco, catalog number: A10492-01), store at RT
24. Ethanol, 96% v/v (PanReac Applichem, catalog number: 131085), store at RT
25. Intestinal organoid growth medium or ENR_CV medium (see Recipes section, Table 1)
26. Intestinal subepithelial myofibroblast digestion medium (see Recipes section, Table 2)
27. Intestinal subepithelial myofibroblast culture medium (see Recipes section, Table 3)
28. Boosting medium (see Recipes section, Table 4)
29. Basic-medium (see Recipes section, Table 5)
30. 70% v/v Ethanol (see Recipes section, Table 6)

Equipment

1. Surgical tweezers (Biolab, catalog numbers: 711198 and 711199)
2. Surgical scalpel (Paramount, catalog number: PCS23)
3. Surgical scissors (Biolab, catalog number: 005064)
4. 37 °C water bath (Fisher Scientific, Polytest 20)
5. Milli-Q water purification system (Millipore, Q-POD)
6. Neubauer counting chamber (BRAND, catalog number: 718605)
7. Micropipettes (Gilson, models: P20, P200, P1000)
8. Pipette aid (Eppendorf, Easypet)
9. Cell culture benchtop centrifuge (Beckman Coulter, model: Allegra X-12R)
10. 37 °C humidified incubator with 5% CO₂ (Thermo Scientific, Steri-Cult 3141)
11. Laminar flow hood (Telstar, BIO II A)
12. Platform Shaker (Heidolph, model: Unimax 1010)
13. Incubated benchtop orbital shaker (Thermo Scientific, 4518 S/N 15150-131)
14. Bright field microscope (Nikon, Eclipse Ts2)
15. Epithelial voltohmmeter with STX3 electrodes (World Precision Instruments, EVOM2)
16. Ice machine (Scotsman, MF56)

Procedure

A. Intestinal crypt isolation and culture (Figure 1A)

Note: Perform until Step A8 on the bench and from then onwards you might continue the protocol under a laminar flow hood to favor sterility.

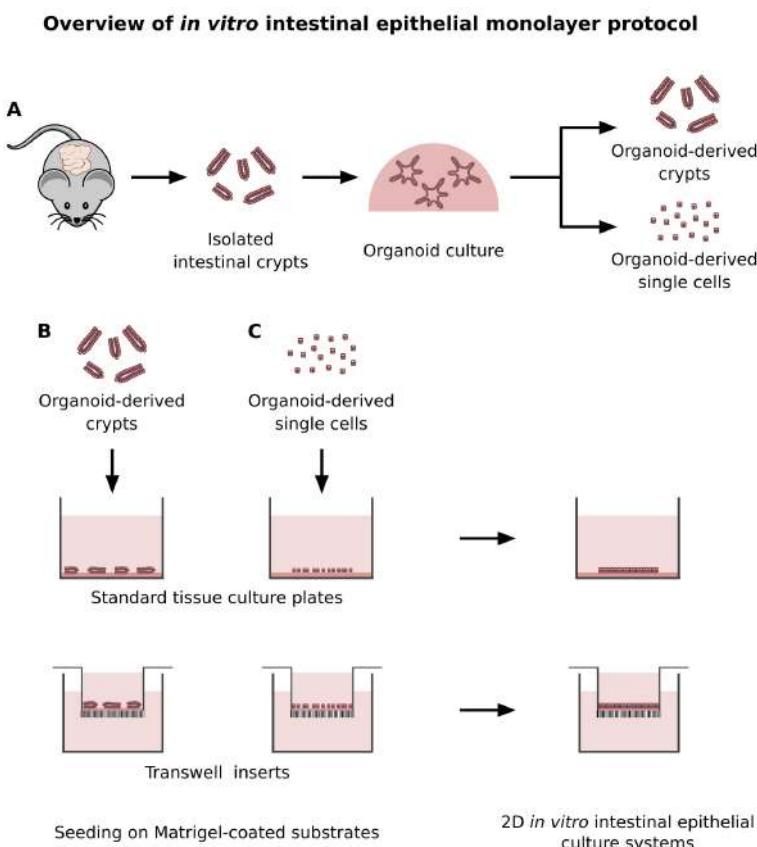


Figure 1. Overview of the developed methodology for the generation of *in vitro* intestinal epithelial monolayer. A. Intestinal crypts are isolated from *Lgr5-EGFP-IRES-creERT2* mouse and grown in Matrigel drops to obtain the organoid culture. Organoids are dissociated into B. crypt pieces or C. single cells and seeded on Matrigel coated standard tissue culture plates or Transwell inserts to obtain a 2D *in vitro* intestinal epithelial culture system.

1. Harvest the small intestine from *Lgr5-EGFP-IRES-creERT2* mouse.
2. Place the intestine into a Petri dish with PBS and remove the fat and mesentery from the tissue using surgical tweezers and a scalpel or surgical scissors.
Note: Be careful not to rupture the intestinal tissue.
3. Flush the mouse small intestine with PBS using a 10 ml syringe and a 23 G 1" needle.
4. Cut up the tissue longitudinally using surgical scissors.
Note: Make sure that when you cut open the tissue the mucosal side is facing upwards.
5. Remove the villi mechanically by scraping carefully the intestinal mucosa with a glass coverslip.

Note: Be careful not to apply too much pressure to preserve the crypts.

6. Rinse 3 times with PBS and place the intestine into a new Petri dish.
7. Cut the tissue into pieces of 1-4 mm thick using surgical scissors (see Figure 2).

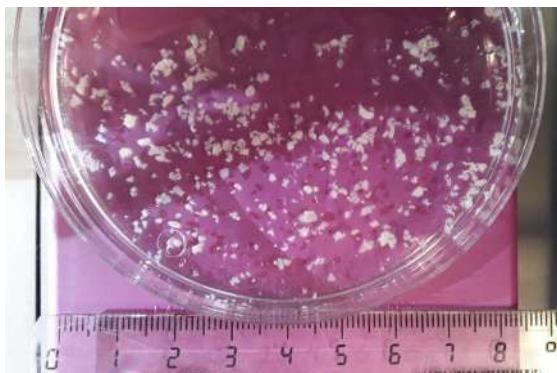


Figure 2. Picture showing the appearance (size and shape) of the tissue pieces obtained after cutting the intestine using surgical scissors.

8. Transfer the pieces into a sterile 50 ml Falcon tube under the laminar flow hood.
9. Add 10 ml ice-cold PBS and pipette up and down 5-6 times using a 10 ml pipette. Let the pieces settle and discard the supernatant.

Note: It is very important that the PBS is ice cold and that the inner pipettes walls are previously wetted with PBS. If not, the tissue pieces might stick to the pipette.

10. Add fresh 10 ml ice-cold PBS and repeat the Step A9 for 10-20 times until the supernatant becomes almost clear.

Note: The supernatant might still be a bit cloudy after 20 washes, proceed with the next step anyways.

11. Add 25 ml of 2 mM EDTA prepared in cold PBS and incubate it for 30 min at 4 °C. Agitate vigorously the Falcon tube during incubation on a platform shaker.

12. After the incubation, let the tissue pieces settle to the bottom of the Falcon tube and discard the supernatant.

13. Add 10 ml of ice-cold PBS with 10% FBS to the tissue pieces using 10 ml pipette. It is recommended to wet the inner wall of the pipette with ice cold-PBS with 10% FBS to avoid tissue pieces sticking to the pipette.

14. Pipette up and down 3-5 times the tissue pieces using a 10 ml pipette. Let the pieces to settle down and filter the supernatant through a 70 µm pore cell strainer placed on top of a new 50 ml Falcon tube. The eluted solution will correspond to the first crypt elution fraction.

15. Repeat the Steps A13 and A14 for 4-5 times more to obtain the following crypt elution fractions. Use new 70 µm pore cell strainers and new 50 ml Falcon tubes to collect the subsequent crypt elution fractions.

16. Pipette 5 µl of each of the eluted fractions in a Petri dish and analyze them using a bright field microscope, to determine the purity and the size of the crypts in each fraction.

Note: Usually, the first fractions contain a lot of debris and some villi pieces. It is advisable to discard these fractions and keep the rest, which mainly contain small crypt pieces (Figure 3).

17. Pool the selected fractions in a 50 ml Falcon tube and centrifuge the suspension at 55 x g for 5 min at 8 °C.

Note: In the case reported in Figure 3, we would discard fraction 1 and pool fractions 2, 3 and 4.

18. Resuspend the pellet containing the crypts in pre-thawed Matrigel by gently pipetting 2-3 times using a P1000 micropipette and cold 1,000 µl pipette tips. The resuspension volume will depend on the number of eluted fractions pooled and therefore the size of the obtained pellet. For example, in the case reported in Figure 3, in which we have pooled fractions 2, 3 and 4 (Figure 3A) and obtained the pellet of cells shown in Figure 3B we would use 500 µl of Matrigel to resuspend the pellet.

Note: Thaw an aliquot (1 ml) of Matrigel on ice at least 1 h before using it. At this step of the protocol you must work always on ice to prevent Matrigel from solidifying.

19. Pipette carefully a bubble-free 40 µl drop of crypt-Matrigel suspension per well in a pre-warmed 24-well plate. Usually you will obtain around 15 drops. Place the plate into a 37 °C incubator for 5-10 min to let the Matrigel solidify.

Note: Place the 24-well plate into the incubator at 37 °C for 10 min prior to seeding. This helps the drops to form properly.

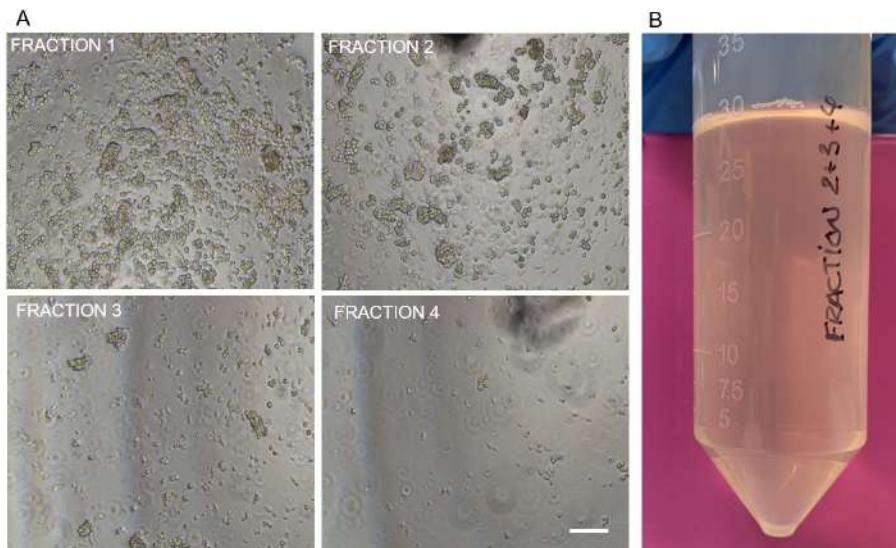


Figure 3. Elution process of the different crypt fractions. A. Bright field microscopy pictures showing 4 different crypt elution fractions of a given intestinal crypt isolation. Scale bar: 100 µm. B. Image showing the pellet of cells obtained from pooling crypt eluted fractions 2 + 3 + 4 of the isolation shown in Figure 3A.

20. Add 400 µl of the pre-warmed intestinal organoid growth medium (Table 1) into each well after Matrigel solidifies and transfer them back to the 37 °C humidified incubator with 5% CO₂.

21. Add extra ENR-CV factors (same concentration as in Table 1) every other day, in the current culture medium, during the first 4-5 days of the culture and Rho kinase inhibitor (Y-27632) (10 μ M) every day for the first 4 days to avoid epithelial cells death by anoikis. Change the whole medium every 2-3 days. Alternatively, add 100 μ l fresh medium every other day until passing of the organoids.

Note: Typically, crypts will start budding after 2-3 days in culture (Figure 4). Passage outgrow crypts after one week in culture. In case crypt confluence is very high, an earlier passage of the budding crypts will be required for a proper organoid formation. Typically, in the original drops we obtain around 100-125 organoids per drop.

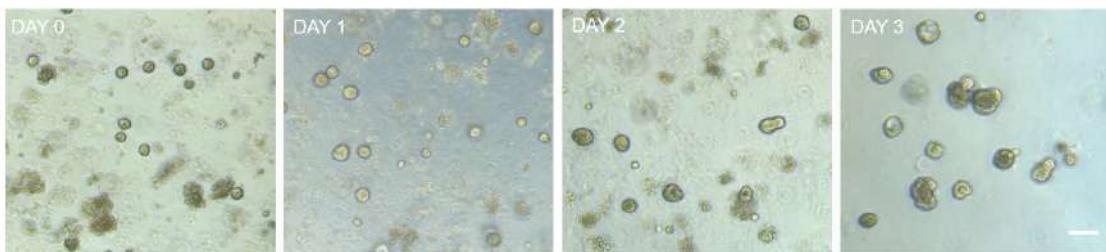


Figure 4. Bright field microscopy images showing the growth and budding of freshly isolated intestinal crypt over time (up to 3 days). Notice that intestinal crypts start to bud 2-3 days in culture after isolation. Scale bar: 100 μ m.

B. Passaging crypts after organoid formation

1. Thaw Matrigel at least 1 h before the experiment by leaving the aliquots on ice.
2. Prepare and pre-warm the organoid growth medium (Table 1) and 24-well plates at 37 °C.
3. Remove the organoid growth media from the wells containing full-grown organoids (see Figure 5, left panels) add 300 μ l TrypLE Express 1x to each well and gently detach and disrupt the Matrigel drops by scraping it off using a P1000 micropipette. This step is performed at RT.

Notes:

- a. You can use 300 μ l TrypLE Express 1x volume to recover organoids from 2 drops.
- b. TrypLE Express 1x is used in this step to obtain crypts with more homogeneous size and favor Matrigel digestion.

4. Transfer the Matrigel suspension to a 15 ml Falcon tube already placed on ice.
5. Pass the suspension through a 1 ml syringe with a 23 G 1" needle 3-4 times to further mechanically disrupt the organoids. This step is performed on ice.

Note: The number of times you pass the sample through the syringe and the force you apply will depend on the size of the full-grown organoids you intend to break. Be aware that a too gentle procedure would not break the organoids and too harsh will over-digest the crypts.

6. Add 1 ml of 2% FBS in PBS for every 300 μ l of suspension and agitate manually the tube to mix well the content.

Note: This step might be repeated in case a further wash is required.

7. Centrifuge at 72 x g for 3 min at 8 °C.

8. Remove all the supernatant and resuspend the pellet in fresh Matrigel by gently pipetting using a micropipette. Resuspension volume will depend on the number of initial drops digested.

Note: Splitting ratio is usually 1:4-1:5.

9. Plate the Matrigel containing crypts drops (40 μ l/drop) in pre-warmed 24-well plates as explained before.

10. Polymerize the Matrigel by incubating at 37 °C for 5-10 min.

11. Add 400 μ l of the intestinal organoid growth medium (Table 1) to each well and transfer the plate back to a 37 °C humidified incubator with 5% CO₂.

Note: See Figure 5 (middle panels) for a representative bright field microscope image of freshly dissociated intestinal organoid-derived crypts.

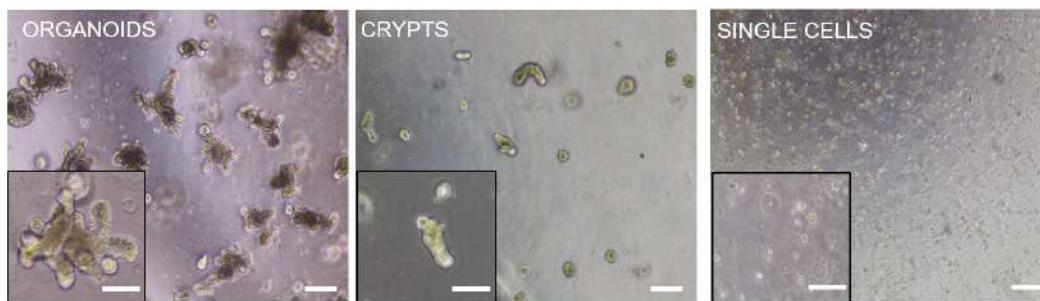


Figure 5. Representative bright field microscope images of full-grown intestinal organoids (left panels), freshly dissociated intestinal organoids-derived crypts (middle panels) and freshly digested intestinal organoids-derived single cells (right panels), grown in Matrigel drops. Insets show a magnification of the corresponding images. Please notice that due to the 3D culture not all cellular structures are in the same focus. Scale bars: 100 μ m, 50 μ m (inset).

C. Preparation of Matrigel-coated substrates

1. Place the ibidi 8-well μ -Slides or Transwell inserts and P20 micropipette tips into the freezer beforehand.

Note: This step is especially important for obtaining homogeneous Matrigel coating.

2. Thaw Matrigel at least 1 h before the experiment by leaving the aliquots on ice.
3. Place the pre-cooled ibidi plate on ice. Pipette 10 μ l of Matrigel using a P20 micropipette and pre-cooled micropipettes in each of the ibidi plate wells or in each of the Transwell insert. Make sure that the 10 μ l of Matrigel are uniformly spread throughout the substrate surface by moving the pipette tip while releasing the Matrigel (see Video 1).

Note: Remove the excess Matrigel that accumulates at the Transwell insert border.



Video 1. Preparation of Matrigel-coated substrates

4. Repeat the Step C3 for all the wells and inserts required.

Note: Perform Steps C3 and C4 as fast as possible for a homogeneous coating.

5. Incubate the plates or inserts at 37 °C for 1h in the incubator to polymerize the Matrigel.
6. Continue with the seeding (see following Procedure D).

D. Generation of organoid-derived intestinal epithelial monolayers

1. Intestinal organoid-derived crypt seeding (Figure 1B)

- a. Digest organoids to obtain crypts with homogeneous sizes as described in Steps B1-B6.

Note: See Figure 5 (middle panels) for a representative bright field microscope image of freshly dissociated intestinal organoid-derived crypts.

- b. Mix well the Falcon containing the digested organoid suspension, take 3 times 5 µl samples using a P20 micropipette and place them into a Petri dish.
- c. Count and average the number of crypt pieces within the 5 µl volume using a bright field microscope and from that value estimate the total number of crypt pieces in the suspension.
- d. Centrifuge to collect the crypt pieces at 72 x g for 3 min at 4 °C.
- e. Resuspend the pellet in intestinal organoid growth medium (Recipe 1). Calculate the total medium volume needed for a seeding density of 1,500 crypts/cm².

Notes:

- i. *The surface area of one well of ibidi 8-well µ-Slides is 1 cm². Then, the seeding density is 1,500 crypts/sample.*
- ii. *For reference purposes, from one full-grown organoids Matrigel drop (Figure 5, left panels) we obtained approximately 1,000-1,500 crypts.*
- f. Pipette 30-50 µl of crypt suspension into each well of the Matrigel-coated ibidi 8-well µ-Slides (see Procedure C). Try to place the sample as a drop in the center of the well.
- g. Let the crypts attach to the substrate for 3-4 h and add 200 µl more of intestinal organoid medium (Table 1).

- h. Change the medium every other day.
 - i. Inspect the monolayer formation and growth throughout the culture time using a bright field microscope. After seeding, organoid-derived crypts will rapidly refold on themselves to form crypt-like domains containing Lgr5⁺ intestinal stem cells (GFP⁺). Then, cells spread on the substrate and migrate out of the crypt-like domains, forming epithelial monolayers composed on GFP⁻ cells which correspond to the villus-like regions (Figure 6A).
2. Intestinal organoid-derived single cell seeding (Figure 1C)
- a. Follow the organoids digestion protocol as described in Steps B1-B4.
 - b. Pass the suspension through 1 ml syringe with a 23 G 1" needle 5-6 times followed by a 5 min incubation at 37 °C. Agitate the cell suspension every minute to disrupt well the cell aggregates. After 5 min of incubation, place the sample on ice, pipette 5 µl of the digestion on a Petri dish and inspect the sample under the microscope. If cell aggregates are still observed increase the incubation time (37 °C) for some more min (up to a total of 8 min).
Note: See Figure 5 (right panels) for a representative bright field microscope image of freshly digested intestinal organoid-derived single cells.
 - c. Once single cell suspension is achieved, place the tube on ice and add 1 ml of PBS with 2% FBS for every 300 µl of suspension and agitate manually the tube to mix well the content.
Note: This step might be repeated in case a further wash is required.
 - d. Count the number of cells in the suspension using a Neubauer counting chamber.
 - e. Centrifuge at 110 x g for 4 min at 4 °C.
 - f. Resuspend the pellet in organoid growth medium (Table 1). Calculate the total medium volume needed for a seeding density of 10⁵ cells/cm².
- Notes:*
- i. *The surface area of one well of ibidi 8-well µ-Slides is 1 cm². Then, the seeding density is 10⁵ cells/sample.*
 - ii. *For reference purposes, from one full-grown organoids Matrigel drop (Figure 5, left panels) we obtained approximately 1.5 x 10⁵-1.7 x 10⁵ single cells.*
- g. Seed the Matrigel-coated ibidi 8-well µ-Slides (see Procedure C) with 10-20 µl of single cell suspension.
Note: Place the single cell suspension drop into the center of the well and be careful to not to disrupt it.
- h. Let the cells attach for 1 h, add 200 µl more of intestinal organoid medium (Table 1) plus Rho kinase inhibitor Y-27632 at 10 µM final concentration.
 - i. Change the medium every other day.
 - j. Inspect the monolayer formation and growth using a bright field microscope. After seeding, organoid-spontaneously self-assemble to form crypt-like domains containing Lgr5⁺ ISCs, closely mimicking the *in vivo* arrangement at the crypt bases. Then, cells start to spread out generating epithelial monolayers composed of GFP⁻ giving rise to the villus-like regions (Figure 6B).

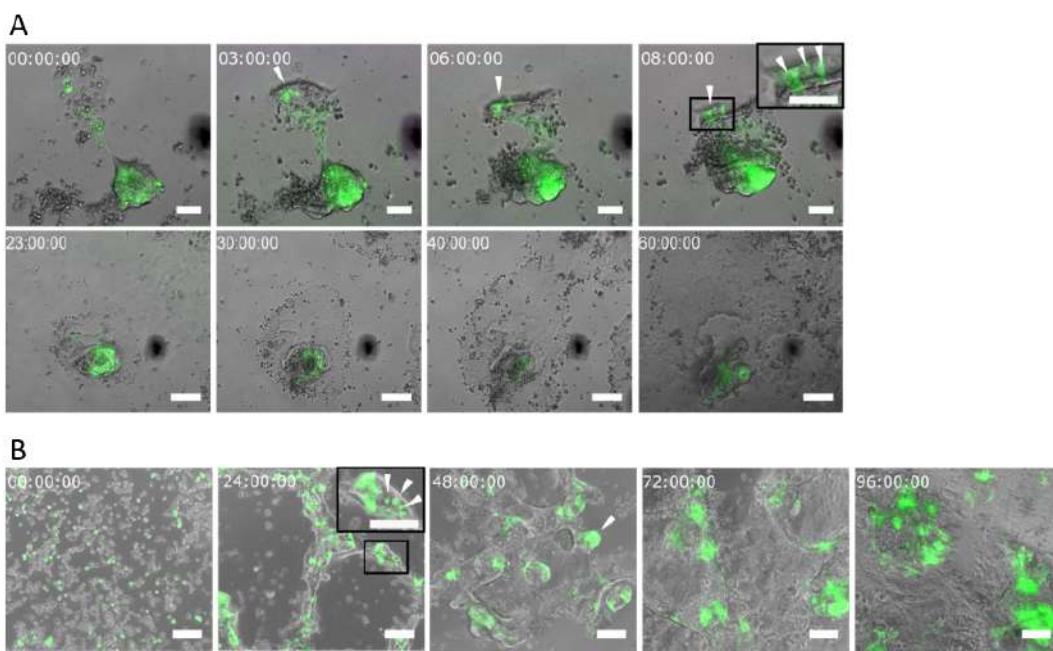


Figure 6. Stem cells self-assemble in crypt-like domains and originate epithelial monolayers. (A) Live-imaging sequence of overlapped bright field and GFP signal corresponding to 60 hours after seeding organoid-derived crypts and (B) 96 hours after seeding organoid-derived single cells on a thin film of Matrigel. The corresponding time for each snapshot is shown in each panel. White arrow heads indicate Lgr5-GFP⁺ cells. Scale bars: 50 µm (A, upper row) and 100 µm (A, lower row, and B). Figure adapted from the original publication (Altay *et al.*, 2019, under Creative Commons Attribution 4.0 International License, <http://creativecommons.org/licenses/by/4.0/>).

E. Intestinal subepithelial myofibroblast isolation and culture

1. Collect the tissue left from the crypt digestion and elution described in Step A15.
2. Add 10 ml of 3 mM EDTA in PBS and incubate for 10 min at 37 °C and 200 rpm in an incubated benchtop orbital shaker.
Note: Pre-warm the EDTA solution to 37 °C.
3. After incubation, let the pieces settle down and discard the supernatant. Repeat Step E2 two more times.
4. Discard the supernatant and rinse the pellet with 20 ml of PBS. Repeat this step 2 times more.
Note: This step is important to remove all EDTA.
5. Discard the supernatant and add intestinal epithelial myofibroblast digestion medium (Table 2).
6. Incubate for 40 min in an incubated benchtop orbital shaker at 37 °C and 200 rpm.
7. After the incubation, pipette the suspension up and down during 1 min using 10 ml pipette.
8. Centrifuge at 160 x g for 5 min.
9. Resuspend the pellet with 10 ml of ACK lysing Buffer (pre-warmed at 47 °C).
10. Centrifuge at 160 x g for 5 min.
11. Resuspend the pellet with 10 ml intestinal epithelial myofibroblast culture medium (Table 3) and

culture in 25 cm² cell culture flask.

Note: Tissue pieces will eventually attach to the flask and myofibroblasts will grow out and spread though the flask. Replace the medium every 3-4 days. Usually, 2-3 weeks after isolation flask will be confluent. Figure 7 shows representative bright field microscopy images of this process.

12. Cell are passaged by normal trypsinization for 5 min. Splitting ration 1:3.

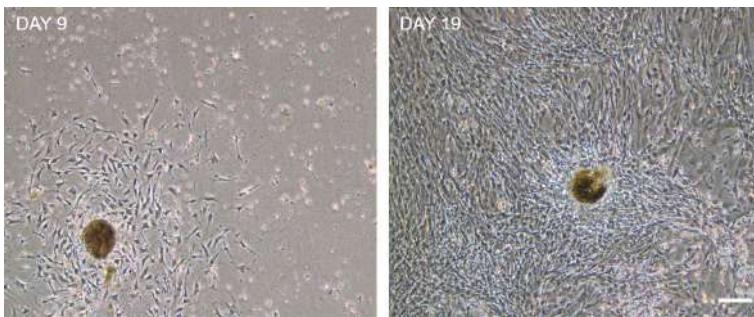


Figure 7. Representative bright field microscopy images of ISEMF growth on tissue culture flasks at day 9 (left panel) and day 19 (right panel) after seeding. Notice that digested tissue pieces attach to the plastic and emerging fibroblast spread through the flask until reaching confluence. This process might take 21 days. Scale bar: 200 μm.

F. Intestinal subepithelial myofibroblast conditioned medium (ISEMF_CM) production

1. Grow 60-80% confluent ISEMF cultures for 4-6 days without changing the medium (Figure 8).
2. Harvest the medium and centrifuge it at 160 x g for 5 min to remove debris.

Note: Add fresh intestinal epithelial myofibroblast culture medium (Table 3) to the plates and continue the culture. Passage the cells if required (Step E12).

3. Collect the supernatant and filter it using a 0.22 μm pore size filter and a 10 ml syringe.
4. Store the filtered ISEMF_CM at -80 °C until use.
5. Repeat Steps F1-F4 until cells become senescent (usually after 5-6 passages).
6. Pool all ISEMF_CM fraction collected from the same ISEMF isolation and prepare the boosting medium as described in Table 4.

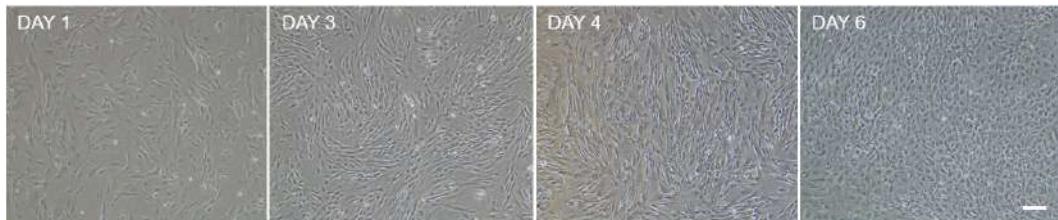


Figure 8. Bright field microscopy images showing the growth of ISEMF during 6 days after splitting. ISEMF_CM is collected during this period when ISEMF confluence is around 60 to 80%.

G. Evaluating the epithelial monolayer formation and barrier integrity with transepithelial electrical resistance (TEER) measurements

1. Prepare Matrigel-coated Transwell inserts as explained in Procedure C.
2. Follow the steps described in Step D1a until Step D1d to dissociate the organoids to crypts.
3. Resuspend the pellet in basic-medium (Table 5). Calculate the total medium volume needed for a seeding density of 3,000-3,500 crypts/cm².

Notes:

- a. *The surface area of one 6.5 mm diameter Transwell insert is 0.33 cm².*
- b. *For reference purposes, from one full-grown organoids Matrigel drop (Figure 5, left panels) we obtained approximately 1,000-1,500 crypts.*

4. Seed each of the Matrigel-coated Transwell inserts with 30-50 µl of crypt suspension.

Note: Leave 2-3 Matrigel-coated Transwell inserts without seeding as controls for TEER measurements.

5. Let the crypts attach for 1-2 h and add 200 µl more of basic-medium (Table 5) at the apical (upper) side of the Transwell.

6. Prepare the boosting medium (Table 4) and add 500 µl to the basolateral (bottom) side of the Transwell.

7. Add the corresponding medium to the control (no cells) inserts as well.

8. Place the plate in a 37 °C humidified incubator with 5% CO₂. Change the medium of both apical and basolateral sides every other day.

9. After changing medium, let the plate under the hood at RT for 5-10 min so that the medium temperature equilibrates.

10. In the meanwhile, sterilize the EVOM2 epithelial voltohmmeter STX3 electrodes by submerging them into 70% Ethanol (Table 6) for 15 min.

11. Rinse the electrodes with PBS and keep them in the solution until starting the measurements.

12. Turn on the voltohmmeter, plug the electrodes and introduce one to the apical and the other to the basolateral side of the Transwell. Record the value measured.

13. Remove the electrodes and reinsert to measure again. Repeat this step one more time to obtain 3 different measurements from the same sample.

14. Wash the electrodes in between measurements by submerging into PBS and then into basic medium.

15. After finishing the measurements, sterilize the electrodes by submerging them into 70% Ethanol for 15 min. Rinse them with sterile Milli-Q, let them air-dry under the cell culture hood and store.

16. Measure the TEER of the monolayers every 2 days, up to 20 days of culture.

17. Calculate the electrical resistance corresponding to epithelial monolayers by subtracting the intrinsic resistance (values measured on control samples without cells) from the total resistance measured (samples with the cells) and corrected for surface area (0.33 cm²) expressed as ohm·cm².

Note: For reference purposes, TEER values progressively increase up to approximately 60 ohms·cm² after 20 days in culture.

Data analysis

For statistical comparison, we used two-tailed, unequal variance t-test and we considered $P < 0.05$ to be significant. We repeated monolayer formation protocol in at least three independent experiments. We performed three independent experiments with two technical replicas per condition per experiment for TEER measurements. The detailed explanation of data analysis performed, can be found in the Materials and Methods, and Supplementary Information sections of the original article (Altay *et al.*, 2019).

Recipes

1. Intestinal organoid growth medium or ENR_CV medium

Table 1. Component list for the formulation of the intestinal organoid growth medium or ENR_CV medium. Stock and final concentration are indicated, as well as the solvent employed (when required). Volumes for each of the components (at stock concentration) for a final volume of 20 ml of medium are shown.

Reagent	Stock concentration	Stock solvent	For 20 ml final volume (use immediately)	Final concentration
Glutamax	100x		200 µl	1% v/v
HEPES	1 M		200 µl	1% v/v (10 mM)
Normocin	50 mg/ml		40 µl	100 µg/ml
B-27	50x		400 µl	2% v/v
N-2	100x		200 µl	1% v/v
N-Acetyl-L-cysteine	500 mM	Milli-Q	50 µl	1.25 mM
Advanced DMEM/F12			18,910 µl	
EGF	100 µg/ml	PBS	20 µl	100 ng/ml
Noggin	100 µg/ml	PBS	20 µl	100 ng/ml
R-Spondin 1	100 µg/ml	PBS	40 µl	200 ng/ml
CHIR99021	10 mM	DMSO	6 µl	3 µM
Valproic acid	1 M	Milli-Q	20 µl	1 mM

If not prepared fresh, this medium (without adding the ENR_CV) can be stored at 2-8 °C up to 2 weeks. ENR_CV must be added just before use.

2. Intestinal subepithelial myofibroblast digestion medium

Table 2. Component list for the formulation of the intestinal subepithelial myofibroblast digestion medium. Stock and final concentration are indicated, as well as the solvent employed (when required). Volumes for each of the components (at stock concentration) for a final volume of 20 ml of medium are shown.

Reagent	Stock concentration	Stock solvent	For 20 ml final volume (use immediately)	Final concentration
FBS			4,000 µl	20% v/v
Collagenase	100 mg/ml	PBS	75 µl	0.37 mg/ml (100 U/ml)
DMEM			16,000 µl	

Add the enzyme just before using the medium.

3. Intestinal subepithelial myofibroblast culture medium

Table 3. Component list for the formulation of the intestinal subepithelial myofibroblast culture medium. Stock and final concentration are indicated, as well as the solvent employed (when required). Volumes for each of the components (at stock concentration) for a final volume of 20 ml of medium are shown.

Reagent	Stock concentration	For 20 ml final volume (store at 2-8 °C up to 2 weeks)	Final concentration
FBS		2,000 µl	10% v/v
Penicillin-streptomycin	100x	200 µl	1% v/v
MEM-NEA	100x	200 µl	1% v/v
Normocin	50 mg/ml	40 µl	100 µg/ml
DMEM		17,560 µl	

4. Boosting medium

Table 4. Components list for the formulation of the boosting medium. Stock and final concentration are indicated, as well as the solvent employed (when required). Volumes for each of the components (at stock concentration) for a final volume of 20 ml of medium are shown.

Reagent	Stock concentration	Stock solvent	For 20 ml final volume (use immediately)	Final concentration
Glutamax	100x		200 µl	1% v/v
HEPES	1 M		200 µl	1% v/v (10 mM)
Normocin	50 mg/ml		40 µl	100 µg/ml
B-27	50x		400 µl	2% v/v
N-2	100x		200 µl	1% v/v
N-Acetyl-L-cysteine	500 mM	Milli-Q	50 µl	1.25 mM
ISEMF_CM			18,910 µl	
EGF	100 µg/ml	PBS	20 µl	100 ng/ml
Noggin	100 µg/ml	PBS	20 µl	100 ng/ml
R-Spondin 1	100 µg/ml	PBS	40 µl	200 ng/ml
CHIR99021	10 mM	DMSO	6 µl	3 µM
Valproic acid	1 M	Milli-Q	20 µl	1 mM
Wnt3a	100 µg/ml	Milli-Q	5 µl	25 ng/ml

If not prepared fresh, this medium (without adding the ENR_CV and Wnt3a factor) can be stored at 2-8 °C up to 2 weeks. ENR_CV and Wnt3a factor must be added just before used.

5. Basic-medium

Table 5. Components list for the formulation of the basic-medium. Stock and final concentration are indicated, as well as the solvent employed (when required). Volumes for each of the components (at stock concentration) for a final volume of 20 ml of medium are shown.

Reagent	Stock concentration	Stock solvent	For 20 ml final volume (store at 2-8 °C up to 2 weeks)	Final concentration
Glutamax	100x		200 µl	1% v/v
HEPES	1 M		200 µl	1% v/v (10 mM)
Normocin	50 mg/ml		40 µl	100 µg/ml
B-27	50x		400 µl	2% v/v
N-2	100x		200 µl	1% v/v
N-Acetyl-L-cysteine	500 mM	Milli-Q	50 µl	1.25 mM
Advanced DMEM/F12			18,910 µl	

6. 70% v/v Ethanol

Table 6. Components list for the preparation of 70% v/v Ethanol. Volumes for each of the components (at stock concentration) for a final volume of 1 L are shown.

Reagent	Stock concentration	For 1 L final volume (store at RT)	Final concentration
Ethanol	96% v/v	230 ml	70% v/v
Milli-Q		770 ml	

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the staff from the IBEC communication department for their assistance in the video recording and editing.

Competing interests

The authors declare no financial or non-financial competing interests.

Ethics

All experimental protocols involving mice were approved by the Animal care and Use Committee of Barcelona Science Park (CEEA-PCB) and the Catalan government and performed in accordance to their relevant guidelines and regulations. Approval ID of the experimentation project: 9162, validity period: 12/2018-12/2023.

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Mesenchymal Stromal Cells Derived from Bone Marrow and Adipose Tissue: Isolation, Culture, Characterization and Differentiation

Gileade P. Freitas¹, Alann T. P. Souza¹, Helena B. Lopes¹, Rayana L. B. Trevisan¹, Fabiola S. Oliveira¹, Roger R. Fernandes¹, Fernanda U. Ferreira², Felipe A. Ros², Marcio M. Belotti¹ and Adalberto L. Rosa^{1,*}

¹Bone Research Lab, School of Dentistry of Ribeirão Preto, University of São Paulo, Ribeirão Preto, SP, Brazil; ²Hemotherapy Center of Ribeirão Preto, University of São Paulo, Ribeirão Preto, SP, Brazil

*For correspondence: adalrosa@fcrp.usp.br

[Abstract] Since their discovery, mesenchymal stromal cells (MSCs) have received a lot of attention, mainly due to their self-renewal potential and multilineage differentiation capacity. For these reasons, MSCs are a useful tool in cell biology and regenerative medicine. In this article, we describe protocols to isolate MSCs from bone marrow (BM-MSCs) and adipose tissues (AT-MSCs), and methods to culture, characterize, and differentiate MSCs into osteoblasts, adipocytes, and chondrocytes. After the harvesting of cells from bone marrow by flushing the femoral diaphysis and enzymatic digestion of abdominal and inguinal adipose tissues, MSCs are selected by their adherence to the plastic tissue culture dish. Within 7 days, MSCs reach 70% confluence and are ready to be used in subsequent experiments. The protocols described here are easy to perform, cost-efficient, require minimal time, and yield a cell population rich in MSCs.

Keywords: Adipose tissue, Adipocyte, Bone, Bone marrow, Cell culture, Chondrocyte, Mesenchymal Stromal cell, Osteoblast

[Background] The concept of stem cells dates back to the 19th century, but their existence was confirmed in the 1960s and 1970s following experiments by Friedenstein and collaborators, which showed the presence of stem cells in the bone marrow (Friedenstein, 1970; Bianco *et al.*, 2008). Afterward, Caplan (1991) named them as mesenchymal stem cells (here, called mesenchymal stromal cells—MSCs) and proposed their use in regenerative medicine. In the bone marrow, the percentage of MSCs is estimated to be 0.001 to 0.01% of the total mononuclear cells. Because of their scarcity, alternative sources have been described, although bone marrow remains as the main source of MSCs (Nancarrow-Lei *et al.*, 2017). Adipose tissue is a very promising source because it contains a large number of MSCs that are relatively easy to harvest with minimal discomfort and risk for donors (Zuk *et al.*, 2001). The protocols used to harvest and culture MSCs from either bone marrow (BM-MSCs) or adipose tissue (AT-MSCs), may vary among different species or even among different strains of the same species. The most commonly used methods for obtaining MCSs involve using flow cytometry (Schrepfer *et al.*, 2007), multipotent adult progenitor cell media (Harting *et al.*, 2008), the ficoll-paque gradient centrifugation method (Pierini *et al.*, 2012), and immunomagnetic beads (Wadajkar *et al.*, 2014). Here, we describe cost-efficient protocols that are relatively easy and fast to perform and can be used

to obtain cell populations rich in MSCs from bone marrow and adipose tissues. These protocols can be used to study several cellular and molecular aspects of MSCs, such as their proliferation, differentiation, and signaling pathways (Abuna *et al.*, 2016; Fideles *et al.*, 2019), the biological effects of growth factors and drugs on MSCs (Oliveira *et al.*, 2012; Zhang *et al.*, 2017), the interactions between MSCs and natural or synthetic biomaterials (Hu *et al.*, 2018; Lopes *et al.*, 2019), and the application of MSCs in regenerative medicine strategies (Almeida *et al.*, 2019; Freitas *et al.*, 2019).

Materials and Reagents

1. Sterile surgical drape
2. Aluminum foil
3. Coat (ProtDesc, catalog number: 80404440020), storage temperature: RT
4. Mask (ProtDesc, catalog number: 80404440006), storage temperature: RT
5. Cap (ProtDesc, catalog number: 80404440004), storage temperature: RT
6. Gloves (Maxitec, Kevenol, catalog number: 80748910002), storage temperature: RT
7. 20-ml syringe (BD Plastipak, catalog number: 990687), storage temperature: RT
8. 21G needle (BD PrecisionGlide, catalog number: 300054), storage temperature: RT
9. Glass tissue culture dish (Pyrex, catalog number: HX0004-00376), storage temperature: RT
10. Corning® 75 cm², U-Shaped canted neck cell culture flask with vent cap (Corning, catalog number: 430641U), storage temperature: 15/30 °C
11. 24-well cell culture plates (Corning, catalog number: 3524), storage temperature: 15/30 °C
12. 12-well plates (Corning, catalog number: 3512), storage temperature: 15/30 °C
13. 6-well culture plates (Corning, catalog number: 3335), storage temperature: 15/30 °C
14. 50-ml conical tube (Sarstedt, catalog number: 62.547.254), storage temperature: 15/30 °C
15. Microtube 1.5-ml (Eppendorf, catalog number: Z606340), storage temperature: RT
16. Micropipette tips (Eppendorf, catalog numbers: 0030000811/0030000854/0030000870/0030000919), storage temperature: RT
17. Ultra-low attachment, 96-well (Costar, catalog number: CLS7007), storage temperature: 15/30 °C
18. Alpha minimum essential medium (α-MEM) (Thermo Fisher Scientific, catalog number: 12000-022), storage temperature: 2/8 °C
19. Dulbecco's modified Eagle's medium (D-MEM) (Thermo Fisher Scientific, catalog number: 12100-046), storage temperature: 2/8 °C
20. Dulbecco's phosphate-buffered saline (PBS) (Thermo Fisher Scientific, catalog number: 21600-010), storage temperature: 15/30 °C
21. Sodium bicarbonate (Sigma-Aldrich, Sigma, catalog number: S5761-1KG), storage temperature: 15/30 °C
22. Gentamycin reagent solution (Thermo Fisher Scientific, catalog number: 15710-064), storage temperature: -20/-5 °C

23. Penicillin-Streptomycin (Thermo Fisher Scientific, catalog number: 15140-122), storage temperature: 15/30 °C
24. Dexamethasone (Sigma-Aldrich, catalog number: D8893), storage temperature: 2/8 °C
25. FBS qualified fetal calf serum (Thermo Fisher Scientific, catalog number: 12657-029), storage temperature: -10 °C
26. Amphotericin B 250 µg/ml (Thermo Fisher Scientific, catalog number: 15290-018, storage temperature: -20/-5 °C)
27. 0.25% Trypsin (1x) (Thermo Fisher Scientific, catalog number: 15050-057), storage temperature: -20/-5 °C
28. Collagenase type II lyophilized (Thermo Fisher Scientific, catalog number: 17101-015), storage temperature: 2/8 °C
29. 2.5% Chlorhexidine (Bioflora Manipullarium), storage temperature: RT
30. β-Glycerophosphate disodium salt pentahydrate 98.0% (NT) (Sigma-Aldrich, catalog number: 50020-100G), storage temperature: 2/8 °C
31. L-Ascorbic acid (Sigma-Aldrich, catalog number: 33034-100G), storage temperature: 15/30 °C
32. Ethanol 96% (Merck, catalog number: 100971), storage temperature: 5/30 °C
33. Formaldehyde solution 37% (Merck, catalog number: 104002), storage temperature: 15/25 °C
34. Isopropanol (Merck Millipore, catalog number: 1096341000), storage temperature: 5/30 °C
35. Alizarin red S (Sigma-Aldrich, catalog number: A5533-25G), storage temperature: 15/30 °C
36. Acetic acid (Merck, catalog number: 199061), storage temperature: 15/25 °C
37. 3-Isobutyl-1-methylxanthine (Sigma-Aldrich, catalog number: I7018-1000MG), storage temperature: -20 °C
38. Methanol (Merck, catalog number: 1.06009), storage temperature: 5/30 °C
39. Insulin human (Sigma-Aldrich, catalog number: I2643-50MG), storage temperature: -20 °C
40. Hydrochloric acid fuming 37% (Merck, catalog number: 1.00317), storage temperature: 5/30 °C
41. Indomethacin (Sigma-Aldrich, catalog number: I7378-5G, storage temperature: 15/30 °C)
42. Oil red O (Sigma-Aldrich, catalog number: O0625-25G, storage temperature: 15/30 °C)
43. Trichome stain (Masson) Kit (Sigma-Aldrich, catalog number: HT15-1KT, storage temperature: RT)
44. Sodium pyruvate (Sigma-Aldrich, catalog number: S8636, storage temperature: 2/8 °C)
45. Human albumin (Institute Grifols, catalog number: A4AFC03441, storage temperature: 2/25 °C)
46. Transforming growth factor-β3 (Peprotech Inc., catalog number: 100-36E, storage temperature: -20 °C)
47. 4% Paraformaldehyde (Electron Microscopy Sciences, catalog number: 157-4-100), storage temperature: 2/8 °C
48. Xylene (LabSynth, catalog number: X1001.01.BJ), storage temperature: 16/26 °C
49. Paraffin (EasyPath, catalog number: EP-21-20068A), storage temperature: 15/30 °C
50. Eosin (Sigma-Aldrich, catalog number: HT110132), storage temperature: RT
51. Monoclonal anti-rat antibody: anti-CD29 (BD Biosciences, catalog number: 562154, storage

- temperature: 4 °C)
52. Monoclonal anti-rat antibody: anti-CD31 (BD Biosciences, catalog number: 555027, storage temperature: 4 °C)
 53. Monoclonal anti-rat antibody: anti-CD34 (Invitrogen, catalog number: 11-0341-81, storage temperature: 4 °C)
 54. Monoclonal anti-rat antibody: anti-CD45 (BD Biosciences, catalog number: 554878, storage temperature: 4 °C)
 55. Monoclonal anti-rat antibody: anti-CD90 (BD Biosciences, catalog number: 554898, storage temperature: 4 °C)
 56. Monoclonal anti-rat antibody: anti-CD106 (BD Biosciences, catalog number: 559229, storage temperature: 4 °C)
 57. Transport medium (see Recipes)
 58. Collagenase solution (see Recipes)
 59. Trypsin solution (see Recipes)
 60. Ascorbic acid and β-Glycerophosphate solution (see Recipes)
 61. Growth medium (10% MEM) (see Recipes)
 62. Osteogenic differentiation medium (see Recipes)
 63. Chondrogenic differentiation medium (see Recipes)
 64. Adipocyte differentiation medium (see Recipes)
 65. Dexamethasone stock solution (200 μM) (see Recipes)
 66. Ascorbic acid stock solution (20 mM) (see Recipes)
 67. TGF-β3 (see Recipes)
 68. Oil red O staining (see Recipes)

Equipment

1. Scissors (Quinelato, catalog number: QT.109.14)
2. Forceps (Quinelato, catalog number: QC.301.14)
3. Erv-Mount® (EasyPath, catalog number: EP-51-05041), storage temperature: 20 °C
4. Micropipette (Eppendorf, catalog numbers: 4921000028/4921000044/4921000079/4921000109/4921000117/4921000150)
5. Analytical balance M214A (BEL, catalog number: BL0003)
6. RT basic series magnetic stirrers (Thermo Fisher Scientific, catalog number: 88880009)
7. Bench meter for pH (Hanna, catalog number: HI5522-01)
8. Stericup quick release vacuum driven disposable filtration system (Merck, catalog number: S2GPU05RE)
9. Vacuum pump and compressor (Prismatec, catalog number: 132)
10. Airstream class II biohazard safety cabinet (Esco Micro Pte.Ltd., model: AC2-4E8)
11. Microprocessor water bath (Quimis, catalog number: Q215M)

12. CO₂ incubator (Panasonic, Panasonic/Sanyo, model: MCO-19AIC)
13. Eppendorf® Centrifuge 5702 (Sigma-Aldrich, catalog number: Z606936)
14. Axiovert 25 inverted microscope for advanced routine (Carl Zeiss)
15. Compact digital microplate shaker (Thermo Fisher Scientific, catalog number: 88880023)
16. Epoch 2 microplate spectrophotometer (BioTek, catalog number: BTEPOCH2)
17. Centrifuge 5418 R (Eppendorf, catalog number: 5401000013)
18. Gas exhaust chapel (Lutech, catalog number: LCE-15)
19. Vertical freezer, 231 liters (Consul, catalog number: CVU26EB)
20. Refrigerator frost free, 342 liters (Consul, catalog number: CRB39AB)
21. Ultra-low freezer (Panasonic, catalog number: MDF-U500VXC-PA)
22. FACSCantoTM II (BD Biosciences, catalog number: 338962)
23. Paraffin dispenser (Oma, catalog number: IO-88)
24. Microtome (Micron, GMI, catalog number: 8243-30-0001)

Software

1. Gen 5 TS 2.06 (BioTek Instruments Inc./BioTek, <https://www.biotek.com/products/software-robotics-software/gen5-microplate-reader-and-imager-software/>)
2. BD FACSDiva™ Software v8.0.3 (<https://www.bdbiosciences.com/en-us/instruments/research-instruments/research-software/flow-cytometry-acquisition/facsdiva-software>)
3. StepOne Software v2.3 (Thermo Fisher Scientific/Applied Biosystems, <https://www.thermofisher.com/br/en/home/technical-resources/software-downloads/StepOne-and-StepOnePlus-Real-Time-PCR-System.html>)

Procedure

A. Surgical procedure

1. Euthanize the rat using isoflurane according to the local regulations.
2. Disinfect the rat by completely bathing with 1% iodized ethanol (Figure 1A) and wipe the abdomen and lower limbs with 2.5% chlorhexidine.
3. Transfer the rat in a sterile surgical drape.
4. Wear a sterile coat, mask, cap, and gloves.
5. To prevent contamination, use sterile scissors and forceps, to make a small bilateral incision in the skin of the femorotibial joint region (Figure 1B).
6. Use this incision as an access point to perform a bilateral divulsion toward the abdominal and inguinal region.
7. Make a horizontal cut to join the two previously made incisions (Figure 1B).
8. Using a #15 scalpel blade attached to cable #3, cut the patellar tendons, and the lateral and medial collateral ligaments bilaterally to expose the joint capsules.

9. Perform joint capsule divulsion bilaterally.
10. Remove the muscle tissue to expose the anterior part of the femur (Figure 1C).
11. Cut the remaining ligaments and disarticulate the femoral hip joint.
12. Remove the femur, and quickly clean off the majority of muscle and connective tissues attached to the bone.
13. Transfer the femur to a 50-ml conical tube containing 15 ml of transport medium.
14. Retract the skin from the abdominal and inguinal regions (Figure 1D).
15. Carefully remove all adipose tissue without puncturing the abdominal wall and transfer it to a 50-ml conical tube containing 15 ml of transport medium.
16. Take the conical tubes containing the fat tissue and femurs to the laminar flow hood.

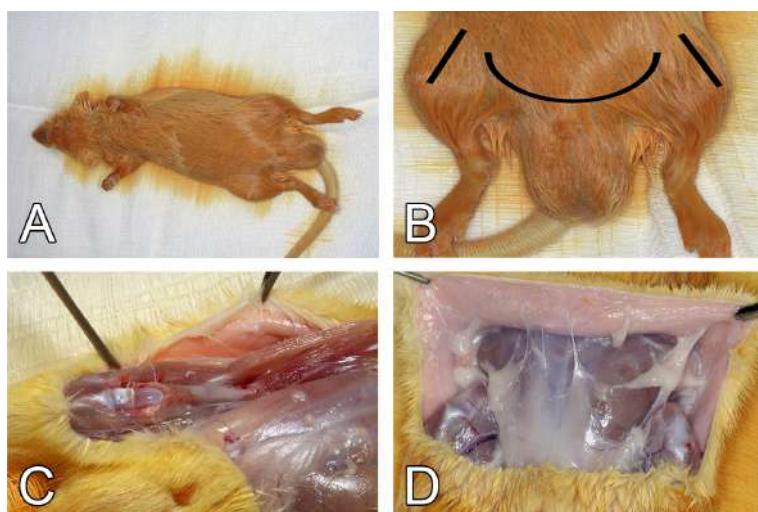


Figure 1. Surgical procedures for harvesting the femur and adipose tissue of a Wistar rat weighing 150-200 g. A. Disinfection of the animal with 1% iodized ethanol after euthanasia. B. Schematic representation of the incisions. C. Muscle removal and femur exposure. D. Skin retraction and exposure of the abdominal and inguinal adipose tissues.

B. BM-MSC isolation and culture procedures

1. Transfer the femurs from the conical tubes to a glass tissue culture dish filled with 70% ethanol.
2. Within 1 min, remove the remaining connective tissue with sterile scissors, forceps and a #15 scalpel blade.
3. Transfer the femurs to a new glass tissue culture dish filled with 2.5% chlorhexidine.
4. Within 1 min, clean the remaining connective tissue with sterile scissors, forceps and a #15 scalpel blade (Figure 2A).
5. Transfer the femurs to new conical tubes containing 15 ml of transport medium and incubate for 15 min at RT.
6. Again, transfer the femurs to a new conical vial containing 15 ml transport medium and incubate for 15 min at RT.

7. Lastly, transfer the femurs to a new conical vial containing 15 ml transport medium and incubate for 15 min at RT.
8. Transfer the contents of this conical tube to a glass tissue culture dish.
9. Fill a 20-ml syringe with growth medium and attach a 21G needle.
10. Hold the femur with tweezers and cut the epiphyses using sterile scissors (Figure 2B).
11. Insert the needle of the syringe filled with growth medium into the diaphysis and flush all bone marrow into a new 50-ml conical tube (Figure 2C).
12. Centrifuge this tube for 5 min at 600 x g at RT.
13. Discard the supernatant and resuspend the pellet in new growth medium (2 ml per femur).
14. Transfer 2 ml of this suspension in a 75 cm² cell culture flask filled with 10 ml of growth medium.
15. Incubate this flask in an incubator at 37 °C in a humidified atmosphere containing 5% CO₂ and 95% air.
16. After 24 h, gently rinse the flask three times with 1x PBS and replace with fresh growth medium.
17. Change the culture medium every 2 days until the cells grow to 70% confluence.

Note: After 7 days of culture in growth medium, approximately 5 × 10⁶ MSCs were generated from each femur of each animal.

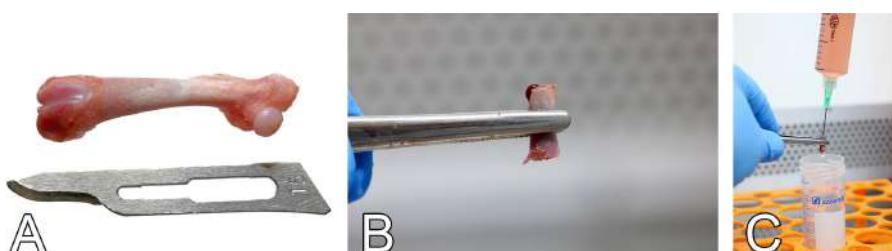


Figure 2. The harvesting of bone marrow from femur. A. Cleaned femur. B. Marrow cavity exposure after epiphyseal sectioning. C. Bone marrow flushing with growth medium using a needle and a syringe.

C. AT-MSC isolation and culture procedures

1. Transfer the adipose tissue from the 50-ml conical tube to a glass tissue culture dish filled with 1x PBS to rinse the tissue.
2. Transfer the adipose tissue to a new glass tissue culture dish (Figure 3A).
3. Use sterile scissors to mince the adipose tissue into small pieces, around 1-2 mm³ (Figure 3B).
4. Transfer the minced pieces to a 50-ml conical tube containing 20 ml of collagenase solution (Figure 3C).
5. Place the tube in a water bath for 40 min at 37 °C, with shaking.
6. Add 20 ml of growth medium to the 50-ml conical tube containing the adipose tissue and collagenase solution.
7. Centrifuge the conical tube containing the adipose tissue, collagenase solution, and growth medium for 5 min at 600 x g.

8. Discard the supernatant and resuspend the pellet in new growth medium (5 ml per adipose tissue removed from 1 animal).
9. Transfer 5 ml of this suspension into a 75 cm² cell culture flask filled with 10 ml of growth medium.
10. Incubate this flask in an incubator at 37 °C in a humidified atmosphere containing 5% CO₂ and 95% air.
11. After 24 h, gently wash the flask three times with 1x PBS and replace with fresh growth medium.
12. Replace the culture medium every 2 days until cells are 70% confluent.

Notes:

- a. *After 7 days of culture in growth medium, approximately 5 × 10⁶ MSCs were generated from the adipose tissue of each animal.*
- b. *Typically, within 7 days, MSCs reach 70% of confluence and are ready to be used in subsequent experiments (Figure 4).*



Figure 3. The enzymatic digestion of abdominal and inguinal adipose tissue. A. Harvested adipose tissue. B. Mincing of adipose tissue into small pieces using sterile scissors and tweezers. C. Transfer adipose tissue pieces to collagenase type II solution for enzymatic digestion and cell isolation.

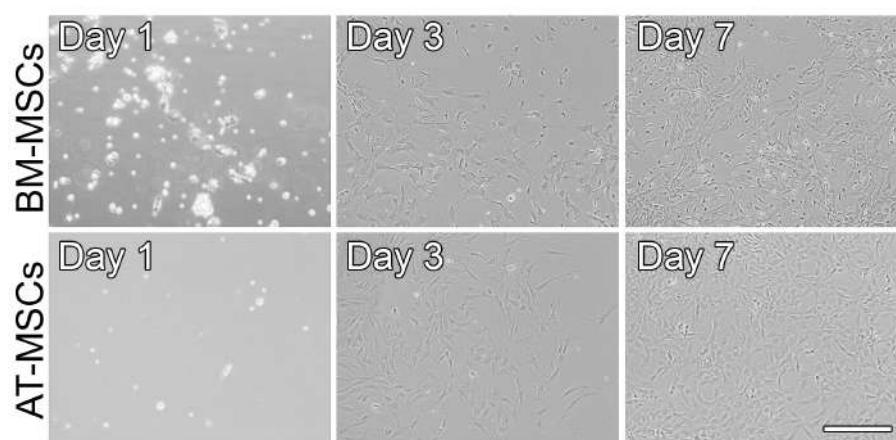


Figure 4. Phase-contrast micrographs showing the morphology of BM-MSCs and AT-MSCs that were cultured in growth medium and on polystyrene dishes for up to 7 days. After 24 h, both BM-MSCs and AT-MSCs have attached to the polystyrene dish, and their morphology is round/oval. As the cells were cultured, they proliferated and became elongated, polygonal, and spindle-shaped. Scale bar = 100 μm.

D. Characterization of BM-MSCs and AT-MSCs

1. Wash the flask three times with 1x PBS.
2. Add 5 ml of trypsin solution into the flask and incubate for 5 min at 37 °C.
3. Add 2.5 ml of fresh growth medium into the flask, transfer the cell suspension into a 50-ml conical tube, and centrifuge for 5 min at 600 x g.
4. Discard the supernatant.
5. Wash the cell pellet once with 1x PBS.
6. Centrifuge the cell suspension for 5 min at 600 x g.
7. Discard the supernatant.
8. Add 5 ml of 1x PBS to the cell pellet and mix the cell suspension.
9. Count the cells in a hemocytometer (Neubauer Chamber).
10. Adjust the concentration of the cell suspension to obtain a density of 2×10^5 cells/ml with 1x PBS.
11. Add 1 ml of cell suspension to each flow cytometer tube (one tube for each specific antibody, one tube for isotype control, and one tube with cells that will not be labeled with antibody).
12. Centrifuge the flow cytometer tubes for 5 min at 600 x g.
13. Discard the supernatant.
14. Add 100 µl of 1x PBS to the cell pellet and mix by flicking/tapping the tube.
15. Incubate each tube for 30 min at RT in the dark with 2 µl of the following monoclonal anti-rat antibodies: anti-CD29, -CD31, -CD34, -CD45, and -CD106, directly conjugated with a fluorophore (antibody final dilution: 1:50). For monoclonal anti-rat antibody -CD90 directly conjugated with a fluorophore: dilute the antibody 1:5 in 1x PBS and then add 2 µl to the cell suspension (antibody final dilution: 1:250).
16. Add 2 µl of isotype control to the corresponding tube.
17. Wash the cells with 2 ml of 1x PBS.
18. Centrifuge for 5 min at 600 x g.
19. Discard the supernatant.
20. Add 0.5 ml of formaldehyde solution (4%) diluted to 1% in 1x PBS.
21. Analyze the cells by flow cytometry (Figures 5 and 6).

BM-MSCs

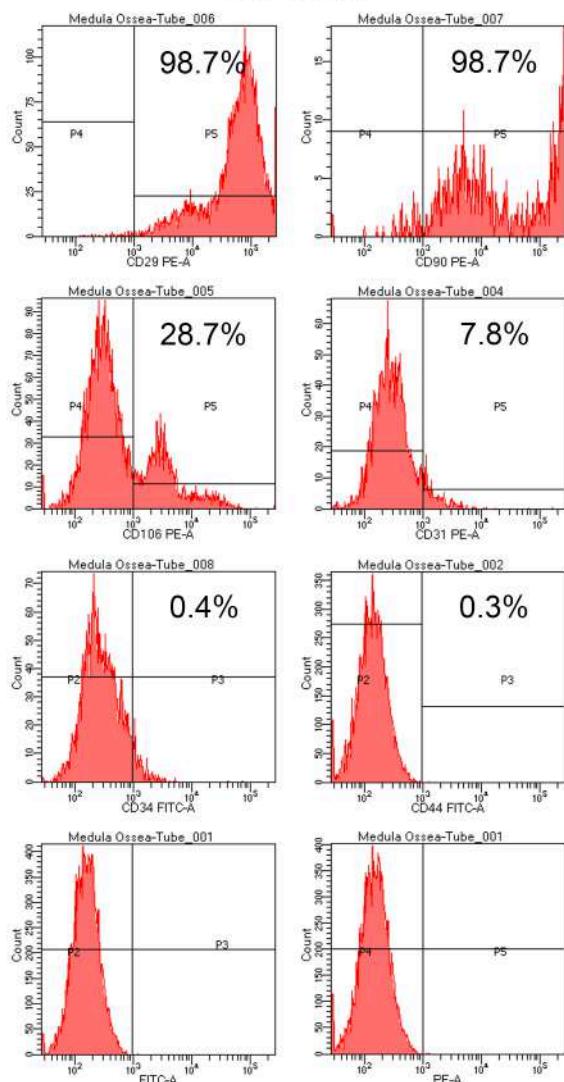


Figure 5. Flow cytometry analysis of BM-MSCs cultured in growth medium on a polystyrene culture dish for 7 days. Histograms show the expression of the surface markers CD29, CD90, CD106, CD31, CD34, and CD44 after incubation with the respective antibodies. Cells were also incubated with the isotypes FITC-A and PE-A, which were used as negative controls. A high percentage of BM-MSCs expressed CD29, CD90, and CD106 (98.7%, 98.7%, and 28.7%, respectively) and a low percentage expressed CD31, CD34, and CD44 (7.8%, 0.4%, and 0.3%, respectively).

AT-MSCs

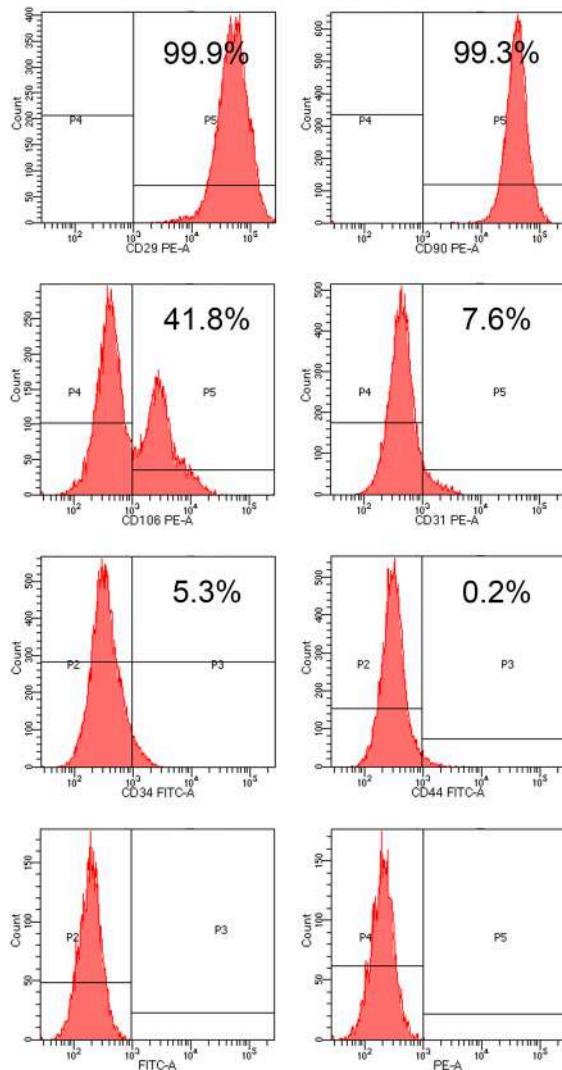


Figure 6. Flow cytometry analysis of AT-MSCs cultured in growth medium on a polystyrene dish for 7 days. Histograms show the expression of the surface markers CD29, CD90, CD106, CD31, CD34, and CD44 after incubation with the respective antibodies. Cells were also incubated with the isotypes FITC-A and PE-A, which were used as negative controls. A high percentage of AT-MSC expressed CD29, CD90, and CD106 (99.9%, 99.3%, and 41.8%, respectively) and a low percentage expressed CD31, CD34, and CD44 (7.6%, 5.3%, and 0.2%, respectively).

E. Osteoblast differentiation

1. When BM-MSCs or AT-MSCs reach 70% confluence, remove the growth medium.
2. Wash the flask three times with 1x PBS.
3. Add 5 ml of trypsin solution into the flask and incubate for 5 min at 37 °C.
4. Add 2.5 ml of fresh growth medium into the flask, transfer the cell suspension to a 50-ml conical tube, and centrifuge for 5 min at 600 x g.

5. Discard the supernatant and resuspend the cell pellet in new growth medium.
6. Count the cells and plate them at a cell density of 2×10^4 cells/well in 24-well culture plates in 1 ml of osteogenic medium or 1×10^5 cells/well in 6-well culture plates in 2 ml of osteogenic medium.
7. Incubate the plates in an incubator at 37 °C in a humidified atmosphere containing 5% CO₂ and 95% air during the time-course of the experiment.
8. Replace the culture medium every 2 days.
9. The extracellular matrix mineralization can be observed after 21 days in culture.

F. Alizarin red staining

To confirm osteoblast differentiation, one of the methods we used is the detection of mineralized extracellular matrix by alizarin red staining.

1. Remove the culture medium from each well and gently wash the cells 3 times with 1x PBS.
2. Add 10% formalin and incubate at 4 °C for 24 h (24-well plates–500 µl; 12-well plates–1 ml; 6-well plates–2.4 ml).
3. Remove the 10% formalin and dehydrate the cells using increasing concentrations of ethanol (30%, 50%, 70%, and 96%) for 1 h each (24-well plates–500 µl; 12-well plates–1 ml; 6-well plates–2.4 ml).
4. Remove the 96% ethanol and incubate at RT until the wells are dry.
5. Cover the well with alizarin red staining and incubate at RT for 10 min.
6. Wash once with deionized water and incubate at RT until the wells are dry.
7. Take macroscopic (Figure 7) and microscopic photos of the wells.

Note: Typically, BM-MSCs are more committed to osteoblast differentiation compared to AT-MSCs, as we previously observed (Abuna et al., 2016).

Extracellular Matrix Mineralization

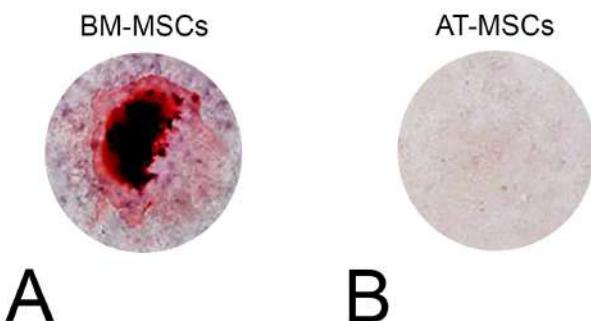


Figure 7. Mineralized extracellular matrix detected by alizarin red staining in BM-MSC and AT-MSC cultures after 21 days of culture in osteogenic medium on polystyrene dishes

G. Chondroblast differentiation

1. When BM-MSC and AT-MSC cultures reach 70% confluence, remove the growth medium.
2. Wash the flask three times with 1x PBS.

3. Add 5 ml of trypsin solution into the flask and incubate for 5 min at 37 °C.
4. Add 2.5 ml of fresh growth medium into the flask.
5. Transfer this cell suspension to a 50-ml conical tube and centrifuge for 5 min at 600 x g.
6. Discard the supernatant.
7. Resuspend the cells in chondroblast differentiation medium at a density of 1.25×10^6 cells/ml.
8. Using a pipette, dispense 200 µl aliquots of the cell suspension (2.5×10^5 cells) into each well of polypropylene 96-well plates.
9. Centrifuge the plates at 500 x g for 5 min.
10. Add 200 µl of 1x PBS into the empty wells to minimize the evaporation of the culture medium.
11. Incubate the plates in an incubator at 37 °C in a humidified atmosphere containing 5% CO₂ and 95% air during the time-course of the experiment.
12. After 24 h of incubation, cell aggregates were visible, and the chondrogenic phenotype was observed after 30 days in culture.
13. Replace the culture medium every 2 days by carefully aspirating the expired medium using a sterile 200 µl pipette and adding 200 µl of fresh chondrogenic medium to each well.

H. Trichrome staining

To confirm chondroblast differentiation, one of the methods we used is the detection of collagen fibers with trichrome staining.

1. Using a micropipette, remove the chondroblast differentiation medium.
2. Add 200 µl of 1x PBS into each well.
3. Using a micropipette, remove the PBS.
4. Add 200 µl of 4% paraformaldehyde for 5 min at RT.
5. Remove the paraformaldehyde.
6. Wash each well with 1x PBS, 2 times for 3 min each.
7. Stain the cell aggregates with eosin for 5 min at RT.
8. Remove the eosin and wash with 1x PBS, 2 times for 3 min each.
9. Using a 1,000 µl micropipette, harvest the aggregates and transfer them to 1.5-ml microtubes.
10. Dehydrate the cells aggregates in 300 µl of a graded ethanol series (70%, 80%, 90%, 95%, and 100%, 5 min each) by placing them into 1.5-ml microtubes.
11. Remove the 100% ethanol and perform three clarification steps in 300 µl xylene for 3 min each.
12. Paraffin-embed the aggregates into a mold in a hot surface for 5 min then transfer each mold to a cold surface.
13. Cut adjacent 5 µm sections using a microtome.
14. Deparaffinize the sections overnight in an incubator at 60 °C.
15. Deparaffinize the sections in three steps of xylene for 5 min each, and rehydrate the sections by incubation in an ethanol series (100%, 95%, 90%, 80%, and 70%, 3 min each).
16. Wash the sections with deionized water for 5 min.
17. Stain with acid fuchsin (HT15-1) for 5 min at RT.

18. Wash with water for 5 min.
19. Stain with a working solution of phosphomolybdic acid (HT15-3) and phosphotungstic acid (HT15-2) for 5 min at RT.
20. Stain the sections with an aniline blue solution for 5 min at RT.
21. Remove the excess aniline blue, and add a 1% acetic acid solution for 3 min.
22. Rinse with sections with tap water for 3 min.
23. Dehydrate sections in a graded ethanol series (70%, 80%, 90%, 95%, and 100%, 1 min each), followed by three clarification steps in xylene for 1 min each.
24. Mount the slides using Erv-Mount®.
25. Take microscopic photos of the histological sections (Figure 8).

Note: Cytoplasm is stained in red, and collagen fibers are stained in blue.

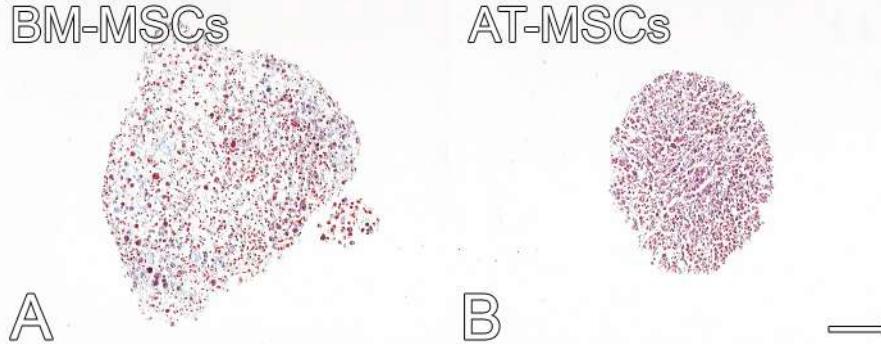


Figure 8. Collagen fibers (blue) and cytoplasm (red) detected by trichrome staining in BM-MSCs and AT-MSCs cultured in chondrogenic medium on ultra-low cluster 96-well plates for 30 days. Scale bar = 100 μm.

I. Adipocyte differentiation

1. When BM-MSC and AT-MSC cultures reach 70% confluence, remove the growth medium.
2. Wash the flask three times with 1x PBS.
3. Add 5 ml of trypsin solution into the flask and incubate for 5 min at 37 °C.
4. Add 2.5 ml of fresh growth medium into the flask.
5. Transfer this cell suspension to a 50-ml conical tube and centrifuge for 5 min at 600 x g.
6. Discard the supernatant and resuspend the cell pellet in fresh growth medium.
7. Count the cells and plate them at a cell density of 2×10^4 cells/well in 24-well culture in 1 ml of adipogenic medium plates or 1×10^5 cells/well in 6-well culture plates in 2 ml of adipogenic medium.
8. Incubate the plates in an incubator at 37 °C in a humidified atmosphere containing 5% CO₂ and 95% air during the time-course of the experiment.
9. Replace the culture medium every 2 days. The intracytoplasmic lipid droplets were observed after 10 days in culture.

J. Oil red O staining

To confirm adipocyte differentiation, one of the methods we used is the detection of intracytoplasmic lipid droplets with oil red O staining.

1. Remove the culture medium from each well.
2. Add 10% formalin and incubate for 5 min at RT (24-well plates–500 µl, 12-well plates–1 ml, 6-well plates–2.4 ml).
3. Discard the 10% formalin and add the same volume of fresh 10% formalin. Incubate for at least 1 h.

Note: Cells can be kept in formalin for a couple of days before staining. Wrap parafilm around the plate to prevent the cells from drying out and cover the plate with aluminum foil.

4. Remove the 10% formalin using a small transfer pipette.
5. Wash the wells with 60% isopropanol (24-well plates–500 µl, 12-well plates–1 ml, 6-well plates–2.4 ml).
6. Let the wells dry completely.
7. Add the oil red O staining working solution for 10 min (do not touch walls of the wells).
8. Remove the oil red O staining, and immediately add deionized water (repeat this step 4 times).
9. Remove all deionized water and incubate at RT to dry.
10. Image the wells using a phase-contrast microscope (Figure 9).

Note: Typically, AT-MSCs are more committed to adipogenic differentiation compared to BM-MSCs, as we previously observed (Abuna et al., 2016).

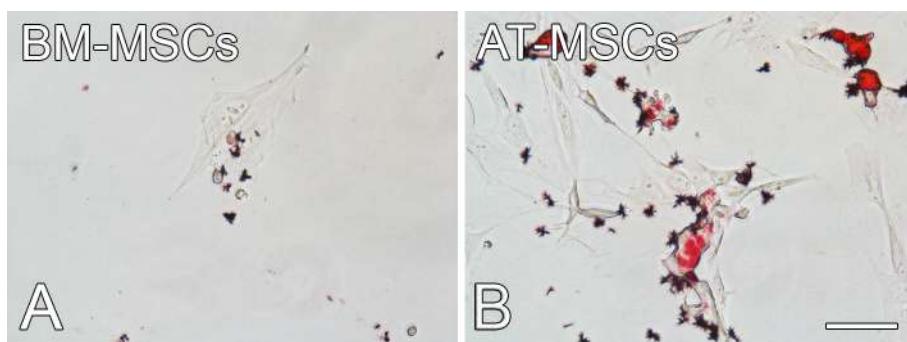


Figure 9. Intracytoplasmic lipid droplets detected by oil red O staining in BM-MSCs and AT-MSCs cultured in adipogenic medium on a polystyrene dish for 10 days. Scale bar = 100 µm.

Recipes

1. Transport medium

Note: Prepared fresh just prior to use and kept at 37 °C.

57 ml of alpha minimum essential medium (α-MEM)

3 ml of gentamycin (50 µg/ml)

720 µl of amphotericin B (0.3 µg/ml)

2. Collagenase solution

Note: Prepared fresh just prior to use and placed at RT.

15 mg of type II collagenase (0.075%)

20 ml of 1x PBS

Filter this solution in the laminar flow hood into a 50-ml conical tube using a 20-ml syringe and 0.2 μ m filter

3. Trypsin solution

Note: Prepared fresh just prior to use and placed at RT.

19 ml of trypsin (0.25%)

500 μ l of type II collagenase (1.3 mg/ml)

1 ml of EDTA (1 mM)

4. Ascorbic acid and β -Glycerophosphate solution

Note: Previously prepared, kept at 4 °C for up to 7 days.

5 mg of ascorbic acid (5 μ g/ml)

2.16 g of β -glycerophosphate (7 mM)

10 ml of deionized water

Filter this solution in the laminar flow hood into a 50-ml conical tube using a 20-ml syringe and 0.2 μ m filter

5. Growth medium (10% MEM)

Note: Previously prepared, kept at 4 °C for up to 30 days.

360 ml of α -MEM

40 ml of fetal calf serum

2 ml of gentamycin (50 μ g/ml)

500 μ l of amphotericin B (0.3 μ g/ml)

6. Osteogenic differentiation medium

Note: Previously prepared, kept at 4 °C for up to 30 days.

400 ml of growth medium

4 ml of dexamethasone (10^{-7} M)

1% ascorbic acid and β -glycerophosphate solution

Note: The ascorbic acid and β -glycerophosphate solution are added immediately before using the medium.

7. Chondrogenic differentiation medium

Note: Previously prepared, kept at 4 °C for up to 30 days.

100 ml of D-MEM

100 μ l of sodium pyruvate (100 mM)

100 μ l of dexamethasone (1 mM)

250 μ l of ascorbic acid (20 mM)

1 ml of human albumin (0.02%)

20 μ l/ml of transforming growth factor β 3 (TGF- β 3, 1 μ g/ml).

Note: The TGF- β 3 is added immediately before using the medium.

8. Adipocyte differentiation medium

Note: Previously prepared, kept at 4 °C for up to 30 days.

180 ml of D-MEM

20 ml of fetal calf serum (10%)

2 ml of gentamycin (50 μ g/ml)

250 μ l of amphotericin B (0.3 μ g/ml)

2 ml of dexamethasone (10^{-6} M)

2 ml of 3-isobutyl-1-methylxanthine (0.5 mM)

260 μ l of indomethacin (0.1 M)

150 μ l of insulin (10 mg/ml)

9. Dexamethasone stock solution (200 μ M)

Note: Previously prepared, kept at -20 °C.

Dexamethasone is dissolved at 200 μ M in absolute ethanol and deionized water

10. Ascorbic acid stock solution (20 mM)

Note: Previously prepared, kept at 4 °C for up to 7 days.

A stock solution of 20 mM ascorbic acid is prepared in 1x PBS

11. TGF- β 3

Note: Prepared fresh just prior to use and placed at RT.

A 1 μ g/ml stock solution of TGF- β 3 is prepared in 1x PBS and 0.5% human albumin.

12. Oil red O staining

Oil red O stock solution

Note: Previously prepared, kept at RT up to 6 months.

Oil red O (700 mg) is added to 200 ml of isopropanol, stirred overnight, and then passed through a 0.2 μ m filter.

Oil red O work solution

Mix 6 parts of oil red O stock solution with 4 parts deionized water and incubate at RT for 20 min.

Acknowledgments

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Competing interests

The authors declare no conflict of interest.

Ethics

All procedures performed were conducted in accordance with the ethical standards of the international, national, and/or institutional animal care guidelines. The Committee of Ethics in Animal Research of the School of Dentistry of Ribeirão Preto, University of São Paulo (#2018.1.30.58.8) reviewed and approved all animal procedures we have done here.

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A Modified Semisolid Clonal Culture for Identification of B-1 and B-2 Progenitor Colony Forming Ability of Mouse Embryonic Hemogenic Endothelial Cells

Michihiro Kobayashi and Momoko Yoshimoto*

Center for Stem Cell and Regenerative Medicine, Institute of Molecular Medicine, McGovern Medical School, University of Texas Health Science Center at Houston, Houston, TX 77030, USA

*For correspondence: Momoko.Yoshimoto@uth.tmc.edu

[Abstract] The search for the origin of the first hematopoietic stem cells (HSCs) in the mouse embryo has been a hot topic in the field of developmental hematopoiesis. Detecting lymphoid potential is one of the supportive evidence to show the definitive hematopoietic activity of HSCs. However, the first B-lymphoid potential in the mouse embryos are reported to be biased to innate-like B-1 cell lineage that can develop from hemogenic endothelial cells (HECs) independently of HSCs. On the other hand, conventional adaptive immune B cells (B-2) cells are considered to be exclusively derived from HSCs. Therefore, segregating B-1 and B-2 progenitor potential is important to understand the developmental process of HSCs that are also produced from HECS through intermediate precursors referred to as pre-HSCs. Both HECS and pre-HSCs show endothelial surface phenotype and require stromal support to detect their hematopoietic activity. The method utilizing stromal cell culture followed by modified semisolid clonal culture enables us to detect the number of colony forming units for B-1/B-2 progenitors originally derived from HECS/pre-HSCs, which will reflect the potential of B-1 biased or multi-lineage repopulating HSCs.

Keywords: B-cell colony forming assay, OP-9, B-1 and B-2 lymphoid progenitors, Mouse embryo, Pre-HSC, Hemogenic endothelial cell, AGM region

[Background] Semisolid clonal culture (methylcellulose colony forming assay) is a traditional method to detect the number of hematopoietic progenitor cells. One colony is considered to be derived from a single progenitor cell (clonal origin). Added cytokines play important roles in the formation of yield colonies. For example, Epo enhances the colony forming unit of erythrocytes (CFU-E) or the burst forming unit of erythrocytes (BFU-E), while G-CSF/GM-CSF will enhance CFU-G (Granurocytes), M (Macrophages) or GM (Granulo-macrophages). SCF stimulate hematopoietic progenitor activity. The formation of B-lymphocyte progenitor colonies from mouse BM requires SCF, IL-7 and Flt3-ligand. Importantly, this assay can detect only the activity of CD45⁺ (or CD41⁺ in case of embryo-derived cells) hematopoietic progenitor cells, thus, cannot detect the hematopoietic activity of CD45⁻CD144⁺ hemogenic endothelial cells (HECs) that produce various hematopoietic cells in the mouse embryo. The detection of hematopoietic activity of HECS heavily depends on the stromal support or organ culture method. Therefore, it is challenging to determine the number of lymphoid progenitors produced from HECS. Recently, Montecino-Rodriguez *et al.* (2016) developed a modified semisolid clonal culture using S-17 stromal cells to detect B-1 and B-2 potential of hematopoietic progenitors in embryonic (E) day 10

yolk sac cells and fetal liver cells.

B-1 progenitor cells are mainly detected in the fetal liver and neonatal BM. B-1 cells belong to innate-like B-lymphocytes that produce natural IgM antibodies, while conventional adaptive immune B cells are referred to as B-2 cells. Montecino-Rodriguez *et al.*, identified B-1 cell-specific progenitors in the mouse fetal liver and neonatal BM as lin⁻IgM⁻CD19⁺B220⁻ (CD19 single positive) cells whereas B-2 specific progenitors (Pro-B cell) are lin⁻IgM⁻CD19⁻B220⁺ (B220 single positive) cells. These two populations quickly become CD19⁺B220⁺ double positive *in vitro* culture, thus, it is difficult to determine if B cells produced from embryonic tissues in the co-culture with stromal cells belong to B-1 or B-2 cells. This modified semisolid clonal culture enables us to detect B-1 and/or B-2 progenitor colonies derived from HECs/pre-HSC by utilizing stromal cells that support B lymphopoiesis (Kobayashi *et al.*, 2019). We used OP9 stromal cells to support B-lymphopoiesis in the semisolid clonal culture, with which we have been successful to induce B lymphocytes from HECs (Yoshimoto *et al.*, 2011; Kobayashi *et al.*, 2019). Since B-2 progenitor colony forming ability seems to be one of the HSC activity, this method may be utilized to evaluate the HSC activity derived from HECs/pre-HSCs in the mouse embryo.

Materials and Reagents

1. T-25 culture Flask (Corning, catalog number: 353109)
2. 6-well culture plates (Corning, Costar, catalog number: 3516)
3. 96-well Flat culture plates (Corning, Costar, catalog number: 3595)
4. Falcon Round bottom polypropylene14 ml tube (Corning, catalog number: 352059)
5. 1.5 ml Eppendorf tube
6. 35 mm Petri dish (Falcon, catalog number: 351008)
7. 15 mm Petri dish
8. 3 ml syringe (BD, catalog number: 309657)
9. 5 ml syringe
10. 18 G needles (BD, catalog number: 305185)
11. 10 μ l pipette tip
12. Bottle top filter system (0.22 μ m pore size)
13. C57BL/6 male and females
14. α -MEM (powder) (Gibco, catalog number: 11900-024)
15. Fetal Bovine Serum

Note: We used FBS from Atlanta Biological for this publication. However, we always perform lot check from 5-10 test FBS samples to find the best FBS for OP9 maintenance culture. We usually purchase 20 bottles to maintain the same equality of experiments for 2-3 years.

16. Fresh Milli-Q water
17. PBS (Fisher Scientific, catalog number: BP665-1)
18. 2-Mercaptoethanol (Fisher Scientific, catalog number: O3446I-100)
19. 500 mM EDTA pH 8.0

20. IMDM (Gibco, catalog number: 12440061)
21. Heparin (Sigma, catalog number: H3149-100KU)
22. Endothelial mitogen 50 mg (Biomed Tech, catalog number: BT-203)
23. Penicillin-Streptomycin 10,000 U-10,000 µg/ml (Gibco, catalog number: 15140122)
24. X-VIVO 20 (Lonza, catalog number: 04-448Q)
25. Methylcellulose-base medium for mouse pre-B lymphoid progenitor cells (Stemcell Technologies, Methocult™, catalog number: M3630)
26. Recombinant murine IL-7 (Peprotech, catalog number: 217-17)
27. Recombinant murine Flt3-ligand (Peprotech, catalog number: 250-31L)
28. 0.05% Trypsin/EDTA (Gibco, catalog number: 25300054)
29. 0.25% Collagenase Type I (Stemcell Technologies, catalog number: 07902)
30. DNase I Solution (1mg/ml) (Stemcell Technologies, catalog number: 07900)
31. Cell Dissociation Buffer (Hank's balanced) (Gibco, catalog number: 13150-016)
32. Antibodies for sorting and detecting B cell colonies (Table 1)

Table 1. Antibody list for flow cytometry

Name	Clone	Color	Company	Catalog No.
CD144 (VE-Cad)	11D4.1	AlexaFlour647	BD	562242
CD201 (EPCR)	eBio1560	PE	eBioscience	12-2012-82
CD45	30-F11	FITC	eBioscience	11-0451-82
Ter119	Ter119	PerCP-Cy5.5	Biolegend	116228
CD11b (Mac1)	M1/70	ACP-Cy7	Biolegend	101226
CD93 (AA4.1)	AA4.1	APC	Biolegend	136510
CD19	1D3	PE	Biolegend	152408
B220	RA3-6B2	PE-Cy7	Biolegend	103222

33. OP9 stromal cells [obtained from Dr. Shin-Ichi Nishikawa (retired from Riken, Kobe, Japan), can be obtained from ATCC CRL-2749 (ideally < 30 passage)]
34. Akt-expressing AGM-ECs (AGM-ECs): endothelial cells (ECs) obtained from aorta-gonod-mesonephros (AGM) region in the mouse embryo was established and induced Akt-overexpression by Dr. Brandon Hadland, Fred Hutchinson Cancer Center, Seattle, USA) (Hadland *et al.*, 2015)
35. Gelatin from porcine skin (Sigma, catalog number: G1890)
36. α-MEM (see Recipes)
37. 2-mercaptoethanol (2-ME) stock (0.1 M) (see Recipes)
38. OP9 maintenance medium (α-MEM + 20% FBS) (see Recipes)
39. AGM-EC maintenance medium (see Recipes)
40. Differentiation medium (α-MEM +10% FBS + 5 x 10⁻⁵ M 2ME) (see Recipes)
41. Staining buffer (PBS + 5% FBS + 2 mM EDTA) (see Recipes)

42. 0.1% Gelatin (see Recipes)

Equipment

1. P1000 pipette
2. Microcentrifuge
3. FACS Sorter and analyzer with more than 6 fluorescent detectors are required. We used BD FACSAria for cell sorting, BD LSRII and BD LSR-Fortessa for analysis
4. Standard cell culture CO₂ incubator (CO₂: 5%, O₂: 20%)

Procedure

A. Maintaining OP9 stromal cells and AGM-EC stromal cells

Maintaining OP9 stromal cells

1. Maintaining OP9 stromal cells in a good condition is an important key to obtain successful results. Make fresh OP9 maintenance medium from the powder every month. Test several lots of FBS for good growth of OP9 cells.
2. Thaw OP9 in T25 Flask in freshly made OP9 medium. Split every 3 days, 1:3.

Notes:

- a. *OP9 cells are flat round or square shapes (Figure 1) if they become spindle shape, they are not good OP9 anymore because spindle shape cells tend to be transforming and losing B cell supporting ability.*



Figure 1. The morphology of OP9 cells (100x). Red arrows indicate good OP9 and blue arrows indicate bad OP9. Scale bar = 100 μm.

- b. *Split one confluent OP9 in T25 flask into 3 flasks every 3 days. If they are not confluent in 3 days, the lot of FBS is not good for them.*

- c. The density of OP9 needs to be appropriate (around 4×10^5 /T25 flask at the starting point). When OP9 cells are confluent in T25 flask, the cell number is around 1.0×10^6 - 1.5×10^6 .
- d. For splitting, aspirate medium, wash with PBS 2 times, and treat them with 0.05% Trypsin/EDTA for 3 min at 37 °C. OP9 cells will be peeled off from the flask and add medium and pipette to make a single cell suspension with 5 ml serological pipette (important). Make sure well pipetting and single cells, because leaving cell clumps may induce bad condition of OP9 cells at later passage. Spin 400 x g for 5 min, aspirate supernatant, loosen the pellets by tapping the bottom of the tube (important), add 1 ml OP9 maintenance medium and pipette very well with P1000 pipette to ensure single cell suspension. Add more OP9 medium and plate them into 3 x T25 flasks.
- e. OP9 must be maintained within 3 days (usually every 3 days). Even if they are not confluent in 3 days, they should be passed (1 to 1 or 1 to 2 to increase the cell density). If they are left confluent without passage, they can be used for co-culture, but they will not expand anymore.
- f. If OP9 cells are not in good condition, it is hard to obtain B cells from early stage of embryos (< E9.0-9.5).
- g. OP9 cells should not be maintained for more than 1 month.
- h. OP9 cells show good contact inhibition. If you need irradiation to stop the over-cell growth of OP9, they are already transformed and not good OP9 anymore.

Maintaining AGM-EC stromal cells

1. Coat 0.1% gelatin T25 Flask for more than 30 min.
2. Aspirate gelatin before plating cells.
3. Thaw AGM-EC on gelatin-coated T25 Flask in AGM-EC maintenance medium (near 5×10^5 /flask). Pass every 3-4 days, 1:1-3 (Hadland *et al.*, 2015).

B. Harvest embryo tissues

1. Set up timed mating of C57BL/6 male and females after 3:00 PM. The following early morning, check vaginal plugs of the female and separate plugged females into a different cage. Noon on this day is considered as embryonic (E) day 0.5.
2. About 7 days before harvesting embryos, thaw OP9 and AGM-ECs in T25 flask and maintain.
3. One day before harvesting embryos, prepare OP9 (1×10^4 /well) and AGM-ECs (5×10^3 /well) in 96-well plates respectively. In addition, keep maintaining OP9 in T25 Flask as needed for plating methylcellulose at 5-7 days later.
4. Ten days later after timed mating (at E10.5), harvest embryos from pregnant mother (follow the procedure under the approved AICUC protocol at your institute) (Morgan *et al.*, 2008).
5. Under the stereomicroscope, open the uterus, separate yolk sac (YS) from embryos (Morgan *et al.*, 2008). After removal of the YS, confirm the embryonic stage by counting somite pairs. Collect caudal half of embryos, containing AGM region into 14 ml tubes filled with 10 ml medium.

6. Centrifuge the AGM tissues in the medium at $450 \times g$ for 5 min and aspirate supernatant. Suspend the AGM tissues in 0.25% Collagenase Type I + DNase I (final 20 $\mu\text{g/ml}$) respectively [use 0.5-1 ml per embryo equivalent (e.e)].
7. Incubate the tissue suspension at 37°C . Pipette every 5 min and confirm dissociation under microscope. Once you see good single cells, stop the collagenase reaction by adding the same volume of Cell Dissociation Buffer. Usually this incubation will take 15-30 min depending on the age of embryos (the earlier embryo, the shorter incubation time).
8. Add differentiation medium and filter them using 70 μm strainer, and centrifuge the cells at $450 \times g$ for 5 min.
9. Aspirate supernatant, suspend single cell pellets in 1 ml staining buffer, and count cell number. Normally around 5×10^5 cells /embryo will be harvested.

C. Sorting HECs/pre-HSCs and co-culture

When examining the B-lymphoid hematopoietic potential of HECs/pre-HSC, it is challenging to detect B-progenitor colony forming ability directly from these cells, since most of them are endothelial phenotype. In order to induce hematopoietic progenitors, sort HECs/pre-HSCs and plate them on OP9 (expand hematopoietic progenitors) or on AGM-ECs (let pre-HSCs mature into HSCs).

Staining and cell sorting

1. Suspend embryonic cells up to 1×10^7 cells in 100 μl staining buffer in 1.5 ml Eppendorf tube, take 5% volume first each for negative control and FMO staining.
2. Add antibodies; anti-mouse CD45, CD144, c-kit, EPCR (CD201), and Ter119 at a ratio of 0.3 $\mu\text{l}/10^6$ cells and incubate them for 30 min on ice.
3. Add 1 ml staining buffer for wash and centrifuge them at $800 \times g$ for 2 min at microcentrifuge.
4. Suspend the cells in staining buffer and sort Ter119 \cdot CD144 $^+$ c-kit $^+$ EPCR $^+$ cells on FACSaria (Figure 2).
5. (optional) mix sorted Ter119 \cdot CD144 $^+$ c-kit $^+$ EPCR $^+$ cells with 1×10^5 OP9 cells in Methocult with IL-7 and Flt3-ligand, and plate them onto a 35 mm Petri dish. However, direct plating of these cells will yield only a few B cell colonies.
6. Plate 100-200 Ter119 \cdot CD144 $^+$ c-kit $^+$ EPCR $^+$ cells at one well of 96-well plate confluent with OP9 in differentiation medium or AGM-EC cells in X-vivo 20 with added IL-7 and Flt3-ligand (final concentration: 10 ng/ml for both).
7. Five to seven days after co-culture, harvest all cells and stain them with anti-mouse CD45 and CD11b antibody.
8. After staining, cells are washed with staining buffer and are suspended in staining buffer and sort CD11b \cdot CD45 $^+$ cells from the co-cultured cells on FACSaria as shown in Figure 2B.

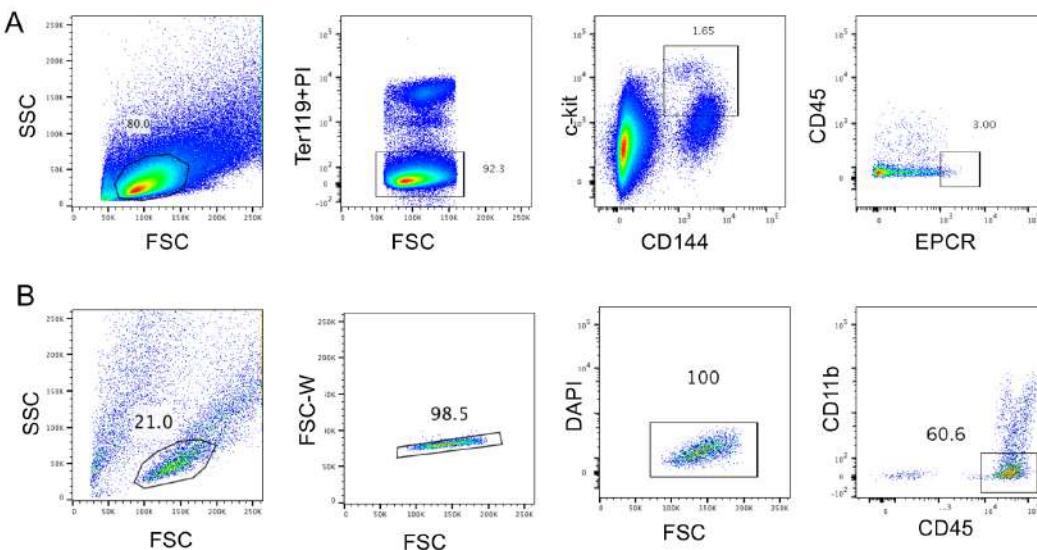


Figure 2. Gating strategy for sorting pre-HSCs (A) and CD45⁺CD11b⁻ cells after co-culture with AGM-ECs(B)

D. Plating semisolid culture

1. Thaw Methocult at room temperature or 4 °C over night.
2. Always prepare N+1 samples for methylcellulose assays. Mix 200-600 CD45⁺ cells after co-culture with 1 x 10⁵ OP9 cells/dish. For triplicates, mix 800-2,400 CD45⁺ cells and 4 x 10⁵ OP9 cells in 14 ml round bottom tube and spin them down, aspirate the medium, and loosen the cell pellets by tapping the tube. Add 4 ml Methocult M3630 using 5 ml syringe with 18 G needle to the cell pellet (This way, you can plate 200-600 CD45⁺ cells with 1 x 10⁵ OP9 cells per 35 mm dish.). In this scale, 20-80 colonies/dish will be expected.
3. Add IL-7 (final 10 ng/ml) and Flit3-ligand (final 10 ng/ml), mix well by vortexing and leave it until all the bubbles are gone.
4. Plate 1.1 ml of methylcellulose medium including cells into each 35 mm Petri dish x 3 dishes using 18 G needle with 3 ml syringe.
5. Place 3 Petri dishes containing the Methocult and 1 Petri dish containing sterile H₂O (preventing the dry out the medium) in a 15 mm Petri dish.
6. Incubate them in a 5% CO₂ incubator for 8-11 days and count colony numbers. The pictures in Figure 3 are representative B-cell colonies and their FACS analysis.

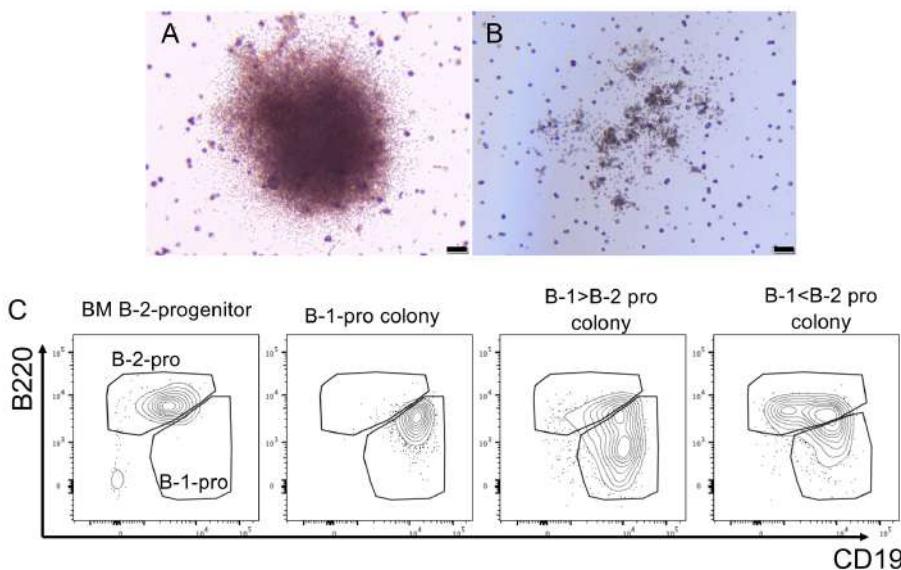


Figure 3. B cell colonies and FACS analysis of picked-up colonies. A. B-1 progenitor (B-1-pro) colony. B. B-2 progenitor (B-2-pro) colony, confirmed by FACS analysis below. Scale bar = 200 μ m. C. FACS analysis of each picked up colony. Based on the gating of BM B-2 progenitors, same gating is applied to the analysis of each sample.

E. Determine each colony as to B-1 or B-2 progenitors

1. Prepare 10 μ l pipette and 1 ml differentiation medium in sterile Eppendorf tubes. For picked up colonies, prepare 1.5 ml Eppendorf tubes containing 1 ml staining buffer. Prepare the same number of tubes as the colony number.
2. Before picking up the colony, wet the 10 μ l pipette tip with differentiation medium by pipetting several times to avoid the methylcellulose sticking to the inside of the pipette tips. Set 8 μ l to pick up a colony. Under the microscope, pick up each colony using 10 μ l pipette with a wet tip, and transfer each colony to the 1.5 ml tube containing staining buffer.
3. Once all the colonies are picked up into each tube, centrifuge the tubes at 800 $\times g$ for 2 min in the microcentrifuge and aspirate the supernatant.
4. Add 50 μ l of staining buffer and vortex to make the pellet a single cell suspension.
5. Stain them with the antibodies: anti-mouse AA4.1-APC, CD19-PE, B220-PEcy7, and Mac1-FITC at a ratio of 0.3 μ l/10⁶ cells for 20-30 min on ice.
6. Wash with 1 ml staining buffer and centrifuge at 800 $\times g$ for 2 min.
7. Aspirate the supernatant and suspend the cell pellet with staining buffer with PI or DAPI for dead cell exclusion and apply the samples to flow cytometry.
8. Determine whether the colonies contain B-1 and/or B-2 progenitors (Figure 3) (Kobayashi *et al.*, 2019). Prepare adult BM cells as a positive control for gating B-progenitors (Montecino-Rodriguez *et al.*, 2006).

Data analysis

Data analysis and expected outcome

Based on the flow cytometric analysis, each dish should contain 1) only B-1 progenitors, 2) only B-2 progenitors, and 3) both B-1 and B-2 progenitors. Count number of colonies that contain B-1, B-2, or both B-1 and B-2 progenitors per plate and calculate mean and SD values for the results of three dishes.

Usually, only B-2 colonies are expected from BM progenitors. B-1 progenitor colonies are dominant from embryonic tissues co-cultured with OP9, while B-1+B-2 progenitor colonies become dominant when these embryonic tissues are co-cultured with AGM-ECs or organ cultured (developing to HSCs) (Kobayashi *et al.*, 2019).

Notes

1. AGM-ECs can support the transition of pre-HSCs to HSCs, but they do not support B cell colony formation when they are plated together in Methylcellulose culture.
2. The number of B cell colony forming cells varies depending on the age of the embryo and the condition of the OP9.
3. Ter119⁻CD144^{+c-kit}⁺EPCR⁺ cells are very rare, therefore, Ter119⁻CD144^{+c-kit}⁺ should also be able to produce B cell colonies by support of OP9.
4. Do not add SCF in the co-culture nor methylcellulose culture. Good OP9 support B-lymphopoiesis without SCF and SCF may enhance macrophage differentiation.

Recipes

Culture medium:

1. α-MEM (with penicillin/streptomycin 50 U-50 µg/ml)
 - a. Add powder α-MEM to 1,000 ml fresh Milli-Q water and stir until it is completely dissolved
 - b. Add 2.2 g H₂CO₃ and stir until completely dissolved
 - c. Add 5 ml Penicillin-Streptomycin
 - d. Filter using 0.22 µm filtering system
2. 2-mercaptoethanol (2-ME) stock (0.1 M)
Mix 70 µl of 2-ME to 10 ml α-MEM and filter using 0.22 µm syringe filter
3. OP9 maintenance medium (α-MEM + 20% FBS)
Mix 400 ml α-MEM and 100 ml FBS and filter using 0.22 µm filtering system
4. AGM-EC maintenance medium
IMDM 400 ml
FBS (Hyclone, heat inactivate)100 ml
Pen/Strep (100x) 5 ml

- Heparin 5 ml (10 mg/ml stock prepared fresh in IMDM)
- L-glutamine (100x) 5 ml
- Endothelial mitogen 50 mg
- Dissolve in 10 ml of above media mix
 - Filter using 0.22 µm filtering system
- Differentiation medium (α -MEM +10% FBS + 5×10^{-5} M 2-ME)
 - 450 ml α -MEM
 - 50 ml FBS
 - 250 µl of 0.1 M 2-ME
 - Filter using 0.22 µm filtering system
 - Staining buffer (PBS + 5% FBS + 2 mM EDTA)
 - 500 ml PBS 500 ml
 - 2.5 ml FBS + EDTA (final 2 mM)
 - Filter using 0.22 µm filtering system
 - 0.1% Gelatin
 - 0.5 g Gelatin
 - Milli-Q water 500 ml
 - Autoclave

Acknowledgments

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Competing interests

There is no conflict of interest.

Ethics

The procedure of harvesting mouse embryos and other related animal works follows the Animal Welfare Committee (AWC) protocol approved by the Center for Laboratory Animal Medicine and Care (CLAMC) at UTHealth (AWC16-0124, 2016-2019).

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***In vivo* Mouse Mammary Gland Formation**

Heng Sun, Xin Zhang, Un In Chan, Sek Man Su, Sen Guo, Xiaoling Xu and Chuxia Deng*

Faculty of Health Sciences, University of Macau, Macau SAR, China

*For correspondence: cxdeng@um.edu.mo

[Abstract] For years, the mammary gland serves as a perfect example to study the self-renew and differentiation of adult stem cells, and the regulatory mechanisms of these processes as well. To assess the function of given genes and/or other factors on stemness of mammary cells, several *in vitro* assays were developed, such as mammospheres formation assay, detection of stem cell markers by mRNA expression or flow cytometry and so on. However, the capacity of reconstruction of whole mount in the cleared fat pad of recipient female mice is a golden standard to estimate the stemness of the cells. Here we described a step-by-step protocol for *in vivo* mammary gland formation assay, including preparation of “cleared” recipients and mammary cells for implantation, the surgery process and how to assess the experimental results. Combined with manipulation of mammary cells via gene editing and /or drug treatment, this protocol could be very useful in the researches of mammary stem cells and mammary development.

Keywords: Mammary gland, Whole mount, Mammary stem cells, Tissue reconstruction, Cell transplantation

[Background] As one of the most typical organ of mammals, the mammary gland (MG) is an exocrine gland and responsible for lactation. The development of MG is under the control of certain sexual hormones, whose levels precisely regulate the structure, cellular composition and functional changes of MG in different developmental stages (Hennighausen and Robinson, 2005). Many genetic and environmental factors are involved in the regulation of mammary stem cells and the MG development. To study the functions and mechanisms of these factors, several methods have been developed, especially for the assessment of stemness of mammary cells. Previous studies demonstrated that only the basal cells instead of luminal cells of MG are able to reconstruct the epithelial tree in the cleared fat pads of recipient female mice, indicating the mammary stem cells only exist in the basal lineage (Van Keymeulen et al., 2011). Later, many studies including ours, uncovered several markers for mammary stem cells (Prater et al., 2014; Wang et al., 2015; Sun et al., 2018), whose function in stemness maintenance was confirmed by the *in vivo* MG formation assay. In this protocol, we described in detail how to prepare the donor mammary cells, how to remove the endogenous epithelia from the fat pads of recipients female mice, how to perform the implantation, as well as how to assess the experimental results. Besides of verifying mammary stem cell markers, this protocol could be also very useful in other researches regarding mammary stem cells and mammary development when combined with manipulation of mammary cells via gene editing, gene overexpression or knockdown, and/or drug treatment.

Materials and Reagents

1. 50 ml centrifuge tubes (Corning, catalog number: CLS430829)
2. 1.5 ml micro-tubes (Axygen, catalog number: MCT150-C-S)
3. Pipette tips 10 µl, 200 µl and 1,000 µl (Axygen, catalog numbers: T-400, T-200-Y and T-1000-B)
4. FACS tubes (FALCON, catalog number: 352058)
5. 40 µm cell strainer (FALCON, catalog number: 352340)
6. Cell counting chambers (Nexcelom Bioscience, catalog number: CHT4-SD100)
7. 0.2 µm Nalgene syringe filter (Thermo Fisher Scientific, catalog number: 725-2520)
8. 50 ml syringe (TERUMO, catalog number: SS-50LE)
9. 1 ml syringe without needle (TERUMO, catalog number: SS-01T)
10. 27G x ½" needle (TERUMO, catalog number: NN-2713R)
11. Microscope slides (Thermo Fisher Scientific, catalog number: J1801ASH)
12. Cover glass (Thermo Fisher Scientific, catalog numbers: 102222 and 102240)
13. Three-month-old female FVB mice for preparation of donor cells (as donors, provided by Animal facility core, Faculty of Health Sciences, University of Macau)
14. Three-week-old virgin female nude mice (as recipients, provided by Animal facility core, Faculty of Health Sciences, University of Macau)
15. DMEM/F12 medium (Thermo Fisher Scientific, catalog number: 11330032)
16. Fetal Bovine Serum (FBS) (Thermo Fisher Scientific, catalog number: 26140079)
17. HBSS (Thermo Fisher Scientific, catalog number: 14140112)
18. Hydrocortisone (Sigma-Aldrich, catalog number: H0888)
19. Insulin (Sigma-Aldrich, catalog number: 91077C)
20. EGF (Thermo Fisher Scientific, catalog number: PHG0313)
21. Cholera toxin (Sigma-Aldrich, catalog number: C8052)
22. Collagenase Type 3 (Worthington Biochemical Corporation, catalog number: LS004183)
23. Hyaluronidase (Sigma-Aldrich, catalog number: H3506)
24. Dispase II (Roche, catalog number: 04942078001)
25. Deoxyribonuclease I (Worthington Biochemical Corporation, catalog number: LS002145)
26. HEPES (1 M) (Thermo Fisher Scientific, catalog number: 15630080)
27. DPBS (Thermo Fisher Scientific, catalog number: 14190250)
28. 0.25% Trypsin-EDTA (Thermo Fisher Scientific, catalog number: 25200056)
29. 1x RBC lysis buffer (Thermo Fisher Scientific, catalog number: 00-4333-57)
30. EasySeq Mouse Epithelial Cell Enrichment kit (STEMCELL Technologies, catalog number: 19868)
31. Trypan blue (STEMCELL Technologies, catalog number: 07050)
32. Anti-mouse CD24-PE-Cy7 antibody (BD Biosciences, catalog number: 560536)
33. Anti-mouse CD29-APC antibody (Biolegend, catalog number: 102216)

34. DAPI solution (1 mg/ml) (Thermo Fisher Scientific, catalog number: 62248)
35. Albumin, Bovine (BSA) (VWR LIFE SCIENCE, catalog number: VWRV0332)
36. EDTA (0.5 M), pH 8.0 (Thermo Fisher Scientific, catalog number: AM9260G)
37. DPBS (Thermo Fisher Scientific, catalog number: 14190250)
38. PBS Tablets (Thermo Fisher Scientific, catalog number: 18912014)
39. 2,2,2-Tribromoethanol (Sigma-Aldrich, catalog number: T48402)
40. 2-Methyl-2-butanol (Sigma-Aldrich, catalog number: 152463)
41. Ethanol (Sigma-Aldrich, catalog number: E7023)
42. Chloroform (Sigma-Aldrich, catalog number: C2432)
43. Acetic acid (Sigma-Aldrich, catalog number: A6283)
44. Carmine (Sigma-Aldrich, catalog number: C1022)
45. Aluminum potassium sulfate (Sigma-Aldrich, catalog number: A7210)
46. Xylenes (Sigma-Aldrich, catalog number: 214736)
47. DPX Mountant (Sigma-Aldrich, catalog number: O6522)
48. Implantation buffer (see Recipes)
49. Anesthetics (Avertin, see Recipes)
50. Carnoy's fix buffer (see Recipes)
51. Carmine-alum staining solution (see Recipes)

Equipment

1. Sterile scissors and forceps
2. Tapes (to restrain the mice)
3. Wound closures applier (RWD, catalog number: 12020-09)
4. 9 mm wound clips (RWD, catalog number: 12022-09)
5. Warming pad (Lab Animal Technology Develop Co., catalog number: LAT-BW2)
6. Pipette
7. Biosafety cabinet for cell culture work
8. Chemical hood
9. Centrifuge with adaptors for 50 ml centrifuge tubes and FACS tubes, for use at room temperature.
10. EasySep Magnet (STEMCELL Technologies, catalog number: 18000)
11. Cell counter (Nexcelom Bioscience, model: Cellometer Auto 2000)
12. BD FACSAria III for cell sorting
13. Leica-M165FC stereo microscope
14. Magnetic stirrer (IKA, RH basic)

Software

1. BD FACSDiva 6.1 for BD FACSaria III
2. ImageJ

Procedure

- A. Prepare donor MG cells (details are described in Sun *et al.*, 2020)
 1. Sacrifice the donor female mice and remove the fourth inguinal MGs under clean and sterile condition.
 2. Digest the MGs into single cells. In brief, the mammary glands were minced, washed in DPBS, and digested in DMEM/F-12, which contained 300 units/ml collagenase III, 100 units/ml hyaluronidase, 5% FBS, 5 µg/ml insulin, 10 ng/ml EGF, 20 ng/ml Cholera toxin, and 500 ng/ml hydrocortisone, for about 1 h at 37 °C, 5% CO₂. The resultant organoid suspension was sequentially re-suspended in DMEM/F-12 supplemented with 5 mg/ml dispase II and 0.1 mg/ml DNase I for 5 min at 37 °C, and then digested with 0.25% trypsin-EDTA for 2 min, and treated with RBC lysis buffer for 3 min to remove the red blood cells before filtration through a 40-µm cell strainer to obtain single-cell suspension.
 3. Enrich the epithelial cells using an EasySeq Mouse Epithelial Cell Enrichment kit according to the manufacturer and stain the MG cells with anti-mouse CD24-PE-Cy7 antibody (1:200), anti-mouse CD29-APC antibody (1:200), and DAPI (1 µg/ml) in FACS buffer in ice for 30 min.
 4. Sort out the DAPI⁻CD24^{Mid}CD29^{Hi} (basal cells) and DAPI⁻CD24^{Hi}CD29^{Lo} (luminal cells) cells separately as donor cells.
 5. Wash the cells with DPBS and re-suspend the cells at given concentrations (10 µl of cells/ fat pad) with implantation buffer (Recipe 1).
- B. Clear the fat pads of recipient female mice
 1. Anesthetize a 3-week-old female nude mouse with Avertin (Recipe 2) (administered *i.p.*, at a dose of 250 mg/kg body weight). Select mice those are < 12 g to ensure that mammary ductal growth has not been initiated.
 2. Firmly restrain the mouse with abdomen up by adhering its limbs into a foam surface using tapes (Figure 1A).
 3. Sterilize the abdomen with 70% alcohol.
 4. Grasp the skin with forceps a few millimeters above the pelvis and lift it up from the abdomen. Make a parallel incision (about 10 mm) along the mouse's abdomen without puncturing the peritoneum with scissors and forceps.
 5. Then make a Y-type incision from the bottom of the midline incision to the hip region just above the legs. Pull the skin back from the peritoneum using forceps to expose the mammary gland (Figure 1B).

6. Cut off the bridge between the fourth and fifth mammary glands to prevent the epithelium of the fifth gland from growing into the fourth cleared fat pads.
7. Divide the fat pad of the fourth mammary gland by cutting across it with scissors just near ventral (the side towards nipple) of the node. Then remove the portion of fat pad with the lymph node and the nipple, and leave the other part as cleared fat pad for MG cells implantation later (Figures 1B-1D).
8. Put the removed mammary piece on a microscope slide for fixing and staining with carmine-alum later to confirm that the entire rudimentary epithelial bud has been removed.
9. Repeat the Steps B6-B8, if desired, on the contralateral mammary gland.

C. Implant the donor MG cells into the cleared fat pads

1. After clearing the mammary fat pad, directly inject the MG cells (10 μ l) prepared from Procedure A into the remaining part of the fat pad by using 1 ml syringe with 27G x $\frac{1}{2}$ " needle (Figures 1E and 1F).
2. Close the skin incisions with wound clips (Figure 1G).
3. Place the mouse on a warming pad until it regains consciousness. And keep monitoring the mouse for up to 1 week for any sign of infection or other surgical complications.

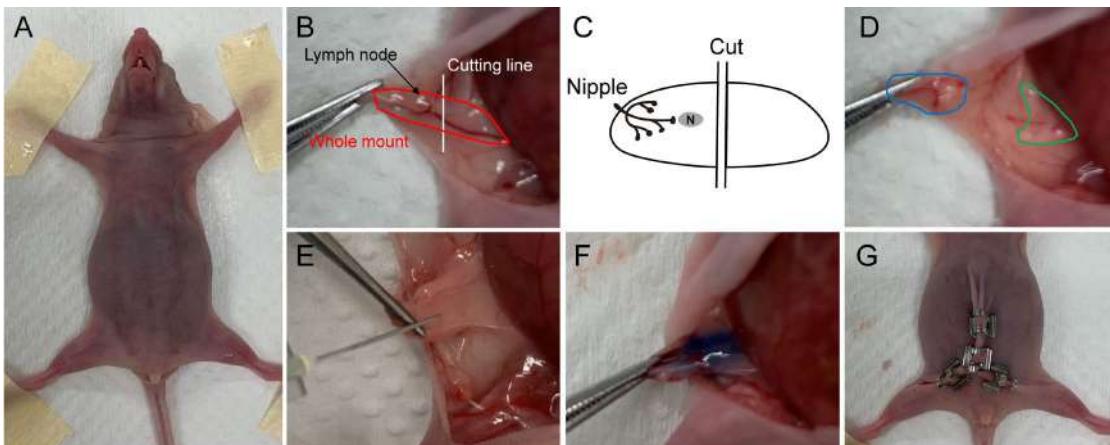


Figure 1. Surgical process. A. The anesthetized mouse was restrained on a foam surface. B. The fourth mammary gland was exposed (the locations of lymph node and cutting line were indicated). C. A schematic diagram shows the locations of nipple, lymph node (labeled as "N") and the cutting line. D. Divide the MG into two parts, the blue circled part was to be removed and the green circled part was the cleared fat pad. E. Injecte the donor cells into the cleared fat pad. F. Cleared fat pad was implanted with MG cells. G. Close the incisions with wound clips.

D. Remove, fix and stain the MG

1. About 8 to 10 weeks after implantation, sacrifice the recipient mouse, dissect out the fourth MG (similar operation as in Procedure B, just remove all the fat pad).
2. Transfer the entire MG on a microscope slide, and use blunt end forceps to spread out the MG

over the glass slide as much as possible without damaging the tissue.

3. Keep the MG on the slide for 5 min at room temperature to allow the MG to stick to the glass surface.
4. Submerge the slide in a 50 ml centrifuge tube containing Carnoy's fix buffer (Recipe 3), and fix the tissue overnight at room temperature.
5. Wash the slide with 70% ethanol for 30 min, then wash it with 50%, 30% ethanol and PBS for 10 min of each step.
6. Stain the MG with carmine-alum staining solution (Recipe 4) at 4 °C overnight.
Note: The staining can be continued for several days.
7. Dehydrate the MG by washing the slide with a gradient ethanol (70%, 95%, 100%, 15 min for each concentration).
8. Submerge the MG in xylene overnight at room temperature in the chemical hood, to remove the fat tissue.
9. Mount the slide with Permout in the chemical hood for long-term storage.

E. Assess the MG formation efficiency

1. Take pictures of the stained whole mounts by using a Leica-M165FC stereo microscope.
2. Measure the size of the reconstructed epithelia and whole mounts by analyzing the picture with ImageJ.
3. Calculate the ratio of epithelial tree size to whole mount size, to indicate the MG formation efficiency.

Data analysis

The mammary gland formation efficiency can be assessed by the branching extent of the mammary epithelial tree. The pictures of stained whole mount could be analyzed by ImageJ. Gate the area of epithelial tree and calculate the size as A (Figure 2); then gate the area of the whole mount and calculate the size as B. Then the MG formation efficiency can be quantified by the ratio of A/B, which ranges from 0 to 1 (0 means no MG formation and 1 means the entire reconstruction of MG). Figure 2 shows three examples of reconstructed MG with different efficiencies.

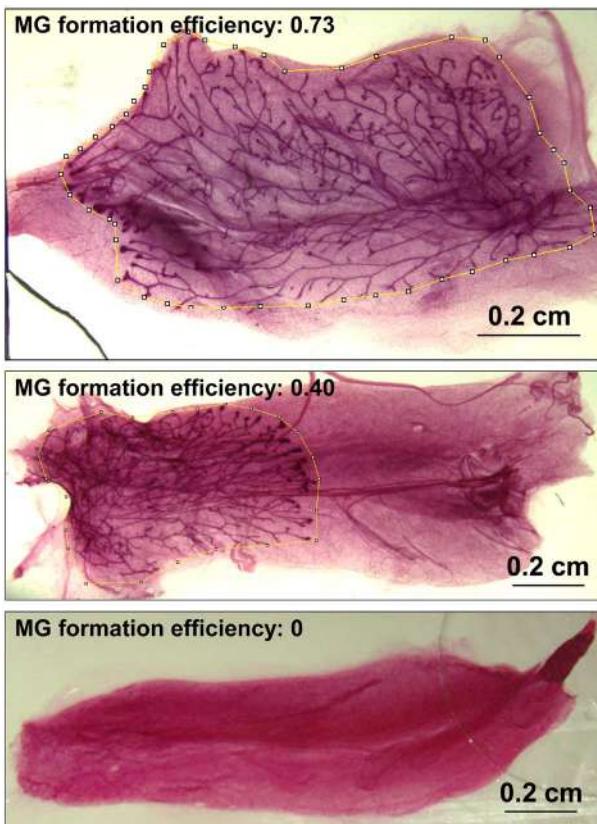


Figure 2. Examples of reconstructed mammary gland. MG formation efficiency was calculated as the ratio of epithelial tree size to whole mount size. The area of the epithelial tree was indicated as yellow circle. The top 2 MG were reconstructed from implantation of 2,000 basal cells, and the bottom MG showed no epithelial tree formation after implantation of 2,000 luminal cells. The recipient mice were kept for 8 weeks after the implantation.

Notes

1. The age and body weight of the recipient mice are very important for the experiment. Select 3-week-old virgin female mice with body weight less than 12 g to ensure that mammary ductal growth has not been initiated.
2. The implantation buffer and the cell type could greatly affect the MG formation efficiency. We found that adding matrigel (30%-50%) to replace FBS could improve the MG formation efficiency.
3. Adjust the MG cell concentration according to your requirement, as only about 10-20 μ l of cells can be implanted in the cleared fat pad. For example, if 2,000 cells will be implanted, make the cell concentration as 2×10^5 cells / ml in implantation buffer, and inject 10 μ l cells per pad.
4. Stain the MG with carmine-alum staining solution at 4 °C overnight to several days, depending on the thickness of MG. Staining could be terminated when the red dye penetrated whole mount.

Recipes

1. Implantation buffer (1 ml)
0.6 ml DMEM/F12 medium
0.3 ml FBS
0.1 ml 0.4% trypan blue
The trypan blue was used to indicate whether the cells were successfully injected as the volume was quite limited
2. Avertin (200 ml)
 - a. Dissolve 2.5 g 2,2,2-Tribromoethanol with 5 ml 2-Methyl-2-butanol by using a magnetic stirrer in the dark overnight
 - b. Then add 195 ml PBS and mix well. Filter the mixture through a 0.2 µm Nalgene syringe filter
 - c. The Avertin could be kept in the dark at 4 °C for several months
3. Carnoy's fix buffer (500 ml)
300 ml ethanol
150 ml chloroform
50 ml acetic acid
4. Carmine-alum staining solution (500 ml)
 - a. Add 1 g carmine and 2.5 g aluminum potassium sulfate to 500 ml demineralized H₂O
 - b. Boil the mixture and let it cool down to room temperature before use
 - c. The carmine-alum staining solution could be kept at room temperature and re-used up to three times

Acknowledgments

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Competing interests

The authors declare that they have no conflict of interest.

Ethics

This protocol involving mice has been approved by the University of Macau Animal Ethics

Committee under protocol UMAEC-050-2015.

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Generation of T cells from Human and Nonhuman Primate Pluripotent Stem Cells

Akhilesh Kumar¹, Saritha S. D'Souza¹, Gene Uenishi¹, Mi Ae Park¹

Jeong Hee Lee¹ and Igor I. Slukvin^{1, 2, 3, *}

¹Wisconsin National Primate Research Center, University of Wisconsin, Madison, WI 53715, USA;

²Department of Cell and Regenerative Biology, School of Medicine and Public Health, University of Wisconsin, Madison, WI 53707, USA; ³Department of Pathology and Laboratory Medicine, University of Wisconsin, Madison, WI 53792, USA

*For correspondence: islukvin@wisc.edu

[Abstract] Pluripotent stem cells (PSCs) have the potential to provide homogeneous cell populations of T cells that can be grown at a clinical scale and genetically engineered to meet specific clinical needs. OP9-DLL4, a stromal line ectopically expressing the Notch ligand Delta-like 4 (DLL4) is used to support differentiation of PSCs to T-lymphocytes. This article outlines several protocols related to generation of T cells from human and non-human primate (NHP) PSCs, including initial hematopoietic differentiation of PSC on OP9 feeders or defined conditions, followed by coculture of the OP9-DLL4 cells with the PSC-derived hematopoietic progenitors (HPs), leading to efficient differentiation to T lymphocytes. In addition, we describe a protocol for robust T cell generation from hPSCs conditionally expressing ETS1. The presented protocols provide a platform for T cell production for disease modeling and evaluating their use for immunotherapy in large animal models.

Keywords: Human pluripotent stem cells, Human embryonic stem cells, Non-human primate pluripotent stem cells, Hemogenic endothelium, Hematopoietic progenitor, T cells, Hematopoietic differentiation

[Background] T lymphocyte (T cells) play a key role in cell-mediated immune responses and are involved in monitoring and killing tumor cells. Throughout the last decades, several strategies have been developed to redirect, culture and/or enhance T lymphocytes against cancer (Houot *et al.*, 2015; June *et al.*, 2018) and utilize them for T cell-based adoptive immunotherapies. Recent clinical trials have shown outstanding outcomes in relapsed and refractory lymphoma patients treated with chimeric antigen receptor (CAR)-T cells (Riviere and Sadelain, 2017).

Human pluripotent stem cells (hPSCs), including embryonic (hESCs) and induced (hiPSCs), provide a promising resource to produce T cells for adoptive cellular immunotherapies, which can be coupled with genetic engineering technologies to generate off-the-shelf supplies of CAR T cells. In addition, generating hPSCs from antigen (Ag)-specific cytotoxic T lymphocytes (CTLs) and redifferentiating them into functional CTLs could enable the scalable production of rejuvenated CTLs (Minagawa and Kaneko, 2014; Kaneko, 2016). Several reports have demonstrated T cell generation from hPSCs (Nishimura *et al.*, 2013; Vizcardo *et al.*, 2013) and the feasibility of hiPSC based CAR T cell therapies (Themeli *et al.*, 2013). However, there is still a need to improve the efficacy of T cell generation and expansion from hPSCs. In addition, further advances in hPSC-based T cell therapies will require their preclinical

evaluation in large animal models. Since macaques are physiologically and immunologically similar to humans, including possessing orthologous MHC genes (Adams *et al.*, 2001), and similarities in killer cell immunoglobulin-like receptors (KIR) with humans (Bimber *et al.*, 2008; Parham *et al.*, 2010), nonhuman primates (NHPs) will be the most appropriate model to address the therapeutic efficacy, safety and immunogenicity of PSC-derived T cells.

Here, we describe an improved method for the derivation of T cells from human and NHP-PSCs with a higher efficiency and shorter time (as soon as 3 weeks) than existing protocols. Differentiation of T cells from hPSCs involves two major steps: induction of hematopoietic progenitor cells (HPs) from hPSCs and their subsequent differentiation into T cells. Our lab previously reported well-established protocols on the induction of hematopoietic lineages from hPSCs on OP9 feeders and in defined feeder- and serum-free conditions (Vodyanik *et al.*, 2005; Vodyanik and Slukvin, 2007; Uenishi *et al.*, 2014). We showed that hemogenic progenitors from different stages of differentiation or different sources were cocultured on OP9-DLL4 to differentiate into T cells (Kumar *et al.*, 2019b). We have also reported a protocol for the induction of hematopoietic lineages from NHP-PSCs (D'Souza *et al.*, 2016). T cells differentiation from both hPSCs or NHP-PSCs proceeds through a CD5⁺CD7⁺ progenitor stage that eventually transitions into CD8⁺CD4⁺ double-positive cells. Altogether, the protocol used for the PSC-derived T cells presents a platform for T cell production to evaluate their utility for adoptive immunotherapies and preclinical testing in large animal models.

Related Information

Hematopoietic differentiation from human PSCs in an OP9 co-culture system

Hematopoietic differentiation of hPSCs on mouse stromal OP9 feeders is performed in serum-containing medium without addition of any cytokines (Vodyanik *et al.*, 2005). In this system, hPSCs undergo stepwise progression toward APLNR⁺PDGFR α ⁺ primitive posterior mesoderm with hemangioblast colony forming cells (HB-CFCs) that reflects primitive hematopoiesis, KDR^{hi}PDGFR α ^{lo}/VEC-hematovascular mesodermal progenitors with definitive hematopoietic potential, immature VE-cadherin (VEC)⁺CD43⁻CD73⁻ HE, which specify into DLL4⁺ arterial hemogenic endothelium (HE) with definitive hematopoietic potential, and DLL4⁻ non-arterial-type HE with mostly primitive hematopoietic potential; and CD34⁺CD43⁺ hematopoietic progenitors (HPs) that include CD43⁺CD235a⁺CD45⁺⁻ HPs, enriched in erythromegakaryocytic progenitors and CD43⁺CD235a/41a⁻ multipotent HPs with a lin⁻CD34⁺CD90⁺CD38⁻CD45RA⁻ hematopoietic stem progenitor cells phenotype, and lymphomyeloid potential (Vodyanik *et al.*, 2006; Choi *et al.*, 2009a and 2009b; Choi *et al.*, 2012; Kumar *et al.*, 2019a and 2019b; Uenishi *et al.*, 2018) (Figure 1). CD43⁺ HPs generated in this coculture can be collected on days 8-9 of differentiation and subsequently cultured on OP9-DLL4 in lymphoid conditions to generate T cells (Kumar *et al.*, 2019b). We also demonstrated that T cells can be generated directly from definitive hemogenic progenitors collected from earlier stages of development (hematovascular mesoderm or HE stage) and cultured in lymphoid conditions on OP9-DLL4 (Kumar *et al.*, 2019b).

Hematopoietic differentiation from human PSCs in a chemically defined system

We have reported defined feeder- and serum-free conditions for generation of blood from hPSCs in chemically defined conditions. In this differentiation system, hPSCs follow stages of development similar to those described in hPSCs cocultured on OP9 feeders, including the formation of VE-Cadherin⁺CD73⁻CD235a/CD43⁻ HE and CD34⁺CD43⁺ HPs with myeloid and T lymphoid potential (Uenishi *et al.*, 2014) (Figure 1). We typically use collagen IV coated plates for differentiation in defined conditions to reduce cost. However, more expensive matrix Tenascin C can be used instead of collagen IV to improve hematopoietic differentiation and promote development of HPs with T cell potential in defined conditions (Uenishi *et al.*, 2014).

Genetic engineering of Doxycycline-inducible iETS1 hPSCs

We have recently reported a protocol for the generation of conditional gene expression of ETS1 under tetracycline responsive element (TRE) promoter along with M2rtTA (reverse tetracycline transactivator) introduced into hPSCs using PiggyBac transposons (Jung *et al.*, 2016; Park *et al.*, 2018a and 2018b). In one vector ETS1 is downstream from the TREtight promoter, along with the zeocin resistance gene driven by the EF1 α promoter, subcloned between the ends of 2 ITRs of the transposon vector. For easy detection of transgene expression upon addition of doxycycline to the culture, ETS1 is linked with Venus through a 2A self-cleaving peptide sequence. The second vector has the M2rtTA promoter linked with a puromycin resistance gene through a 2A peptide sequence subcloned between the ends of 2 ITRs. Using 2 different antibiotic genes facilitates the selection of clones that incorporate both vectors in a single step. The use of a two-vector system allows flexibility to adjust the TRE/M2rtTA ratio to achieve robust doxycycline dependent gene expression in hPSCs while limiting transgene leakage. hPSCs are cultured and then transfected on matrigel plates in mTeSR1 medium. Single hPSC colonies can be picked up from low-density cultures of cell populations (Park *et al.*, 2018a and 2018b).

T cell production from iETS1 hPSCs

We have demonstrated that T cell production from hESCs can be increased through activation of the arterial program at the mesodermal stage of development by overexpression of the transcription factor ETS1. Hemogenic progenitors generated following induction of ETS1 were more than 100-fold enriched in T cell precursors as compared to control (Park *et al.*, 2018b). Doxycycline treatment of differentiation cultures from days 2 to 6, enhances the generation of CD34⁺ HPs with lymphoid potential. HPs collected from day 9 of differentiation cultures in the presence of doxycycline can subsequently be differentiated into T cells in coculture with OP9-DLL4. Although T cell cultures from DOX- and DOX+ conditions generate a similar percentage of CD5⁺CD7⁺ and CD4⁺CD8⁺ T cells, total numbers of T lymphocytes produced per 10⁴ CD43⁺ cells from DOX-treated cultures are dramatically (> 8-fold) greater.

Hematopoietic differentiation of NHP-PSCs on OP9 coculture

Although the defined serum and feeder-free differentiation system described above works well with hPSCs, it does not support efficient generation of CD34⁺CD43⁺ HPs with T cell potential from NHP-

PSCs. That is why we recommend to culture on OP9 feeders to induce efficient production of lymphoid progenitors from NHP-PSCs. OP9 coculture supports blood production from different NHP species, including ESCs and iPSCs derived from rhesus and cynomolgus macaques. However, in contrast to human PSCs, NHP-PCS/OP9 cocultures require addition of GSK3 β inhibitor and VEGF to promote hemogenic mesoderm development and human hematopoietic cytokines to support blood development (D'Souza *et al.*, 2016) (Figure 2). Similar to human, NHP-PSC-derived HPs can be differentiated into T cells in coculture with OP9-DLL4 (D'Souza *et al.*, 2016).

Materials and Reagents

A. Cell lines

1. hESC WA01 and WA09 (WiscBank, WiCell, Madison, WI)
2. Transgene-free iPSCs, DF-19-9-7T and 4-3-7T33 (WiscBank, WiCell, Madison, WI)
3. Mouse embryonic fibroblasts (MEFs, WiCell, Madison, WI)
4. Non-human primate PSCs (NHP-iPSCs), RhF5-iPS 19.1, Cy.F 3L iPS, and Cy0669#1 iPS (D'Souza *et al.*, 2016)
5. OP9 mouse bone marrow stromal cell line (Provided by Dr. Toru Nakano, Osaka University, Japan)
6. Lenti-X™ 293T Cell Line (Clontech, catalog number: 632180)

B. Reagents

1. α -MEM basal medium, powder (Life Technologies, catalog number: 12000-022)
2. DMEM/nutrient mixture F-12, powder (Life Technologies, catalog number: 12400-024)
3. DMEM powder (Life Technologies, catalog number: 12100-046)
4. Iscove's Modified Dulbecco's Medium (IMDEM), powder (Life Technologies, catalog number: 122-00036)
5. Ham's F-12 Nutrient Mixture (F12), powder (Life Technologies, catalog number: 217-00075)
6. Chemically Defined Lipid Concentrate (CDLC) (Life Technologies, catalog number: 119-050-31)
7. GlutaMax (Life Technologies, catalog number: 35050-061)
8. Non-essential Amino Acids (NEAA) (Life Technologies, catalog number: 11140-076)
9. DPBS powdered, without calcium and magnesium (Life Technologies, catalog number: 21600-044)
10. EDTA 0.5 M, pH 8.0 (Life Technologies, catalog number: 15575-038)
11. KnockOut Serum Replacement (KOSR) for hPSCs (Life Technologies, catalog number: 10828-023)
12. TrypLe Select (Life Technologies, catalog number: 12563-029)
13. Collagenase type IV (Life Technologies, catalog number: 17104-019)
14. Puromycin (Life Technologies, catalog number: A1113803)
15. Primate ES cell medium (REPROCELL, catalog number: RCHEMD001)

16. mTeSR1 defined feeder-free medium (StemCell Technologies, catalog number: 05850)
17. TeSR-E8 (E8) medium (Stem Cell Technologies, catalog number: 05990)
18. Vitronectin XF™ (VTN) (Stem Cell Technologies, catalog number: 07180)
19. Sodium Selenite (Millipore Sigma, catalog number: S5261)
20. Tenascin C (Millipore Sigma, catalog number: CC065)
21. Collagen IV (CollIV) (Millipore Sigma, catalog number: C5533)
22. Acetic Acid (Millipore Sigma, catalog number: 537020)
23. Holo-transferrin (Millipore Sigma, catalog number: T0665)
24. Lithium Chloride (Millipore Sigma, catalog number: L9659)
25. 7-Aminoactinomycin D (7-AAD; Millipore Sigma, catalog number: A9400)
26. Human insulin (Millipore Sigma, catalog number: 19278)
27. Monothioglycerol (MTG) (Millipore Sigma, catalog number: S5261)
28. HEPES (Millipore Sigma, catalog number: H4034)
29. Dextrose (Millipore Sigma, catalog number: G8270)
30. 2-mercaptoethanol (Millipore Sigma, catalog number: M7522)
31. Gelatin from porcine skin, Type A (Millipore Sigma, catalog number: G-1890)
32. Hexadimethrine bromide, Polysorbate 20 (Millipore Sigma, catalog number: H9268)
33. Fetal bovine serum, defined (HyClone, catalog number: SH30070.03)
34. Trypsin 0.05%/EDTA 0.5 mM (HyClone, catalog number: SH30236.02)
35. Recombinant Human Flt3-Ligand (Peprotech, catalog number: 300-19)
36. Recombinant Human FGF basic (Peprotech, catalog number: 100-18B)
37. Recombinant Human Stem Cell Factor (SCF) (Peprotech, catalog number: 300-07)
38. Recombinant Human Activin A (ActA) (Peprotech, catalog number: 120-14E)
39. Recombinant Human Interleukin 3 (IL-3) (Peprotech, catalog number: 200-03)
40. Recombinant Human Thrombopoietin (TPO) (Peprotech, catalog number: 300-18)
41. Recombinant Human Interleukin 6 (IL6) (Peprotech, catalog number: 200-06)
42. Recombinant Human Bone Morphogenic Protein 4 (BMP4) (Peprotech, catalog number: 120-05ET)
43. Recombinant Human Vascular Endothelial Growth Factor (VEGF) (Peprotech, catalog number: 100-20)
44. Recombinant Human Interleukin 7 (IL7) (Peprotech, catalog number: 200-07)
45. GSK-3β inhibitor (CHIR99021) (Tocris, catalog number: 4423)
46. Rock inhibitor (Tocris, catalog number: 1254)
47. Human ES cell qualified Matrigel (Corning, catalog number: 354277)
48. Calcium Chloride (CaCl_2) (Alfa Aesar, catalog number: 33296)
49. Potassium Chloride (KCl) (Fisher Scientific™, catalog number: BP366-1)
50. Sodium Chloride (NaCl) (Fisher Scientific™, catalog number: S642-212)
51. Sodium Bicarbonate (Fisher Scientific™, catalog number: S233-500)
52. Sodium azide, NaN_3 (Fisher Scientific™, catalog number: BP922-500)

53. BSA fraction V (Fisher Scientific™, catalog number: BP1600-100)
54. Sodium Phosphate Dibasic Anhydrous (Na_2HPO_4) (Fisher Scientific™, catalog number: BP332-1)
55. Polyvinyl alcohol (PVA) (MP Biomedicals, catalog number: 151-941-83)
56. Doxycycline (DOX) (MP Biomedicals, catalog number: 198955)
57. Antibodies (Table 1)

Table 1. List of antibodies used

Antigen	Fluorochrom	Clone	Company	Cat. No.	Species
CD3	FITC	SK7	BD Biosciences	349201	Human
CD3	APC	S4.1	Caltag-Invitrogen	MHCD0305	Human
CD3	PE	SP34	BD-Bioscience	552127	NHP
CD4	APC	RPA-T4	BD Biosciences	555449	Human
CD4	PE	L200	BD Biosciences	550630	NHP
CD5	PE	UCHT2	BD Biosciences	555353	Human
CD5	APC	UCHT2	Biolegend	300611	NHP
CD7	FITC	M-T701	BD Biosciences	555360	Human
CD7	PE	M-T701	BD Bioscience	555361	NHP
CD8	PE	HIT8a	BD Biosciences	555635	Human
CD8	APC	SK1	Biolegend	344721	NHP
CD43	FITC	1G10	BD Biosciences	555475	Human
DLL4	PE	447506	R&D Systems	FAB1506P	Human
TCR $\alpha\beta$	PE	T10B9.1A-BD	BD Biosciences	555548	Human
TCR $\alpha\beta$	APC	R73	Biolegend	201110	NHP
CD34	PE	563	BD Biosciences	550761	NHP
CD45	FITC	MB4-6D6	Miltenyl Biotech	130-119-764	NHP

C. Materials

1. Cell strainer, 40 μm (Fisher Scientific™, catalog number: 223663547)
2. Cell strainer, 70 μm (Fisher Scientific™, catalog number: 22363548)
3. 9" Pasteur pipets, Flint glass (Fisher Scientific™, catalog number: 1367820D)
4. Serological Pipettes (10 ml, VWR International, catalog number: 89130-898)
5. Serological Pipettes (95 ml, VWR International, catalog number: 89130-896)
6. Nalgene Disposable bottle top filter, Polyethersulfone membrane with 0.2 μm pore size (Fisher Scientific™, catalog number: 595-4520)
7. 0.5 ml microcentrifuge tube, autoclavable (Fisher Scientific™, catalog number: 05-408-120)
8. Serological pipet, 1 ml Nonpyrogenic (Fisher Scientific™, catalog number: 13-678-11B)
9. Borosilicate glass pipets, 5 ml (Fisher Scientific™, catalog number: 1367827F)
10. 5 ml Polystyrene round-bottom tube, 12 \times 75mm, non-sterile (BD Bioscience, catalog number: 352008)
11. MACS separation columns, LS (Miltenyi Biotec, catalog number: 130-042-401)

12. MACS Multistand (Miltenyi Biotec, catalog number: 130-042-303)
13. Pre-separation filters with 30 µm nylon mesh (Miltenyi Biotec, catalog number: 130-041-407)
14. Tissue culture dishes, polystyrene 100 × 20 mm (Thermo Scientific, catalog number: 130182)
15. Tissue culture 6-well plate, Polystyrene flat bottom (Thermo Scientific, catalog number: 130184)
16. 15 ml Polypropylene Conical tubes (Thermo Scientific Nunc, catalog number: 339650)
17. 50 ml Polypropylene Conical tubes (Thermo Scientific, catalog number: 339652)
18. Steriflip Filter Units, 50 ml Vacuum filtration system with 0.22 µm pore size membrane (Millipore Sigma, catalog number: SCGP00525)
19. Serological pipet, 5 ml Nonpyrogenic (VWR, catalog number: 89130-896)
20. Serological pipet, 10 ml Nonpyrogenic (VWR, catalog number: 89130-898)
21. Sterling Nitrile-xtra powder-free exam gloves (Kimberly-Clark, catalog number: 53139)

D. Medium and solutions

1. Human PSC growth Medium (see Recipes)
2. MEF growth medium (see Recipes)
3. NHP-iPSC Primate PSCs Medium (see Recipes)
4. Mouse OP9/OP9-DLL4 bone marrow stromal cell culture medium (see Recipes)
5. Human hematopoietic differentiation (OP9 coculture system) medium (see Recipes)
6. NHP differentiation medium (see Recipes)
7. IF4S Stock Medium (see Recipes)
8. 5x PVA Stock Solution (see Recipes)
9. IF9S Medium (see Recipes)
10. T lymphoid differentiation medium (see Recipes)
11. HBS saline solution (2x) (see Recipes)
12. CaCl₂ solution (2 M) (see Recipes)
13. Gelatin solution (0.1% (wt/vol)) (see Recipes)
14. Magnetic cells sorting (MACS) buffer (see Recipes)
15. Flow cytometry buffer (see Recipes)
16. Reconstitution of cytokines (see Recipes)
17. Collagenase solution (1 mg/ml) (see Recipes)
18. Doxycycline (1 mg/ml) (see Recipes)

Equipments

1. MACSQuant analyzer (Miltenyl Biotech, catalog number: 130-096-343)
2. Cellometer^R Auto 2000 cell Viability Counter (Nexcelom Bioscience, model: 2000)
3. Hemocytometer, Reichert Bright-Line counting chamber (Fisher ScientificTM, catalog number: 02-671-5)
4. MACSmix Tube Rotator (Miltenyi Biotec, catalog number: 130-090-753)

5. Water bath (Fisher Scientific, catalog number: 16-462-10)
6. Thermo IEC Centra CL2 Centrifuge (Thermo Scientific, model: 4992)
7. Microcentrifuge (Eppendorf, model: 5418)
8. Centricon Plus-70 Centrifugal Filter (Millipore Sigma, catalog number: UFC701008)
9. Sterile biosafety cabinet (The Baker Company, model: SG603)
10. 37 °C/5% CO₂ incubator (Thermo Scientific, model: MCO-19A1)
11. Hypoxia incubator (Thermo Scientific, model: MCO-19M-PA)
12. Inverted microscope with objective lenses 4x, 10x and 20x (Olympus, model: IX71)
13. Object marker, Cell dotter for inverted microscope (Nikon, catalog number: MBW10020)
14. Balance (Denver Instrument, model: APX60)
15. Milli-Q water purification system (Millipore, Billerica, MA, USA)
16. Pipet-Aid, Filler/Dispensers (Drummond, model: 4-000-300)
17. Liquid waste disposal system for aspiration
18. Beckman Optima XL-A analytical ultracentrifuge (Beckman Coulter, catalog number: 369005)
19. Bright-Field microscope (Olympus, model: CKX31SF)

Software

1. FlowJo™ software (v10.6.1) (BD Bioscience, <https://www.flowjo.com/>)
2. GraphPad Prism 7 (GraphPad, www.graphpad.com)

Procedure

A. Coating of matrix

1. Prepare gelatin-coated 10 cm culture dish (10 ml/dish) or 6-well plate (2 ml/well) by adding sterile gelatin solution and allowing the gelatin solution to cover the entire plastic surface. Incubate the plasticware at least 3 h at 37 °C in an incubator. Gelatin coated dishes/ plates are used for culture for OP9/OP9-DLL4/MEFs.
2. Prepare VTN or Matrigel-coated 6-well plate by adding 2 ml of the coating solution to each well of the 6-well plate and incubate at 4 °C overnight. The coated plate can be stored in 4 °C for up to 1 month. VTN/Matrigel coated plates are used for culture of PSCs under feeder free conditions.
3. Prepare a TenC or CollIV coating solution by adding 2ml of the coating solution to each well of a 6-well plate and incubate at 4 °C overnight. The coated plate can be stored in 4 °C for up to 1 month. TenC/CollIV coated plates are used for hematopoietic differentiation of PSCs under feeder free conditions.

B. Culture of hPSCs/NHP-PSCs on feeder cells (MEFs)

1. Resuspend inactivated MEFs (provided by WiCell) at 2 × 10⁵ cells/ml in pre-warmed (37 °C)

MEF growth medium.

2. Add 2 ml/well of prewarmed MEF growth medium and then dispense MEF suspension on a gelatin-coated 6-well plate (1 ml/well) and distribute MEFs evenly with a back and forth movement of the plate.
3. Incubate MEF plates in a 5% CO₂ incubator at 37 °C for at least 24 h before adding hPSCs or NHP-PSCs.
4. Aspirate growth medium from 1 well of the 6-well plate of hPSCs or NHP-PSCs.
5. Add 2 ml/well of collagenase IV solution (1 mg/ml) and incubate at 37 °C in CO₂ incubator until the edges of the hPSCs or NHP-PSCs colonies begin to detach (approximately 7-10 min for hPSCs and 4-5 min for NHP-PSCs).
6. Aspirate collagenase, add 2 ml of growth medium and disperse the colonies into small cell aggregates by pipetting gently for couple of times until all cells are in suspension.
7. Transfer the cells to a 15 ml conical tube and centrifuge at 300 × g at room temperature for 3 min.
8. Aspirate the medium gently, resuspend the cells in 4-6 ml growth medium and break hPSC or NHP-PSCs colonies into small aggregates with a glass pipette.
9. Plate the cell suspension onto a confluent MEF grown 6-well plate from Step B3 (Splitting ratio for hPSCs is 1:6 and for NHP-PSCs is 1:4).

C. Culture of hPSCs or iETS1 hPSCs in feeder-free conditions

1. Aspirate VTN or Matrigel coating solution from each well and add 1 ml of TeSR-E8 (E8) in case of a VTN plate or mTeSR1 for a Matrigel plate.
2. To passage the PSCs, aspirate spent media from one well of PSCs culture plate and add EDTA-PBS solution (2 ml/well).
3. Incubate the plate for 3-4 min at 37 °C with 5% CO₂.
4. Carefully aspirate EDTA-PBS solution and add 1 ml of E8 or mTeSR1.
5. Gently pipette to dislodge the cells and collect into a 15 ml conical tube.
6. Adjust the volume to 6 ml with E8 or mTeSR1.
7. Mix well and add cell suspension (1 ml/well of 6-well plate) to VTN or Matrigel-coated plate.
8. Thereafter change spent media to fresh E8 or mTeSR1 media daily.

D. Human DLL4 lentivirus production

1. Subculture 293T cells the day before transfection to 60-70% confluence in 9 ml of DMEM medium with 10% FBS per 100 mm dishes.
2. On day 0, perform Calcium phosphate (CaPO₄) transfection of 293T cells in the morning. For CaPO₄ transfection, for each dish, make a mixture containing 10 µg of expression lentiviral vector containing hDLL4 (pSIN4-EF1α-hDLL4-IRES-Puro), 10 µg of packaging vector, 7.5 µg of envelope vector, 62 µl of 2 M CaCl₂ and make up the volume to 500 µl with deionized autoclaved water.

3. Add 500 μ l of 2x HBS dropwise into this mixture while bubbling using another 1 ml pipette.
4. Bubble the mixture for an additional 5-10 s. Incubate the mixture for 15-20 min at room temperature.
5. Add the mixture to the 293T cells dropwise while continuously swirling the dish.
6. Place the dish into a 5% CO₂ incubator at 37 °C for 6-8 h.
7. Aspirate the medium after 8 h of incubation.
8. Add 8 ml of fresh medium to the dishes and continue the culture for another 2 days.
9. On day 3, harvest the medium containing virus into 50 ml tubes.
10. Centrifuge the supernatant at 700 $\times g$ for 10 min at room temperature.
11. The supernatant containing the virus can be stored at -70 °C or concentrated using ethylene oxide-sterilized Centricon Plus-70 or Amicon Ultra-15 Centrifugal Filter Units.
12. To calculate the amount of Infectious Units (IFU) per ml of viral concentrate, transduce HeLa cells with the virus and select with puromycin (1 μ g/ml).

E. Generation of human DLL4 expressing mouse OP9 stromal cells

1. Aspirate the gelatin solution from the dish. Do not allow dishes to dry.
2. Thaw OP9 cells quickly in a 37 °C water bath.
3. Pipette the cell suspension slowly and transfer the contents to the 15-ml tube containing 5 ml of medium.
4. Centrifuge the cells at 300 $\times g$ for 5 min at 4 °C and resuspend the cells in 10 ml of OP9 medium.
5. Transfer the resuspended cells into a 10-cm dish, and place the dish in a 37 °C incubator with 5% CO₂.
6. For viral transduction, add polybrene (6 μ g/ml) to the culture medium.
7. Add hDLL4 lentivirus to the cells at an MOI of 10 and incubate the culture dish at 37 °C with 5% CO₂ for 24 h.
8. Repeat viral transduction one more time as described in E7-E8.
9. Select the transduced OP9-DLL4 cells by adding 20 ng/ml of puromycin into the culture medium.
10. To confirm the transduction of hDLL4 into OP9, resuspend the cells into 100 μ l FACS buffer and stain with human DLL4 antibody for 20 min at room temperature (Table 1).
11. Wash the stained cells with FACS buffer and perform flow cytometry.

F. Maintenance of OP9/OP9-DLL4 cells

1. To passage the cells, aspirate OP9 growth medium and wash OP9/OP9-DLL4 cells twice with 10 ml of PBS.
2. Add 5 ml of trypsin/EDTA [0.05% (wt/vol)] solution and incubate for 10 min at 37 °C in a 5% CO₂ incubator.
3. Add 5 ml of OP9 growth medium and collect cells by pipetting up and down until single cell suspension is formed.
4. Transfer cell suspension into a 15-ml conical tube and centrifuge for 5 min at 300 $\times g$ at room

temperature.

5. Aspirate supernatant and resuspend the cells in 1 ml of OP9 growth medium.
6. Add 100 μ l of the cell suspension to 10 ml of OP9 growth medium and plate cells onto 10-cm gelatin-coated culture dishes.
7. When cultures are confluent, split one dish for maintenance.
8. 8-9 days old OP9 dishes should be used for hematopoietic differentiation for human ESCs and 5-6 days old OP9 dishes should be used for NHP-iPSCs hematopoietic differentiation.
9. 3-4 days old OP9-DLL4 should be used for subsequent protocol, *i.e.*, T lymphoid differentiation.

G. Hematopoietic differentiation

Human PSCs on OP9

1. From 1 well of a 6-well hPSC plate, aspirate hESC growth medium. Add 2 ml of collagenase IV solution (1 mg/ml) and incubate cells for 10 min at 37 °C (Figure 1).

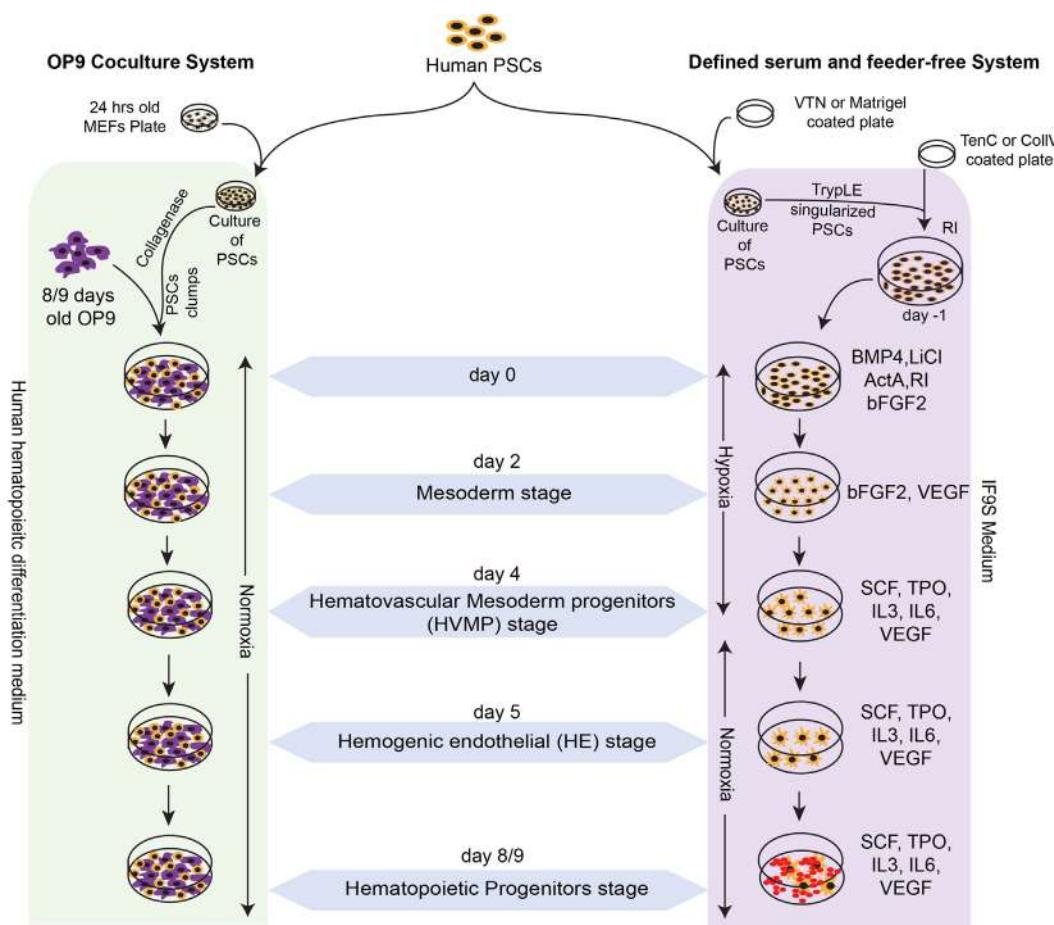


Figure 1. Schematic diagram shows the culture and hematopoietic differentiation of human pluripotent stem cells in serum free chemically defined feeder-free condition and on OP9 coculture system. Use various MACS enriched progenitor from OP9 coculture system and floating cells from feeder free system for T lymphoid differentiation.

2. Aspirate collagenase IV solution and add 2 ml per well of growth medium directly to the well and break up colonies into small cell aggregates by gently pipetting. Transfer cells into a 15-ml conical tube.
3. Centrifuge cells at 300 $\times g$ for 5 min at room temperature. Aspirate the medium gently without disturbing the pellet.
4. Resuspend the pelleted cells into 10 ml of differentiating medium.
5. For coculture, aspirate the OP9 growth medium from overgrown OP9 dishes.
6. Add 10 ml of cell suspension from Step 4 on OP9 dishes.
7. Evenly spread the cells on the OP9 Monolayer by moving the dish in a back/forth and right/left movement twice and keep at 37 °C in a 5% CO₂ incubator.
8. On day 1 aspirate all of the medium and replace it with 10 ml of differentiation medium.
9. On day 4 and day 6, aspirate 5 ml of spent media and add 5 ml of fresh differentiating medium.
10. On day 9, aspirate all of the medium and add 5 ml of collagenase solution to each dish of coculture and incubate for 20 min at 37 °C in a CO₂ incubator.
11. Remove the collagenase solution and keep it on ice in a 50-ml conical tube.
12. Add 5 ml of Trypsin/EDTA solution [0.05% (wt/vol)] to the dish and incubate for 10 min at 37 °C in a 5% CO₂ incubator.
13. Add 5 ml per dish of MACS buffer, suspend cocultured cells by pipetting and transfer to the collection tube from Step 11.
14. Centrifuge the cell suspension at 300 $\times g$ for 5 min at room temperature.
15. Resuspend the pelleted cells with 5 ml of MACS buffer and centrifuge at 300 $\times g$ for 5 min at room temperature.
16. Resuspend cells in 0.5-1 ml of MACS buffer.
17. Stain the cells with anti-human CD43-FITC antibody.
18. Place the tube on the MACS mixer and incubate at 4 °C for 20-25 min.
19. Wash cells with ice-cold MACS buffer as described in Step 15.
20. Resuspend in 80 µl of MACS buffer and add anti- FITC magnetic beads.
21. Repeat Step 18.
22. Wash cells with ice-cold MACS buffer, as described in Step 15, and resuspend in 2 ml of MACS buffer.
23. Filter cells through a 30 µm pre-separation filter.
24. Assemble the MACS-LS separation unit according to the manufacturer's instructions.
25. Rinse the column with 2 ml of MACS buffer.
26. To purify CD43⁺ multipotent progenitors, apply the cell suspension from Step 22 into the LS column and allow cells to pass completely through the column into the collection tube.
27. Wash the column with 2 ml of MACS buffer and collect it in the same collection tube.
28. Discard the cell suspension in the collection tube.
29. Remove the column from the magnet and place it on an empty 15-ml tube.
30. Wash out CD43⁺ cells with 5 ml of MACS buffer using the plunger supplied with the column.

31. Centrifuge cells at $300 \times g$ for 5 min at 4°C .
32. Resuspend cells in 0.2 ml of MACS buffer.
33. The MACS enriched CD43 $^{+}$ multipotent hematopoietic progenitor cells are ready to be used for T lymphoid differentiation.

Human PSCs or iETS1-PSCs in a defined serum and feeder-free system

1. Prepare a single-cell suspension of human ESCs by using TrypLE select. Add 1ml/well of TrypLE solution to 80% confluent ESC plate and incubate for 4 min 37°C with 5% CO₂ (Figure 1).
2. Harvest the cells in 1ml TrypLE solution and collect it into a 15 ml conical tube with 9 ml of E8 medium.
3. Centrifuge $300 \times g$ for 5 min at room temperature.
4. Resuspend the pelleted cells in 6 ml of fresh E8 media and count the cells.
5. Aspirate TenC or CollV coating solution from each well of the coated plate.
6. Add 1 ml of E8 media with 10 μM of rock inhibitor to each well of the coated plate.
7. Transfer the optimized number of cells from Step F4 to a new 15 ml conical tube and adjust the volume to 6 ml.
8. Add 1 ml of E8 containing ESCs into each well of Ten-C coated plates.
9. Incubate overnight at 37°C in 5% CO₂.
10. On day 0, aspirate E8 media and add 2 ml/well of differentiating medium (IF9S) containing Activin A (18 ng/ml), BMP4 (50 ng/ml), bFGF2 (50 ng/ml), LiCl (2 mM) and Rock inhibitor (1 μM).
11. Place the cells in a hypoxia (5% O₂) incubator. During the next two days, do not remove the plate from the incubator.
12. On day 2, aspirate the spent differentiating medium and add 2 ml/well of fresh differentiating media with VEGF (50 ng/ml) and bFGF2 (50 ng/ml). Treat iETS1-PSCs differentiating plate with Doxycycline (2 $\mu\text{g}/\text{ml}$).
13. Place the cells back in the hypoxic incubator, *i.e.*, 37°C with 5% CO₂.
14. On day 4, aspirate the spent differentiating medium and add 2 ml/well of fresh differentiating medium with SCF (50 ng/ml), VEGF (50 ng/ml), TPO (50 ng/ml), IL6 (50 ng/ml), IL3 (10 ng/ml) and FLT3-L (10 ng/ml). Treat iETS1-PSCs differentiating plate with Doxycycline (2 $\mu\text{g}/\text{ml}$).
15. Place the cells in the normoxic incubator.
16. On day 6, add 1 ml/well of fresh differentiating medium with cytokines from Step 14.
17. On days 8-9, collect the floating multipotent HPs by passing through a 70 μm filter and use them for further Lymphoid differentiation.

Nonhuman Primate PSCs on OP9

1. From 1 well of a 6-well NHP-PSCs plate, aspirate NHP-PSC growth medium. Add 2 ml of collagenase IV solution (1 mg/ml) and incubate the cells for 5 min at 37°C .

2. Aspirate collagenase IV solution and add 2 ml per well of growth medium directly to the well and break up colonies into small cell aggregates by gently pipetting. Transfer cells into a 15-ml conical tube.
3. Centrifuge cells at 300 $\times g$ for 5 min at room temperature. Aspirate the medium gently without disturbing the pellet.
4. Resuspend the pelleted cells into 10 ml of Primate differentiating medium.
5. Remove 6-7 days old OP9 dishes prepared for coculture from the incubator and aspirate OP9 growth medium (Figure 2).

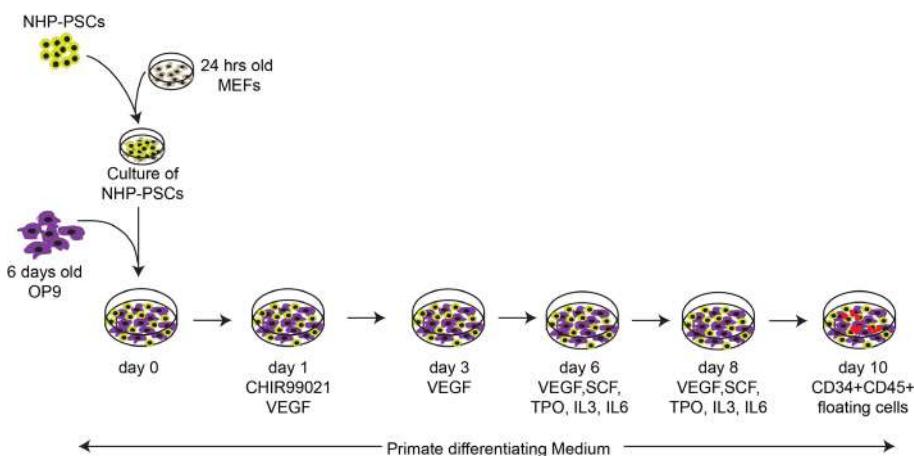


Figure 2. Schematic representation of the established differentiation protocol for induction of mesoderm and blood formation for the NHP iPSCs. Hematopoietic differentiation of NHP-PSCs was induced in coculture with OP9 in the presence of GSK3b inhibitor. Use floating CD34 $^{+}$ CD45 $^{+}$ cells for T lymphoid differentiation.

6. Add the cells from Step 4 to the OP9 dish and move the dish back and forth to uniformly spread the cells on the OP9 monolayer (day 0).
7. On day 1 of coculture, aspirate the medium and add 10 ml of primate differentiation medium with 4 μ M of CHIR99021 and 50 ng/ml of VEGF.
8. On day 3, aspirate the medium and add fresh 10 ml of fresh primate differentiation media with 50 ng/ml of VEGF.
9. On day 6, hematopoietic cytokine cocktail containing 50 ng/ml of VEGF, 50 ng/ml of SCF, 20 ng/ml of TPO, 20 ng/ml of IL-3 and 20 ng/ml of IL-6 in primate differentiating medium is added to the coculture.
10. On day 8 repeat Step 9.
11. On day 10 of coculture, collect the floating multipotent HPs are collected by passing through a 70 μ m filter and use them for further Lymphoid differentiation.

H. Lymphoid differentiation on OP9-DLL4 coculture system

1. Prepare the OP9-DLL4 containing 6-well plates by seeding 0.5×10^5 OP9-DLL4 cells per well

(Figure 3).

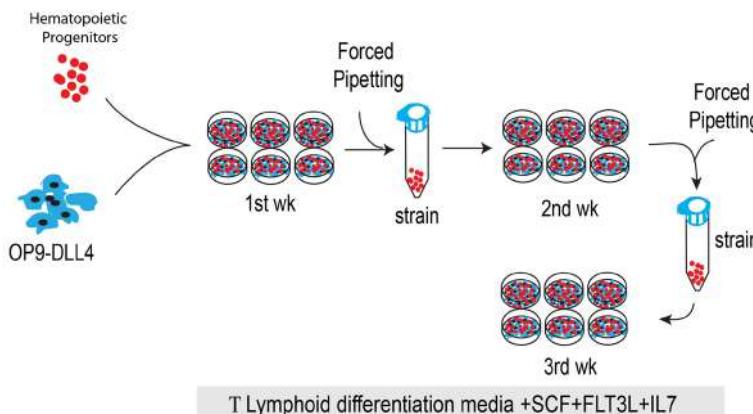


Figure 3. Schematic diagram shows the T lymphoid differentiation of HPs from different sources. Collect and count the HPs from OP9/hPSC or OP9/NHP-PSCs or feeder-free system and culture on OP9-DLL4 to induce T cell differentiation.

2. Culture the cells at 37 °C with 5% CO₂ for 3-4 days or until a monolayer of cells is formed.
3. Once the OP9-DLL4 plate is ready for coculture, seed the HPs cells from different sources (1 x 10⁵-3 x 10⁵ cells/well of 6-well plate) on the OP9-DLL4 with T cell media (OP9 medium containing 20% FBS with FLT3-L (5 ng/ml), IL-7 (5 ng/ml) and SCF (10 ng/ml)).
4. Culture the plates for 3-4 weeks at 37 °C with 5% CO₂ in an incubator and passage weekly.
5. On day 3, add 1ml of fresh T cell differentiation media and prepare a new OP9-DLL4 plate for cell transfer on days 6-7.
6. On days 6-7, dislodge the progenitor cells by vigorously pipetting and collect the cells into a 15 ml conical tube (Figure 3).
7. Filter the cells using a 40 µm strainer.
8. Centrifuge the cells at 300 x g for 5 min at room temperature.
9. Aspirate the supernatant and resuspend the cells in 2 ml of T cell medium and transfer onto a fresh OP9-DLL4 monolayer (Figure 3).
10. On day 10, add 1 ml of fresh T cell differentiation media and prepare a new OP9-DLL4 plate for cell transfer on days 13-14.
11. On days 13-14, repeat Steps H6-H10.
12. On day 18, add 1ml of fresh T cell differentiation media and prepare a new OP9-DLL4 plate for cell transfer on days 20-21.
13. On days 20-21, repeat Steps H6-H10.
14. On day 24, add 1ml of fresh T cell differentiation media and prepare a new OP9-DLL4 plate for cell transfer.
15. On day 27, harvest the cells by vigorously pipetting and straining into a 15 ml conical tube.
16. Centrifuge the cells at 300 x g for 5 min at room temperature. Aspirate the supernatant and resuspend the cells in 1 ml of flow cytometry buffer.

17. Evaluate the T cell differentiation by flow cytometry analysis of T cell markers (CD5, CD7, CD8, CD4, CD3, and TCR $\alpha\beta$) (Table 1) using MACSQuant Analyser 10 (Figure 4).

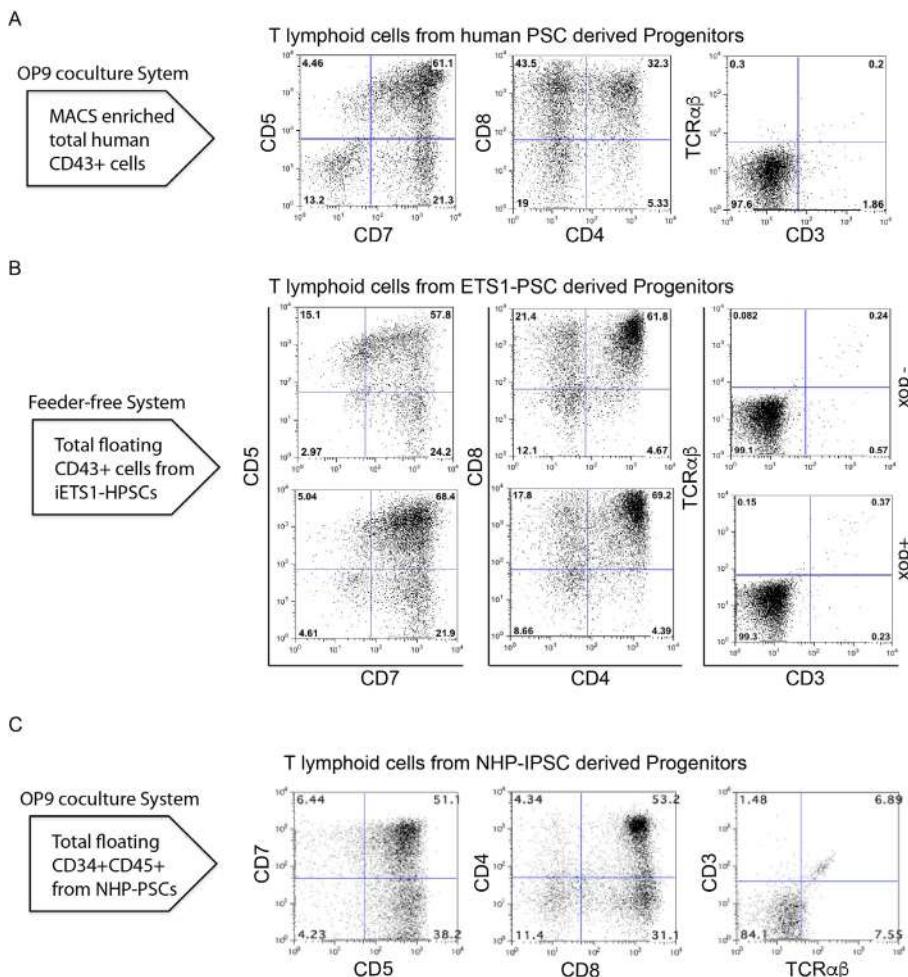


Figure 4. T cell potential of HPs. A. Flow cytometric profile of T cells generated after 3 weeks of culture of total CD43⁺ on OP9-DLL4 from the indicated human PSCs differentiated on OP9 for 8/9 days. B. Flow cytometric profile of T cells generated from iETS1-PSCs. iETS1-PSCs were differentiated with doxycycline or without doxycycline in chemically defined feeder free system for 9 days and floating cells were collected on day 9. C. Flow cytometric profile of T cells generated after 3 weeks of culture of CD34⁺CD45⁺ on OP9-DLL4 from NHP-PSCs.

Data analysis

1. The flow cytometry data was analyzed using FlowJo software (v10.6.1). Live cells were gated based on the 7AAD population to remove the dead cells.
2. The significance of differences between the mean values was determined by one-way ANOVA followed by Tukey post hoc test as appropriate using GraphPad Prism software (GraphPad, San Diego, CA).

Notes

1. hPSCs cultured on MEFs needs to be split once every 6-7 days whereas NHP-PSCs cultures need to be split once every 4 days.
2. Growth medium used to culture PSCs depends on the type of feeder/ matrix used for culture. For hPSCs grown on vitronectin use E8, while for those grown on Matrigel use mTeSR1. For hPSCs grown on MEFs use PSC growth medium, while for NHP-PSCs grown on MEFs use NHP-PSC medium.
3. After collagenase treatment, hPSCs/NHP-PSCs colonies are loosely attached and can be collected by gentle pipetting. Do not use excessive mechanical force or scraping, which can provoke spontaneous differentiation.
4. If spontaneous differentiation of hPSCs/ NHP iPSCs occurs, differentiated colonies should be eliminated during the maintenance; observe PSCs every day before changing the medium. Mark differentiated colonies with an objective marker under the inverted microscope and aspirate marked areas using a glass Pasteur pipette while feeding PSCs with fresh medium.
5. Matrigel is temperature sensitive and should be kept on ice during handling. It is recommended to avoid freeze/thaw cycles by preparing 200 μ l aliquots for four 6-well plates and storing at -20 °C. Specific aliquot volumes vary by lot. Quickly dissolve each aliquot in 48 ml cold PBS and subsequently add 2 ml to each well of a 6-well plate. Incubate at least 1 h at room temperature or store overnight at 4 °C until ready to use. Matrigel-coated plates can be kept for several weeks at 4 °C.
6. Vitronectin is not temperature sensitive and can be thawed at room temperature. Dilute Vitronectin in 1x PBS to reach a final concentration of 10 μ g/ml (2 ml Vitronectin/50 ml 1x PBS) and gently mix by pipetting. Coat 1 ml/well of 6-well tissue-culture plate with diluted Vitronectin. Incubate for at least 1 h at room temperature before use. If it is not used immediately, the coated culture plate must be sealed and can be stored up to 1 week at 4 °C. Stored, coated culture plates must be brought to room temperature 30 min before cells are passaged.
7. hPSCs can be maintained under feeder-free conditions. For the OP9 coculture system, we always prefer to use hPSCs or NHP-iPSCs maintained on MEFs. hPSCs maintained under feeder-free conditions are used for hematopoietic differentiation under chemically defined feeder-free conditions. We observe substantial differences in the efficiency of hematopoietic differentiation of hPSCs/NHP iPSCs maintained on MEFs or in feeder-free cultures in the OP9 coculture system.
8. We found that engineered iETS1 hPSCs retain undifferentiated morphology and efficient hematopoietic differentiation potential when maintained on Matrigel-coated plates. In contrast, cultures on vitronectin-coated plates, iETS1 cells do not exhibit uniform morphology and fail to differentiate efficiently.
9. It is essential to culture OP9/OP9-DLL4 on gelatin-coated plates to prevent spontaneous adipogenesis.

10. Human hematopoietic differentiation on OP9 coculture system prefers over-confluent OP9 (8-9 days) whereas NHP iPSCs hematopoietic differentiation on OP9 coculture system prefers 6-7 day old OP9 cultures.
11. Human hematopoietic differentiation in chemically defined conditions requires specific plating densities for optimal hematoendothelial differentiation. Human ESCs need to be plated around 7,500 cells/cm². Harvesting, counting, and seeding of hPSCs on the Ten-C or ColIV plate should be performed quickly as singularized hPSCs do not survive well until mixed with rock inhibitor and plated.
12. OP9-DLL4 cells should be split every 3-4 days for maintenance/expansion. Proper maintenance of OP9-DLL4 is most critical step for T cell differentiation. Our lab strictly uses the defined FBS without heat inactivation. Heat inactivation does not benefit culture but rather results in a higher adipogenic effect on OP9/OP9-DLL4.
13. We have found that different lots of HyClone-defined FBS provide relatively consistent effects on OP9-DLL4 maintenance and T cell differentiation without substantial adipogenesis. Results from other suppliers are more variable.
14. OP9-DLL4 cells should not get overconfluent as it reduces the T cell differentiation ability. Hence, it is always important to perform passages the cells before they get confluent, which is typically 3-4 days when cultured in an appropriate serum.
15. Cell density in initial HPs/OP9-DLL4 coculture may affect downstream differentiation. A very high number of HPs can cause rapid development of NK cells which could lead to rapid killing of OP9-DLL4 feeders and compromise T cell development.
16. Differentiating HPs on OP9-DLL4 may reach high density by the second week or earlier. If this occurs, split the cells onto fresh OP9-DLL4 plates. Around 1×10^5 T cells progenitors/well of 6-well plates are ideal for differentiation during the second or subsequent weeks. High density cultures may result in reduced T cell differentiation efficiency.
17. There is no strict rule of weekly passaging during T cell differentiation. If the differentiating cells appear highly dense, then it is advised to subculture the cells as soon as possible onto fresh OP9-DLL4.
18. Total number of T cells generated from 1×10^4 CD43⁺ cells obtained from iETS1-hESCs in doxycycline treated and untreated conditions are much higher in comparison to other systems, cell types and sources (Figure 5).

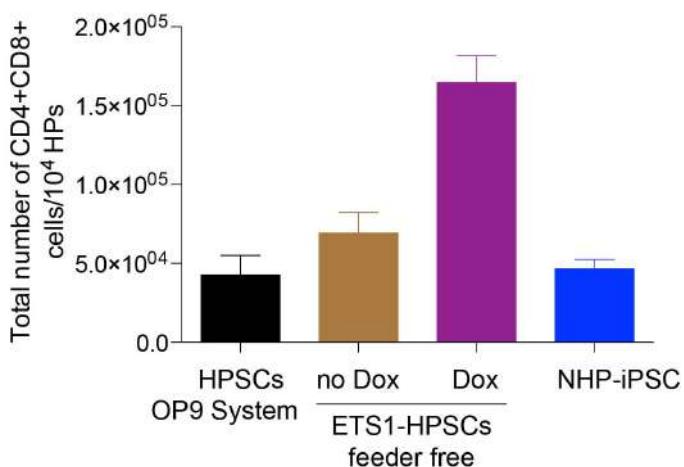


Figure 5. Bar graph shows the total number of T cells generated from 10⁴ HPs obtained from iETS1-hESCs in doxycycline treated and untreated conditions, wild-type NHP iPSCs and hPSCs. Total number of T cells generated is higher from iETS1-PSC in comparison to total HPs generated from different methods and different sources.

Recipes

1. Human PSC growth Medium (Table 2)
 - a. Prepare stocks of DMEM/F-12 from powder according to the manufacturer's instructions
 - b. Sterilize filter using a 0.22 µm membrane filter and store for up to 2 months at 2-8 °C
 - c. Use this basal media to prepare a complete 500 ml of hESC culture medium

Table 2. Human PSC growth medium

Composition	Volume (500 ml)	Final concentration
DMEM/F-12	390 ml	
KO serum replacement	100 ml	20%
NEAA solution (100x;10 mM)	5 ml	100 µM
L-glutamine/2-mercaptoethanol (100 mM)	5 ml	1 mM
Basic FGF-2 (100 µg/ml)	10 µl	4 ng/ml

2. MEF growth medium (Table 3)
 - a. Prepare stocks of DMEM from powder according to the manufacturer's instructions
 - b. Sterilize filter using a 0.22 µm membrane filter and store for up to 2 months at 2-8 °C
 - c. Prepare 500 ml of MEF growth medium by using stocks of DMEM and store up to 3 weeks at 2-8 °C

Table 3. MEF growth medium

Composition	Volume (500 ml)	Final concentration
DMEM	445 ml	
FBS	50 ml	10%
NEAA solution (100x,10mM)	5 ml	100 µM

3. NHP-PSC Medium (Table 4)

Use primate ESC medium from Reprocell to make 500 ml of NHP-PSCs medium

Table 4. NHP-PSC medium

Composition	Volume (500 ml)	Final concentration
Primate ES cell Medium	500ml	
Basic FGF2	20 µl	4 ng/ml

4. Mouse OP9/OP9-DLL4 bone marrow stromal cell culture medium (Table 5)

- Prepare stocks of fresh α-MEM from powder according to the manufacturer's instructions
- Sterilize by filtration using a 0.22-µm membrane filter and store for up to 2 months at 2-8 °C
- Use this basal media to prepare a complete 500 ml of OP9/OP9-DLL4 culture medium

Table 5. OP9/OP9-DLL4 medium

Composition	Volume	Final concentration
α-MEM	400 ml	
Hyclone FBS	100 ml	20%

5. Human hematopoietic differentiation (OP9 coculture system) medium (Table 6)

Use α-MEM basal medium to prepare 500 ml of human hematopoietic differentiation medium

Table 6. OP9 coculture medium for differentiation of hPSCs

Composition	Volume	Final concentration
α-MEM	400 ml	
Hyclone FBS	100 ml	20%
MTG	500 µl	100 µM
Ascorbic acid	500 µl	50 mg/ml

6. NHP differentiation medium (Table 7)

Use α-MEM basal medium to prepare 500 ml of primate differentiation medium

Table 7. NHP-PSC differentiation medium

Composition	Volume	Final concentration
a-MEM	450 ml	
Hyclone FBS	50 ml	10%
2-mercaptoethanol	1.76 µl	50 µM

7. IF4S Stock Medium (Table 8)

Prepare 10x IF4S basal media by using the following components

Table 8. IF4S stock medium

Composition	Volume	Stock concentration
IMDM	1 L x 5	5x
F12	1 L x 5	5x
ddH ₂ O	900 ml	
Sodium Bicarbonate	21 g	21 g/L
Ascorbic acid	640mg	640 mg/L
MTG	400 µl	400 µl/L
Sodium Selenite	120 µl	140 µg/L
CDLC	20 ml	2x

8. 5x PVA Stock Solution (Table 9)

- a. Autoclave 1 L ddH₂O with a stir bar. Once finished, place it on a heated magnetic stirrer
- b. Slowly add PVA in 2.5 g increments
- c. Make sure the PVA is dissolved before adding more and that it does not aggregate in the hot ddH₂O
- d. Once all of the PVA is added, continue stirring overnight at room temperature
- e. Autoclave again the next day to sterilize
- f. Store at room temperature up to 1 year

Table 9. PVA stock solution

Composition	Volume	Final concentration
PVA	50 g	50 g/L
ddH ₂ O	1 L	

9. IF9S Medium (Table 10)

- a. Prepare 500ml of IF9S medium for human hematopoietic differentiation media by using the IF4S medium
- b. Add the following component without PVA into IF4S and sterilize by filtration using a 0.22 µm membrane filter

- c. After filtration add 100 ml of 5x PVA

Table 10. IF9S medium

Composition	Volume	Stock concentration
IF4S (10x)	50 ml	
ddH ₂ O	340 ml	
PVA	100 ml	10 g/L
GlutaMax (100x)	5 ml	1x
NEAA(100x)	5 ml	1x
Holo-transferrin	500 µl	10.6 mg/L
Insulin	1 ml	20 mg/L

10. T lymphoid differentiation medium (Table 11)

- a. Use α-MEM basal medium to prepare T lymphoid differentiation medium
- b. To prepare 100 ml of T lymphoid differentiation medium, add the following component to α-MEM basal medium and sterilize by filtration using a 0.22 µm membrane filter
- c. Alternatively, add SCF (10 ng/ml), IL-7 (5 ng/ml) and FLT3-L (5 ng/ml) to the OP9/OP9-DLL4 culture medium to make T lymphoid differentiation medium

Table 11. T lymphoid differentiation medium

Composition	Volume	Final concentration
α-MEM	80 ml	
Hyclone FBS	20 ml	20%
SCF-1	10 µl	10 ng/ml
IL7	5 µl	5 ng/ml
FLT3L	5 µl	5 ng/ml

11. HBS saline solution (2x)

- a. Prepare 100 ml of 2x HBS solution by adding HEPES (50 mM), KCl (10 mM), dextrose (12 mM), NaCl (280 mM) and Na₂HPO₄ (1.5 mM)
- b. Adjust the pH to exactly 7.05-7.08
- c. Filter the solution through 0.2 µm filter

12. CaCl₂ solution (2 M)

- a. Prepare 50 ml of 2 M of CaCl₂ solution by dissolving 14.702 g of CaCl₂ in 50 ml of distilled water
- b. Filter sterilize the solution using 0.2 µm filter

13. Gelatin solution [0.1% (wt/vol)]

- a. Add 500 mg of gelatin to 500 ml of endotoxin-free reagent-grade distilled water
- b. Solubilize and sterilize by autoclaving for 20 min at 121 °C

- c. Store the solution at 4 °C for up to 6 months
14. Magnetic cells sorting (MACS) buffer (Table 12)
 - a. Prepare 500ml of MACS buffer as shown
 - b. Sterilize MACS buffer by filtration using a 0.22 µm membrane filter and keep at 2-8 °C for up to 6 months

Table 12. MACS buffer

Composition	Volume (500 ml)	Final concentration
DPBS	473 ml	
FBS	25 ml	5%
EDTA (0.5M)	2 ml	2 mM

15. Flow cytometry buffer (FACS) buffer (Table 13)
 - a. Prepare 500ml of FACS buffer as shown
 - b. Filtrate the buffer using a 0.22 µm membrane filter and store at 2-8 °C for up to 6 months

Table 13. FACS buffer

Composition	Volume (500 ml)	Final concentration
DPBS	488 ml	
FBS	10 ml	2%
EDTA (0.5M)	2 ml	2 mM
Sodium azide	0.25g	0.05%

16. Reconstitution of cytokines
 - a. All the cytokines were reconstituted and stored in -80 °C according to manufacturer's recommendations
 - b. Briefly, centrifuge the vials at maximum speed for 1 min to precipitate lyophilized pellets before opening vials
 - c. Dilute with 0.1% BSA/PBS solution for working concentration and store at -80 °C until needed for use
17. Collagenase solution (1 mg/ml)
 - a. Add 50 mg of collagenase to 50 ml of DMEM/F-12 basal medium and sterilize the solution by filtration using a 0.22 µm membrane filter
 - b. Keep the solution at 2-8 °C and use it for up to 1 week
18. Doxycycline (1 mg/ml)
 - a. Dissolve doxycycline to 1 mg/ml in ddH₂O
 - b. Prepare 100 µl aliquotes in amber 1.5-ml microcentrifuge tubes for light protection and store up to 5 days at 4 °C or 2 months at 20 °C

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This protocol was adapted from previous work Vodyanik et al., 2006; Uenishi et al., 2014; D'Souza et al., 2016 Kumar et al., 2019b; Park et al., 2018b.

Competing interests

The authors declare no conflicts of interest.

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***In vitro* Differentiation of Human iPSC-derived Cardiovascular Progenitor Cells (iPSC-CVPCs)**

Agnieszka D'Antonio-Chronowska^{1,*}, Matteo D'Antonio¹ and Kelly A. Frazer^{1,2}

¹Department of Pediatrics, University of California, San Diego, La Jolla, USA; ²Institute for Genomic Medicine, University of California, San Diego, La Jolla, USA

*For correspondence: adantoniochronowska@health.ucsd.edu

[Abstract] Induced pluripotent stem cell derived cardiovascular progenitor cells (iPSC-CVPCs) provide an unprecedented platform for examining the molecular underpinnings of cardiac development and disease etiology, but also have great potential to play pivotal roles in the future of regenerative medicine and pharmacogenomic studies. Biobanks like iPSCORE (Stacey *et al.*, 2013; Panopoulos *et al.*, 2017), which contain iPSCs generated from hundreds of genetically and ethnically diverse individuals, are an invaluable resource for conducting these studies. Here, we present an optimized, cost-effective and highly standardized protocol for large-scale derivation of human iPSC-CVPCs using small molecules and purification using metabolic selection. We have successfully applied this protocol to derive iPSC-CVPCs from 154 different iPSCORE iPSC lines obtaining large quantities of highly pure cardiac cells. An important component of our protocol is Cell confluence estimates (ccEstimate), an automated methodology for estimating the time when an iPSC monolayer will reach 80% confluence, which is optimal for initiating iPSC-CVPC derivation, and enables the protocol to be readily used across iPSC lines with different growth rates. Moreover, we showed that cellular heterogeneity across iPSC-CVPCs is due to varying proportions of two distinct cardiac cell types: cardiomyocytes (CMs) and epicardium-derived cells (EPDCs), both of which have been shown to have a critical function in heart regeneration. This protocol eliminates the need of iPSC line-to-line optimization and can be easily adapted and scaled to high-throughput studies or to generate large quantities of cells suitable for regenerative medicine applications.

Keywords: Human induced pluripotent stem (iPSC), Cardiovascular progenitor cells (CVPCs), cardiomyocytes (CMs), Epicardium cells (EPDCs), Human induced pluripotent stem cell-derived cardiovascular progenitor cells (iPSC-CVPCs), Human induced pluripotent stem cell-derived cardiomyocytes cells (iPSC-CMs), Human induced pluripotent stem cell-derived epicardium derived cells (iPSC-EPDCs), Cardiovascular disease, Heart, Differentiation, Genetic studies, Small molecules, Pharmacogenomics, Regenerative medicine

[Background] Cardiovascular diseases (CVDs) remain the leading cause of death worldwide and account for about 30% of all mortality causes globally. Coronary artery disease (CAD) and myocardial infarction (MI) are among the most common CVDs, and in the USA alone, every 40 s someone suffers a heart attack (Association, 2016; Heron, 2017; WHO, 2018; Benjamin *et al.*, 2019; D'Antonio-Chronowska *et al.*, 2019a). Heart failure results in the death of cardiac muscle cells and is the consequence of morphological and functional changes (cardiac remodeling: necrosis, scar formation,

inflammation, fibrosis, dilation and reshaping) that occur in response to pre-existing cardiac conditions, including CAD, MI, hypertension, cardiomyopathy, myocarditis and abnormal cardiac valve function (Cohn *et al.*, 2000; Reed *et al.*, 2017). Heart failure is commonly treated with beta blockers, ACE inhibitors and aldosterone antagonists which partially reverse cardiac remodeling and thereby improve prognosis (Reis Filho *et al.*, 2015), but does not result in the regeneration of cardiac tissue. There are currently several ongoing clinical trials, including ESCORT and DREAM-HF (Menasche *et al.*, 2015 and 2018; Borow *et al.*, 2019), which are aimed at evaluating the effectiveness of transplanting iPSC-derived Cardiovascular Progenitor Cells (iPSC-CVPCs) or embryonic stem cell derived CVPCs (ESC-CVPCs) as a therapeutic treatment for heart failure. The ability to generate iPSC-CVPCs in large quantities, as is required for regenerative medicine, using biological material obtained directly from the patient would enable autologous transplantations and thereby eliminate the need of immunosuppression. Thus, the development of a robust and cost-effective protocol for generating large amounts of high-quality iPSC-CVPCs without requiring individualized optimization for each iPSC line is imperative for the advancement of future therapeutic treatments of heart failure.

Large collections of iPSC-CVPCs (D'Antonio-Chronowska *et al.*, 2019b) generated from genetically and ethnically diverse individuals could also be used for cost-effective large-scale testing of drugs for cardiotoxicity or proarrhythmic effects. Previous studies (Burridge *et al.*, 2016) and initiatives like CiPA Project (Blinova *et al.*, 2017) have shown the utility of iPSC-CVPCs for testing drugs for cardiotoxicity, which, if scaled to examine large collections of iPSC-CVPCs derived from both healthy or disease bearing individuals, could greatly improve the efficiency of testing new drugs for safety, and in turn decrease the cost of drug development.

We have previously demonstrated the feasibility of using a highly standardized protocol for successfully deriving high quality iPSC-CVPCs from hundreds of iPSC lines reprogrammed from ethnically diverse individuals (D'Antonio-Chronowska *et al.*, 2019b). In this study, we performed 193 differentiations to derive iPSC-CVPCs from 154 iPSCORE iPSC lines (Panopoulos *et al.*, 2017) from 144 individuals. We obtained large numbers of high quality cells, specifically, on average we derived 1.5×10^8 (and up to 6×10^8) cells from a 450 cm^2 culture with median cardiac troponin T (cTnT; TNNT2) positive cells of 89.2%. Importantly, while previous differentiation studies acknowledged cellular heterogeneity and the presence of beating cardiomyocytes and non-contractile cell types, the origin and cellular identity of the non-contractile cells had not been addressed. We characterized the 154 iPSC-CVPC samples using single cell RNA-seq and bulk RNAs-seq and determined that across all the iPSC-CVPC samples there were two distinct fetal-like cardiac cell types: cardiomyocytes (CMs) and epicardium-derived cells (EPDCs), which were present in varying proportions. Of note, both CMs and EPDCs have been shown to contribute to the post-infarction heart regeneration (Bargehr *et al.*, 2019). Moreover, our previous studies have shown how molecular characterization of iPSC-CVPCs can result in the identification of genetic variants that contribute to heart development and cardiac pathologies (Benaglio *et al.*, 2019).

Protocols to derive cardiac cells from ESCs or iPSCs have been developed to mimic the processes naturally occurring during cardiogenesis. Initially, cardiac cells were derived as embryoid bodies cultures,

first as spontaneous differentiations by culturing ESCs in medium containing 20% fetal calf serum or by stimulation with several reagents known to enhance cardiac differentiation like dimethyl sulfoxide, retinoic acid, or 5-aza-2'-deoxycytidine, after which beating cardiac cells were manually or mechanically purified (Maltsev *et al.*, 1993; Burridge *et al.*, 2014). Differentiation efficiency was greatly improved by the development of directed differentiation protocols that incorporated recombinant proteins including fibroblast growth factor 2, transforming growth factor β , superfamily growth factors activin A and BMP4, vascular endothelial growth factor and the WNT inhibitor DKK-1 proteins (Schneider and Mercola, 2001; Marvin *et al.*, 2001; Beqqali *et al.*, 2006; Laflamme *et al.*, 2007), and by modification of the format of cell differentiation from embryoid bodies to monolayer culture (Paige *et al.*, 2010; Lian *et al.*, 2012). Further advancements were made by the introduction of small molecule protocols (Lian *et al.*, 2012 and 2013) and chemically defined differentiation media (Burridge *et al.*, 2014). Finally, by taking advantage of the adaptation of the developing heart to metabolize lactate, we and others were able to eliminate all non-cardiac cells (Burridge *et al.*, 2014; D'Antonio-Chronowska *et al.*, 2019b; Tohyama *et al.*, 2013). Importantly, previous studies have optimized differentiation protocols to derive cardiac cells from a limited number of iPSC or ESC lines, and in most cases utilized small format culture vessels. Here, we present an optimized, cost-effective and highly standardized protocol which we applied to derive iPSC-CVPCs from 154 genetically and ethnically diverse human iPSC lines in large-sized culture flasks. We optimized the concentration of IWP-2 used to drive the cardiac cell differentiation, which resulted in improved formation of a thick cardiac syncytium and strong wave-like beating (Video 1) (D'Antonio-Chronowska *et al.*, 2019b). We also demonstrated that simple mechanical disruption of the cardiac syncytium prior to metabolic purification of iPSC-CVPCs using lactate results in improved selection and virtually pure cardiac cells (CMs and EPDCs). Additionally, we developed Cell confluence estimates (ccEstimate), an automated method for estimation of cell confluency during the monolayer stage. ccEstimate estimates the point in time for each iPSC line when the monolayer will reach 80% of confluency, which is optimal condition at which to initiate iPSC-CVPC differentiation. Thereby ccEstimate overcomes some of the technical issues in standardizing a differentiation protocol across iPSC lines which have widely varying growth rates. The derived iPSC-CVPCs beat synchronously, are positive for multiple cardiac markers and can be used directly for molecular or electrophysiological assays like multielectrode array (MEA), or they can be cryopreserved for future analysis. Our optimized protocol allowed us to derive high quality iPSC-CVPC samples from 154 iPSC lines generated from ethnically diverse individuals under identical culturing conditions without the requirement of any individualized optimization steps.

Materials and Reagents

F. iPSC Cell Culture as described also in detail in D'Antonio-Chronowska *et al.* (2019)

21. 6-well plates (Corning, catalog number: 3506)
22. Syringe filter 0.2 μ m (VWR, catalog number: 28145-501)
23. Soft-Ject[®] 3-Part Disposable Syringe, Air-Tite-3 ml (VWR, catalog number: 89215-234)

24. 5 ml Borosilicate serological pipettes (Fisher Scientific, catalog number: 1367827E)
 25. 5 ml Serological pipettes (Bio Pioneer, catalog number: GEX0050-S01)
 26. 10 ml Serological pipettes (Bio Pioneer, catalog number: GEX0100-S01)
 27. P20 pipette tips sterile with filter (Fisher Scientific, catalog number: 21-403-00) or other equivalent sterile tips with filter compatible with pipettes
 28. P1000 pipette tips sterile with filter (Fisher Scientific, catalog number: 21-403-02) or other equivalent P1000 sterile tips with filter compatible with pipettes
 29. 15 ml conical tubes (Bio Pioneer, catalog number: CNT-15R)
 30. iPSC cells
 31. UltraPure™ DNase/RNase-Free Distilled Water (Thermo Fisher Scientific, catalog number: 10977023)
 32. Corning® Matrigel® Growth Factor Reduced (GFR) Basement Membrane Matrix (Corning, catalog number: 354230)
 33. mTeSR™ 1 (Stem Cell Technologies, catalog number: 85850)
 34. DMEM/F-12 medium (Thermo Fisher Scientific, catalog number: 11330057)
 35. Dispase II, powder (Thermo Fisher Scientific, catalog number: 17105041)
 36. 200 proof Ethanol denatured (*i.e.*, VWR, catalog number: 71002-398)—to prepare 70% ethanol
 37. 70% ethanol (see Recipes: Table 20)
 38. Matrigel solution (Matrigel) (see Recipes: Table 1)
 39. 10 mM ROCK inhibitor, Y-27632 dihydrochloride solution (ROCK Inhibitor) (see Recipes: Table 3)
 40. 10x Dispase (see Recipes: Table 4)
 41. mTeSR™ 1 complete medium (mTeSR) (see Recipes: Table 5)
- B. PSC passaging using Versene–Versene I passage
1. 6-well plates (Corning, catalog number: 3506)
 2. Automated cell counter slides (Bio-Rad Laboratories, catalog number: 1450019) or a hemocytometer (Hausser Scientific, catalog number: 1483) or equivalent
 3. 5 ml Serological pipettes (Bio Pioneer, catalog number: GEX0050-S01)
 4. P20 pipette tips sterile with filter (Fisher Scientific, catalog number: 21-403-00) or other equivalent P200 sterile tips with filter compatible with pipettes
 5. P1000 pipette tips sterile with filter (Fisher Scientific, catalog number: 21-403-02) or other equivalent P1000 sterile tips with filter compatible with pipettes
 6. 15 ml conical tubes (Bio Pioneer, catalog number: CNT-15R)
 7. iPSC cells
 8. UltraPure™ DNase/RNase-Free Distilled Water (Thermo Fisher Scientific, catalog number: 10977023)
 9. Corning® Matrigel® Growth Factor Reduced (GFR) Basement Membrane Matrix (Corning, catalog number: 354230)

10. mTeSR™ 1 (Stem Cell Technologies, catalog number: 85850)
 11. DMEM/F-12 medium (Thermo Fisher Scientific, catalog number: 11330057)
 12. Versene® (EDTA) 0.02% (Lonza, catalog number: 17-711E)
 13. 200 proof Ethanol denatured (*i.e.*, VWR, catalog number: 71002-398)—to prepare 70% ethanol
 14. 70% ethanol (see Recipes: Table 20)
 15. Matrigel solution (Matrigel) (see Recipes: Table 1)
 16. 10 mM ROCK inhibitor, Y-27632 dihydrochloride solution (ROCK Inhibitor) (see Recipes: Table 3)
 17. mTeSR™ 1 complete medium (mTeSR) (see Recipes: Table 5)
- C. iPSC passaging using Versene - Versene II passage
1. 100 mm tissue culture dishes (Corning, catalog number: 430167)
 2. Automated cell counter slides (Bio-Rad Laboratories, catalog number: 1450019) or a hemocytometer (Hausser Scientific, catalog number: 1483) or equivalent
 3. 5 ml Serological pipettes (Bio Pioneer, catalog number: GEX0050-S01)
 4. P20 pipette tips sterile with filter (Fisher Scientific, catalog number: 21-403-00) or other equivalent sterile tips with filter compatible with pipettes
 5. P1000 pipette tips sterile with filter (Fisher Scientific, catalog number: 21-403-02) or other equivalent P1000 sterile tips with filter compatible with pipettes
 6. 15 ml conical tubes (Bio Pioneer, catalog number: CNT-15R)
 7. iPSC cells
 8. UltraPure™ DNase/RNase-Free Distilled Water (Thermo Fisher Scientific, catalog number: 10977023)
 9. Corning® Matrigel® Growth Factor Reduced (GFR) Basement Membrane Matrix (Corning, catalog number: 354230)
 10. mTeSR™ 1 (Stem Cell Technologies, catalog number: 85850)
 11. DMEM/F-12 medium (Thermo Fisher Scientific, catalog number: 11330057)
 12. Versene® (EDTA) 0.02% (Lonza, catalog number: 17-711E)
 13. 200 proof Ethanol denatured (*i.e.*, VWR, catalog number: 71002-398)—to prepare 70% ethanol
 14. 70% ethanol (see Recipes: Table 20)
 15. Matrigel solution (Matrigel) (see Recipes: Table 1)
 16. 10 mM ROCK inhibitor, Y-27632 dihydrochloride solution (ROCK Inhibitor) (see Recipes: Table 3)
 17. mTeSR™ 1 complete medium (mTeSR) (see Recipes: Table 5)
- D. Monolayer plating is also described in detail in D'Antonio-Chronowska *et al.* (2019a)
21. 100 mm tissue culture dishes (Corning, catalog number: 430167)
 22. Automated cell counter slides (Bio-Rad Laboratories, catalog number: 1450019) or a hemocytometer (Hausser Scientific, catalog number: 1483) or equivalent

23. 5 ml Serological pipettes (Bio Pioneer, catalog number: GEX0050-S01)
 24. 10 ml Serological pipettes (Bio Pioneer, catalog number: GEX0100-S01)
 25. P20 pipette tips sterile with filter (Fisher Scientific, catalog number: 21-403-00) or other equivalent P20 sterile tips with filter compatible with pipettes
 26. P200 pipette tips sterile with filter (Fisher Scientific, catalog number: 21-403-01) or other equivalent P200 sterile tips with filter compatible with pipettes
 27. P1000 pipette tips sterile with filter (Fisher Scientific, catalog number: 21-403-02) or other equivalent P1000 sterile tips with filter compatible with pipettes
 28. 15 ml conical tubes (Bio Pioneer, catalog number: CNT-15R)
 29. 50 ml conical tubes (Bio Pioneer catalog number: CNT-50R)
 30. Corning® Matrigel® Growth Factor Reduced (GFR) Basement Membrane Matrix (Matrigel) (Corning, catalog number: 354230)
 31. mTeSR™ 1 (Stem Cell Technologies, catalog number: 85850)
 32. DMEM/F-12 medium (Thermo Fisher Scientific, catalog number: 11330-057)
 33. Accutase (Innovative Cell Technologies, Inc., catalog number: AT 104)
 34. Trypan Blue Solution, 0.4% (Thermo Fisher Scientific, catalog number: 15250061)
 35. ROCK inhibitor, Y-27632 dihydrochloride (Selleck hem, catalog number: S1049)
 36. iPSC cell culture
 37. 200 proof Ethanol denatured (*i.e.*, VWR, catalog number: 71002-398)—to prepare 70% ethanol
 38. 70% ethanol (see Recipes: Table 20)
 39. Matrigel solution (see Recipes: Table 1)
 40. 10 mM ROCK inhibitor, Y-27632 dihydrochloride solution (see Recipes: Table 3)
 41. mTeSR™ 1 complete medium (see Recipes: Table 5)
- E. Estimation of optimal time for initiation of iPSC-CVPCs differentiation using ccEstimate
39. iPSCs monolayer
 40. Marker pen resistant to 70% ethanol
- F. iPSC-CVPCs differentiation
1. T150 tissue culture flasks, vented (Sigma, catalog number: Z707929)
Note: At the time of preparation of this manuscript Z707929 was no longer available. The same flasks are available under the catalog number Z707511-36EA (Sigma, catalog number: Z707511-36EA).
 2. Automated cell counter slides (Bio-Rad Laboratories, catalog number: 1450019) or a hemocytometer (Hausser Scientific, catalog number: 1483) or equivalent
 3. 10 ml Serological pipettes (Bio Pioneer, catalog number: GEX0100-S01)
 4. 25 ml Serological pipettes (Bio Pioneer catalog number: GEX250-S01)
 5. 50 ml Serological pipettes (Bio Pioneer, catalog number: GEX500-S01)
 6. P20 pipette tips sterile with filter (Fisher Scientific, catalog number: 21-403-00) or other equivalent P20 sterile tips with filter compatible with pipettes

- equivalent P20 sterile tips with filter compatible with pipettes
7. P200 pipette tips sterile with filter (Fisher Scientific, catalog number: 21-403-01) or other equivalent P200 sterile tips with filter compatible with pipettes
 8. P1000 pipette tips sterile with filter (Fisher Scientific, catalog number: 21-403-02) or other equivalent P1000 sterile tips with filter compatible with pipettes
 9. Cell scraper (VWR International, catalog number: 179707)
 10. 15 ml conical tubes (Bio Pioneer, catalog number: CNT-15R)
 11. 50 ml conical tubes (Bio Pioneer, catalog number: CNT-50R)
 12. 125 ml Nalgene® PET sterile bottle (Fisher Scientific, catalog number: 342040-0125)
 13. Nalgene® Cryogenic vials (Thermo Fisher Scientific, catalog number: 5000-1020)
 14. iPSCs monolayer
 15. Corning® MatriGel® Growth Factor Reduced (GFR) Basement Membrane Matrix (MatriGel) (Corning®, catalog number: 354230)
 16. RPMI 1640 medium (Thermo Fisher Scientific, catalog number: 11875119)
 17. RPMI 1640 medium, no glucose (Thermo Fisher Scientific, catalog number: 11879020)
 18. 1x Dulbecco's phosphate buffered saline (DPBS; PBS) without calcium and magnesium (Thermo Fisher Scientific, catalog number: 14190250)
 19. B-27™ Supplement, minus insulin (Thermo Fisher Scientific, catalog number: A1895601)
 20. B-27™ Supplement (50x), serum free (Thermo Fisher Scientific, catalog number: 17504044)
 21. FBS (Omega Scientific, catalog number: FB-02) or equivalent, or KnockOut™ Serum Replacement (KOSR) (Thermo Fisher Scientific, catalog number: 10828028)
 22. MEM Non-Essential Amino Acids Solution 100x (Thermo Fisher Scientific, catalog number: 11140050)
 23. Penicillin-Streptomycin (10,000 U/ml) (Thermo Fisher Scientific, catalog number: 15140122)
 24. CHIR-99021 (CT99021) (CHIR99021) HCl (Selleckchem, catalog number: S2924)
 25. IWP-2 (Tocris, catalog number: 3533)
 26. Sodium L-lactate (Sigma, catalog number: 71718-10G)
 27. HEPES sodium salt solution 1M, BioReagent, suitable for cell culture (Sigma, catalog number: H3662-100ML)
 28. Accutase (Innovative Cell Technologies, Inc., catalog number: AT 104)
 29. Trypan Blue Solution, 0.4% (Thermo Fisher Scientific, catalog number: 15250061)
 30. Dimethyl Sulfoxide (DMSO) (Sigma, catalog number: D2650-100ML)
 31. Liquid nitrogen
 32. 200 proof Ethanol denatured (*i.e.*, VWR, catalog number: 71002-398) – to prepare 70% ethanol
 33. 70% ethanol (see Recipes: Table 20)
 34. RPMI Minus (-) medium (see Recipes: Table 6)
 35. RPMI Plus (+) medium (see Recipes: Table 7)
 36. RPMI Lactate medium (see Recipes: Table 8)
 37. iPSC-CVPCs Harvest Medium (see Recipes: Table 9)

38. iPSC-CVPCs 2x freezing medium (see Recipes: Table 10)
39. 10 mM CHIR-992021 solution (see Recipes: Table 11)
40. 5 mM IWP-2 solution (see Recipes: Table 12)
41. 1M Sodium L-lactate solution (see Recipes: Table 13)

G. Flow cytometry

24. 96-well round bottom assay plates (Genesee Scientific, catalog number: 25-224)
25. Corning™ Falcon™ Test Tube with Cell Strainer Snap Cap (Fisher Scientific, catalog number: 352235)
26. Corning™ Costar™ Sterile Disposable Reagent Reservoirs (Fisher Scientific, catalog number: 4870)
27. 5 ml Serological pipettes (Bio Pioneer, catalog number: GEX0050-S01)
28. 10 ml Serological pipettes (Bio Pioneer, catalog number: GEX0100-S01)
29. P20 pipette tips sterile with filter (Fisher Scientific, catalog number: 21-403-00) or other equivalent P20 sterile tips with filter compatible with pipettes
30. P200 pipette tips sterile with filter (Fisher Scientific, catalog number: 21-403-01) or other equivalent P200 sterile tips with filter compatible with pipettes
31. P1000 pipette tips sterile with filter (Fisher Scientific, catalog number: 21-403-02) or other equivalent P1000 sterile tips with filter compatible with pipettes
32. P20 sterile pipette tips without filter (VWR, catalog number: 83009-694) or other equivalent P20 sterile tips without filter compatible with pipettes
33. (Optional) P200 sterile pipette tips without filter (VWR, catalog number: 89495-378) or other equivalent P20 sterile tips without filter compatible with pipettes
34. 1x Dulbecco's phosphate buffered saline (PBS) without calcium and magnesium (Thermo Fisher Scientific, catalog number: 14190250)
35. Methanol, ACS reagent, ≥ 99.8% (Sigma, catalog number: 179337-4L-PB)
36. Bovine Serum Albumin (BSA) (Sigma, catalog number: A2153-100G)
37. (Optional) NaN₃ (Sigma, catalog number: S2002-5G)
38. 37% Formaldehyde (Sigma, catalog number: F-1635-500ML)
39. Triton™ X-100 (Sigma, catalog number: X-100-500ML)
40. Goat serum, New Zealand origin (Thermo Fisher Scientific, catalog number: 16210064)
41. Mouse monoclonal anti-Troponin T, Cardiac Isoform (cTNT; TNNT2) Ab-1 antibody, clone 13-11 (Thermo Fisher Scientific, catalog number: MS-295-P0)
42. Mouse IgG1 antibody (Thermo Fisher Scientific, catalog number: MG100)
43. Goat-anti-Mouse Alexa Fluor™ 488 conjugated antibody (Thermo Scientific, catalog number: A-11001)
44. FACS Buffer (see Recipes: Table 14)
45. FACS-FIX Buffer (see Recipes: Table 16)

Note: For antibody working concentration, see Recipes: Table 21.

H. Immunofluorescence

24. Millicell EZ SLIDE 8-well glass slides (Millipore, catalog number: PEZGS0816)
25. Coverslip glass slides (Fisherbrand, catalog number: 12-545-F [coverslip thickness #1]).
Different coverslip thickness may be used if required
26. 5 ml Serological pipettes (Bio Pioneer, catalog number: GEX0050-S01)
27. 10 ml Serological pipettes (Bio Pioneer, catalog number: GEX0100-S01)
28. P20 pipette tips sterile with filter (Fisher Scientific, catalog number: 21-403-00) or other equivalent P20 sterile tips with filter compatible with pipettes
29. P200 pipette tips sterile with filter (Fisher Scientific, catalog number: 21-403-01) or other equivalent P200 sterile tips with filter compatible with pipettes
30. 1x Dulbecco's phosphate buffered saline (PBS) without calcium and magnesium (Thermo Fisher Scientific, catalog number: 14190250)
31. Bovine Serum Albumin (BSA) (Sigma, catalog number: A2153-100G)
32. Paraformaldehyde (PFA) (Sigma, catalog number: 158127-100G)
33. Tween® 20 (Sigma, catalog number: P9416-100ML)
34. Triton™ X-100 (Sigma, catalog number: X100-500ML)
35. Gelatin from porcine skin (Sigma, catalog number: G1890-100G)
36. Mouse monoclonal anti- α -Actinin (Sarcomeric) antibody, clone EA-53 (Sigma, catalog number: A7811)
37. Rabbit polyclonal anti-Connexin 43 (CX43/GJA1) antibody (Invitrogen, catalog number: 71-0700)
38. Rabbit polyclonal anti-Myosin Light Chain 2 (MYL2; MLC2v) antibody (Proteintech, catalog number: 10906-1-AP)
39. Mouse monoclonal anti-Myosin Atrial Light Chain 2 (MYL7; MLC2a) antibody clone 56F5 (Synaptic Systems, catalog number: 311 011)
40. Donkey anti-Rabbit Alexa Fluor™ 488 conjugated antibody (Invitrogen, catalog number: A21206)
41. Goat anti-Mouse Alexa Fluor™ 568 conjugated antibody (Invitrogen, catalog number: A-11004)
42. ProLong® Gold Antifade Reagent with DAPI (Cell Signaling Technologies, catalog number: 8961)
43. 0.1% Gelatin solution (see Recipes: Table 2)
44. 4% PFA solution in PBS
45. IF Perm Buffer II (see Recipes: Table 17)
46. IF Blocking Buffer II (see Recipes: Table 18)
47. IF Staining Buffer (see Recipes: Table 19)

Note: For antibody working concentration, see Recipes: Table 21.

Equipment

A. iPSC Cell Culture

12. Biosafety cabinet (Labconco, model: Logic+)

13. Incubator with humidity and gas control set to maintain 37 °C and 95% humidity in an atmosphere of 5% CO₂ in air (Panasonic, model: MCO-170AICUVH-PA)
 14. Water bath (Thermo Scientific, model: Precision)
 15. Tissue culture centrifuge with rotors for 15 ml conical tubes and 50 ml conical tubes (Thermo Scientific, model: Legend RT+)
 16. Phase contrast inverted microscope (objectives: x4, x10, x20) (Olympus, model: CKX41SF)
 17. (Optional) Phase contrast inverted microscope with camera (objectives: x4, x10, x20) (Thermo Scientific, model: EVOS XL Core)
 18. Microscope Object marker (Nikon, model: MBW10020)
 19. Pipette aid (Labnet, catalog number: FastPette™ V2 P2000) or other equivalent available pipette aid
 20. P20 Micropipette (Rainin, catalog number: 17014392) or other available P20 pipette
 21. Non-frost-free freezer -20 °C
 22. Refrigerator 2-8 °C
- B. iPSC passaging using Versene (Versene I and Versene II passage)
13. Biosafety cabinet (Labconco, model: Logic+)
 14. Incubator with humidity and gas control set to maintain 37 °C and 95% humidity in an atmosphere of 5% CO₂ in air (Panasonic, model: MCO-170AICUVH-PA)
 15. Tissue culture centrifuge with rotors for 15 ml conical tubes and 50 ml conical tubes (Thermo Scientific, model: Legend RT+)
 16. Phase contrast inverted microscope (objectives: x4, x10, x20) (Olympus, model: CKX41SF)
 17. (Optional) Phase contrast inverted microscope with camera (objectives: x4, x10, x20) (Thermo Scientific, model: EVOS XL Core)
 18. Pipette aid (Labnet, catalog number: FastPette™ V2 P2000) or other equivalent available pipette aid
 19. P20 Micropipette (Rainin, catalog number: 17014392) or other available P20 pipette
 20. P200 Micropipette (Rainin, catalog number: 17014391) or other available P200 pipette
 21. P1000 Micropipette (Rainin, catalog number: 17014382) or other available P1000 pipette
 22. Automated cell counter (Bio-Rad, model: TC20) or a hemocytometer (Hausser Scientific, catalog number: 1483) or equivalent
 23. Non-frost-free freezer -20 °C
 24. Refrigerator 2-8 °C
- C. Monolayer plating
1. Biosafety cabinet (Labconco, model: Logic+)
 2. Incubator with humidity and gas control set to maintain 37 °C and 95% humidity in an atmosphere of 5% CO₂ in air (Panasonic, model: MCO-170AICUVH-PA)
 3. Tissue culture centrifuge with rotors for 15 ml conical tubes and 50 ml conical tubes (Thermo

Scientific, model: Legend RT+)

4. Phase contrast inverted microscope (objectives: x4, x10, x20) (Olympus, model: CKX41SF)
5. (Optional) Phase contrast inverted microscope with camera (objectives: x4, x10, x20) (Thermo Scientific, model: EVOS XL Core)
6. Pipette aid (Labnet, catalog number: FastPette™ V2 P2000 or other equivalent available pipette aid)
7. P20 Micropipette (Rainin, catalog number: 17014392) or other available P20 pipette
8. P200 Micropipette (Rainin, catalog number: 17014391) or other available P200 pipette
9. P1000 Micropipette (Rainin, catalog number: 17014382) or other available P1000 pipette
10. Automated cell counter (Bio-Rad, model: TC20) or a hemocytometer (Hausser Scientific, catalog number: 1483) or equivalent
11. Non-frost-free freezer -20 °C
12. Refrigerator 2-8 °C

D. iPSC-CVPCs differentiation and cryopreservation

16. Biosafety cabinet (Labconco, model: Logic+)
17. Incubator with humidity and gas control set to maintain 37 °C and 95% humidity in an atmosphere of 5% CO₂ in air (Panasonic, model: MCO-170AICUVH-PA)
18. Tissue culture centrifuge with rotors for 15 ml conical tubes and 50 ml conical tubes (Thermo Scientific, model: Legend RT+)
19. Phase contrast inverted microscope (objectives: x4, x10, x20) (Olympus, model: CKX41SF)
20. (Optional) Phase contrast inverted microscope with camera (objectives: x4, x10, x20) (Thermo Scientific, model: EVOS XL Core)
21. Pipette aid (Labnet, catalog number: FastPette™ V2 P2000) or other equivalent available pipette aid
22. P20 Micropipette (Rainin, catalog number: 17014392) or other available P20 pipette
23. P200 Micropipette (Rainin, catalog number: 17014391) or other available P200 pipette
24. P1000 Micropipette (Rainin, catalog number: 17014382) or other available P1000 pipette
25. Automated cell counter (Bio-Rad, model: TC20) or a hemocytometer (Hausser Scientific, catalog number: 1483) or equivalent.
26. Mr. Frosty freezing container (Corning, model: CoolCell® FTS30)
27. Refrigerator 2-8 °C
28. Non-frost-free freezer -20 °C
29. Freezer -80 °C
30. Liquid nitrogen vapor tank

E. Estimation of optimal time for initiation of iPSC-CVPCs differentiation using ccEstimate

1. Phase contrast inverted microscope with camera (objective: x4) (Thermo Scientific, model: EVOS XL Core) or equivalent or automatic imaging system

2. Any computer with R 3.5.1 and the R package EBImage (Pau *et al.*, 2010) installed and 4GB RAM

F. Flow cytometry

9. Pipette aid (Labnet, catalog number: FastPette™ V2 P2000) or other equivalent available pipette aid
10. P20 Micropipette (Rainin, catalog number: 17014392) or other available P20 pipette
11. P200 Micropipette (Rainin, catalog number: 17014391) or other available P200 pipette
12. P1000 Micropipette (Rainin, catalog number: 17014382) or other available P1000 pipette
13. P200 Multichannel micropipette (Rainin, catalog number: 17013805) or other available multichannel P200 pipette
14. Tissue culture centrifuge with rotors suitable to centrifuge 96-well plates (Thermo Scientific, model: Legend RT+)
15. Refrigerator 2-8 °C
16. Non-frost-free freezer -20 °C
17. Flow cytometer (BD Biosciences, model: FACSCanto II) or equivalent.

G. Immunofluorescence

1. Pipette aid (Labnet, catalog number: FastPette™ V2 P2000) or other equivalent available pipette aid
2. P20 Micropipette (Rainin, catalog number: 17014392) or other available P20 pipette
3. P200 Micropipette (Rainin, catalog number: 17014391) or other available P200 pipette
4. P1000 Micropipette (Rainin, catalog number: 17014382) or other available P1000 pipette
5. Refrigerator 2-8 °C
6. Non-frost-free freezer -20 °C
7. Confocal laser scanning fluorescence microscope (Olympus, FluoView1000)

Software

3. FlowJo (Version 10) (FlowJo, LLC, <https://www.flowjo.com/>)
4. FlowView ASW V03.01.03.03 or V4.2a (Olympus Life Science, <https://www.olympus-lifescience.com/en/support/downloads/>)
5. ccEstimate available on request
6. R 3.5.1
7. R package EBImage (Pau *et al.*, 2010)

Procedure

- A. iPSC cell culture is also described in detail in D'Antonio-Chronowska *et al.* (2019a)
3. Thaw iPSC cells
 - n. Prepare 12 ml of mTeSR containing 10 µM ROCK Inhibitor.
 - o. Transfer 9 ml of mTeSR containing 10 µM ROCK Inhibitor into a sterile conical tube labeled with the name of the line.
 - p. Remove vial of cryopreserved cells from liquid nitrogen tank. Keep vial on dry ice.
 - q. Place and shake gently in a 37 °C water bath until a pea-sized ice crystal remains (around 2 min).
 - r. Wipe off excess water from the vial, spray with 70% ethanol before placing in the hood.
 - s. Remove thawed cells from the vial and add gently into 9 ml mTeSR containing 10 µM ROCK Inhibitor in a conical tube. Wash the vial with 1-2 ml of mTeSR containing 10 µM ROCK Inhibitor. Collect all cells in the same conical tube.
 - t. Centrifuge cells for 5 min at 53 x g (500 RPM in a Sorvall 75006445 rotor with 75006441 K buckets) at room temperature.
 - u. Aspirate supernatant, and gently resuspend cell pellet in 2 ml of mTeSR containing 10 µM ROCK Inhibitor (1 cryovial is thawed into 1 well of 6-well plate).

Note: The iPSC cells are cryopreserved in clumps and cannot be counted. Therefore, we recommend to cryopreserve cells from 1 well of a 6-well plate into 1 cryovial, and then to thaw cells from one cryovial onto 1 well of a 6-well plate.
 - v. Label a Matrigel plate with name of line, clone and passage number. Aspirate DMEM/F-12 from Matrigel-coated plate. Add +1 to the passage number after thawing.

Note: Do not add +1 to the passage number if the passage number was increased during cryopreservation of iPSCs.
 - w. Plate cells resuspended in 2 ml into one well of a Matrigel-coated 6-well plate (final volume 2 ml/well).
 - x. 24 h after plating, observe cells. Wash cells gently with DMEM/F-12 (2 ml/well) to remove cell debris and feed using fresh mTeSR medium without ROCK Inhibitor (2 ml/well).
 - y. Daily, observe the iPSCs, remove the differentiated cells, and change the medium with fresh mTeSR (2 ml/well).

Note: It is critical to maintain iPSC culture differentiation free. For details on how to mark and remove differentiated iPSC cells, please refer below to procedure: 2. iPSC passaging using Dispase (Steps A2c-A2d) and Figure 1.
 - z. Cells should reach 80-90% of confluence and be ready for passage in about 5 days.

iPSCs can be passaged using multiple methods, depending on the needs. For routine iPSC culture and expansion, cells should be passaged using Dispase, which cleaves the proteins of extracellular matrix used for iPSC culture (Matrigel). Dispase will dissociate iPSCs into clumps

of cells, which is a gentle method of iPSC expansion and maintenance. We recommend passaging iPSC with Dispase at the ratio 1:2 (one well split onto 2 wells) or 1:3 (one well split onto 3 wells), which allows to easily schedule iPSC cell maintenance. Cells passaged with Dispase should reach about 80% confluence in about 4-5 days. Other ratios also can be used (*i.e.*, 1:1 or 1:4), however we recommend testing it for individual lines.

When iPSC cells have to be expanded at much higher ratios or using a defined cell number, iPSC should be dissociated as single cells using Versene or Accutase. Please note that single cell passage is very stressful for iPSCs and leads to cell apoptosis. To prevent dissociation-induced apoptosis of iPSC, reagents like ROCK inhibitor, Y-27632 dihydrochloride (or others) have to be used. We do not recommend dissociating iPSCs into single cells for routine expansion or prior to cryopreservation.

Versene is a non-enzymatic, gentle method of single cell dissociation which acts by chelating metal ions (mainly magnesium and calcium) required by integrins to maintain cell-cell and cell-extracellular matrix contacts. We have routinely expanded over 150 different iPSC lines with Versene prior to plating large-scale monolayers, however we do not recommend dissociating cells using Versene in more than two consecutive passages. Accutase is an enzymatic reagent that allows for very efficient single cell dissociation. Although Accutase is a gentle reagent, commonly used for iPSC, we recommend using Accutase only for monolayer plating.

Notes:

- a. *It is critical for a successful iPSC-CVPCs differentiation to use healthy and pluripotent iPSC cells and use iPSC which were culture was maintained free of differentiation.*
 - b. *Optimal cell number will vary depending on the scale of differentiation. Differentiation at the scale described in this protocol requires that cells are expanded gradually, by performing two Versene passages (Passages: Versene I and Versene II).*
4. iPSC passaging using Dispase
- p. Prepare 1x (2 mg/ml) Dispase solution by adding 9 ml DMEM/F-12 to 1 ml of 10x Dispase (20 mg/ml).
 - q. Allow 1x Dispase solution to come to room temperature.
- Note: 1x Dispase solution can be stored at 4 °C for maximum 2 weeks.*
- Mark any areas of differentiation in the well(s) to be split, using the Microscope Object marker refer to Figure 1 for representative image of healthy pluripotent colonies (A) and differentiated colonies which need to be removed (B).
- r. Aspirate spent media. Aspirate marked areas of differentiation, if any, by gently tapping a Pasteur pipette within the marked circle (Figure 1C). Wash with 2 ml of DMEM/F-12 per well.

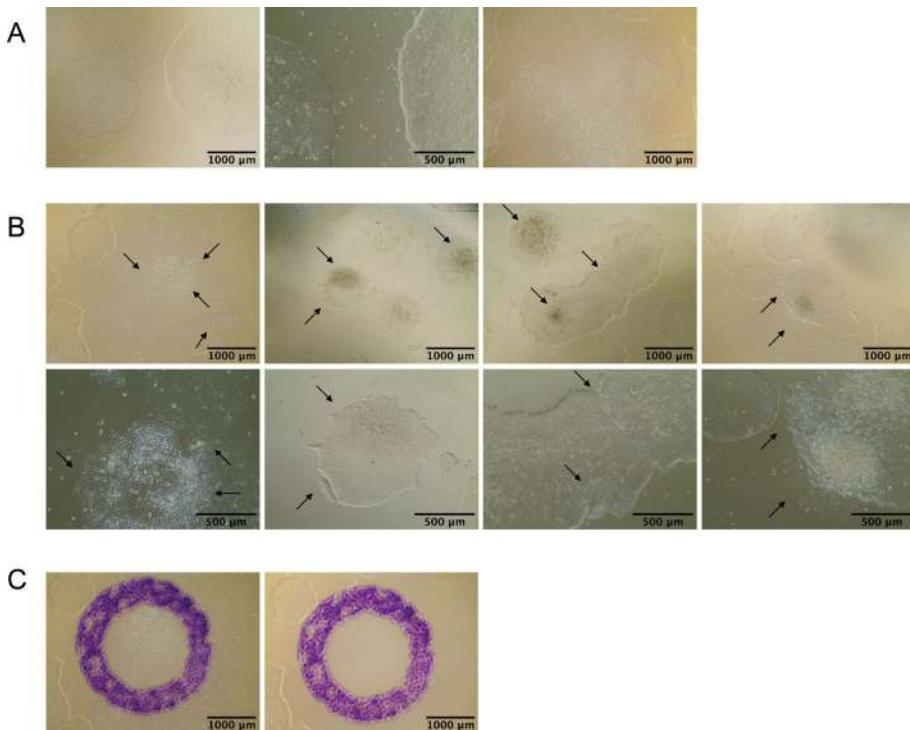


Figure 1. Pluripotent and differentiated iPSC colonies. A. Healthy and pluripotent iPSC colonies fuse into larger colonies: before fusion (left and middle) and after fusion (right). Healthy and pluripotent iPSC colonies have a smooth, flat, colony surface, are very compact with tightly growing cells that have almost invisible cell membranes (borders between cells), and uniform, well defined, colony edges. Cells have high nucleus:cytoplasm ratios with clearly visible nucleoli. Almost any deviation from this morphology may suggest loss of pluripotency and may indicate unwanted differentiation. B. Examples of differentiated iPSC cell which need to be removed from the culture. Black arrows indicate differentiated cells. Cells of various iPSC lines may have diverse morphology upon differentiation and these are only a few representative examples. C. Differentiated colony has been marked in purple dye using Microscope Object marker (left) and aspirated with a Pasteur pipette (right).

- s. Add 1 ml of 1x Dispase in each well to be split. Incubate at 37 °C for 5 min.
- t. Check morphology of colonies after 5 min.

When edges of the colonies are slightly curled up, cells are ready to be passaged. If edges of colonies are not curled up, incubate cells at 37 °C for another 1-2 min. Do not incubate with Dispase for longer than 8 min. Refer to Figure 2A for representative image of colonies with edges curled up.

- u. Aspirate Dispase from all wells.

Note: iPSC colonies passaged with Dispase will remain fully attached to the well. Only the edges of the colonies will be slightly curled up indicating that cells are ready for the next steps.

- v. Rinse the wells gently 3 times with DMEM/F12 (2 ml/well).

- w. Add 1 ml of mTeSR media to each well to be passaged.
- x. Use a glass serological pipette to detach colonies. Hold the pipette at a 90° angle to the surface of the plate. Scrape across the surface of the 6-well plate in the motion outlined in Figure 2B (start from top left side of the well and zig-zag tightly down to bottom-right side, then turn plate clockwise or counterclockwise and scrape again). Scrape until at least 90% of the colonies are detached from the well.
- y. Wash plate with the volume of mTeSR required to bring cells up to the final volume needed to seed a new Matrigel-coated vessel. Calculate the final volume considering 2 ml per each well to be seeded with passaged cells. For example, if cells are to be passaged 1 to 3 the final volume will be 6 ml, therefore the volume of mTeSR used to wash the plate is 5 ml.
- z. Seed cells on a new Matrigel plate plating 1 ml of cell suspension per well and then add 1 more ml of cell suspension to each well (Figure 2C). Plate cells dropwise across the entire surface of the well to ensure uniform plating.
 - aa. Observe seeded cells under microscope to ensure even plating.
 - bb. Place in a 37 °C incubator. Shake the plate in T-shape to homogenously distribute the colonies pieces in the well.
 - cc. Twenty-four hours after plating gently, wash cells with DMEM/F-12 before adding fresh mTeSR medium.

Note: For a healthy and efficient iPSC culture, it is critical to plate cells uniformly. Plate cells uniformly across the entire surface of the well and, when plating multiple wells plate cells uniformly across all wells.

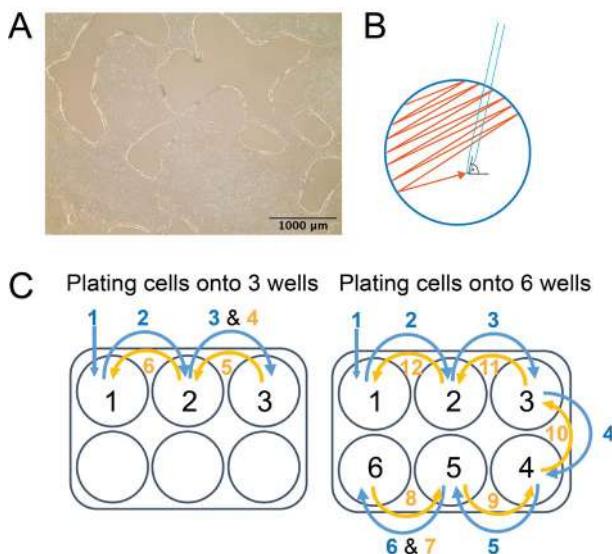


Figure 2. Passaging iPSC with Dispase. A. Edges of the iPSCORE_2_1 iPSC colonies are curled up after 5 min incubation with Dispase. B. Pattern of movement of a glass serological pipette during the iPSCs passaging with Dispase. After scraping the well in one direction, turn the plate clockwise or counterclockwise by 90° and scrape remaining iPSC colonies again. About 90% cells should be detached from the well. C. Example of plating

cells when passaging iPSC cells from 2 wells onto 6 wells. Add 1 ml of cell suspension into each well following a pattern: when plating cells onto three wells: 1-2-3-3-2-1 or when plating cells onto six wells: 1-2-3-4-5-6-6-5-4-3-2-1. Please refer to the section on iPSC passaging using Dispase for details. (Described also in detail in D'Antonio-Chronowska *et al.*, 2019a)

B. Passaging iPSC using Versene (Versene I)

Notes:

- a. *After thawing an iPSC line, passage cells with Dispase at least once before passaging cells with Versene.*
- b. *Optimal cell number will vary depending on the scale of iPSC-CVPC differentiation. Differentiation at the scale described in this protocol requires cells from 1-2 wells for Versene I passage.*

12. Remove 6-well plates from the incubator. When iPSC cells are at around 80% confluence (cells are ready for a passage), iPSC cells are ready for Versene I passage. Mark all differentiated cells, which need to be removed (Figure 1).
13. Aspirate the spent medium. Remove all marked differentiated cells and wash cells with DMEM/F-12 (2 ml/well).
14. Aspirate DMEM/F-12 and add 1 ml of room temperature Versene® (EDTA) 0.02% to well of a 6-well plate. Incubate cell for 5 min at 37 °C.
15. After 5 min of incubation check cells under microscope to ensure the cells are ready—individual cell borders should be visible. (If the cells are not ready, return cells to incubator and allow cells to act for another 30 s-1 min. Do not allow Versene incubation exceed total of 8 min).
16. Gently aspirate the Versene from each well. DO NOT wash cells with DMEM/F12.
Note: Individual cells incubated with Versene will be clearly visible. Cells will remain attached to the surface of the well however the plate should be handled gently.
17. Add 1 ml per well of mTeSR containing 5 µM ROCK inhibitor and re-suspend cells as single cells without scraping plate surface, by pipetting up and down using a P1000 pipette. Pipette cells 10-12 times, turn the dish by 180° (upside down) and pipette 5 more times. Collect cells from all wells in a 15 ml conical tube. You should not see any cell clumps.
18. Wash well (wells) twice with 3 ml of mTeSR containing 5 µM ROCK Inhibitor. Collect all cells in the same conical tube.
19. Centrifuge the cells at 53 x g (500 RPM in a Sorvall 75006445 rotor with 75006441 K buckets) for 5 min at room temperature. Aspirate the supernatant and resuspend cells in 3-5 ml of mTeSR containing ROCK Inhibitor.
20. Mix the pooled cell suspension by inverting 20 times or more if necessary. Perform the live cell count using 0.4% Trypan Blue Solution.
Note: iPSC cell viability should not be lower than 80%.

21. Prepare required number of cells. For a Versene I passage on three wells of a 6-well plate, prepare 7 ml of cell suspension containing 1.05×10^6 cells in a 15 ml conical tube. Mix cell suspension very well by inverting the tube 20 times.
Note: Optimal cell numbers will vary depending on the scale of differentiation. iPSC-CVPCs differentiated at the scale described in this protocol require plating 9.0×10^5 live cells onto three wells of a 6-well plate during the Versene I passage (3.0×10^5 live cells per each well of a 6-well plate).
22. Add 2 ml of cell suspension per each of the three well of a 6-well plate. Add cells dropwise using a 5 or 10 ml pipette.
Note: It is critical to plate cells uniformly on the entire surface of the plate. To help distribute the cells uniformly plate one dish at the time and shake the newly plated dish in a cross shape (T-shape).
23. Place plates in the incubator without stacking the plates. Incubate the cells until next morning, at 37 °C, 5% CO₂.
24. Next day change medium for fresh mTeSR without ROCK inhibitor (2 ml/well). iPSC after Versene I passaged requires culturing cells for about 3-4 days until the culture reaches 80% confluency. Change medium daily with fresh mTeSR. Maintain differentiation-free iPSC culture.

C. Passaging iPSC using Versene (Versene II)

Note: Optimal cell number will vary depending on the scale of differentiation. iPSC-CVPCs differentiated at the scale described in this protocol requires cells from 3 wells for Versene II passage.

1. Remove 6-well plates from the incubator. When iPSC cells are at around 80% confluency (cells are ready for a passage), iPSC cells are ready for Versene II passage. Mark all differentiated cells, which need to be removed (Figure 1).
2. Aspirate the spent medium. Remove all marked differentiated cells and wash cells with DMEM/F-12 (2 ml/well).
3. Aspirate DMEM/F-12 and add 1 ml of room temperature Versene® (EDTA) 0.02% to well of a 6-well plate. Incubate cell for 5 min at 37 °C.
4. After 5 min of incubation check cells under microscope to ensure the cells are ready—individual cell borders should be visible. (If the cells are not ready, return cells to incubator and allow cells to act for another 30 s-1 min. Do not allow Versene incubation exceed total of 8 min).
5. Gently aspirate the Versene from each well. DO NOT wash cells with DMEM/F12
6. Add 1 ml per well of mTeSR containing 5 µM ROCK inhibitor and re-suspend cells as single cells without scraping plate surface, by pipetting up and down using a P1000 pipette. Pipette cells 10-12 times, turn the dish by 180° (upside down) and pipette 5 more times. Collect cells from all wells in a 15 ml conical tube. You should not see any cell clumps.
7. Wash all wells twice with 5 ml of mTeSR containing 5 µM ROCK Inhibitor. Collect all cells in the same conical tube.

8. Centrifuge the cells at $53 \times g$ (500 RPM in a Sorvall 75006445 rotor with 75006441 K buckets) for 5 min at room temperature. Aspirate the supernatant and resuspend cells in 5-7 ml of mTeSR containing ROCK Inhibitor.
9. Mix the pooled cell suspension by inverting 20 times or more if necessary. Perform the live cell count using 0.4% Trypan Blue Solution.
Note: iPSC cell viability should not be lower than 80%.
10. Prepare required number of cells. For a Versene II passage on three 100 mm dishes, prepare 33 ml of cell suspension containing 6.6×10^6 cells in a 50 ml conical tube. Mix cell suspension very well by gently inverting the tube 20 times.
Note: Optimal cell number will vary depending on the scale of differentiation. iPSC-CVPCs differentiated at the scale described in this protocol require plating 6.0×10^6 live cells onto three 100 mm dishes during the Versene II passage (6.0×10^6 live cells per each 100 mm dish).
11. Add 10 ml of cell suspension per each of the three 100 mm dishes. Add cells dropwise using a 10 ml pipette. Before plating each of the 100 mm dishes, mix cells by inverting tubes 5-10 times.
Note: It is critical to plate cells uniformly on the entire surface of the plate. To help distribute the cells uniformly plate one dish at the time and shake the newly plated dish in a cross shape (T-shape).
12. Place dishes in the incubator without stacking the plates. Incubate the cells until next morning, at 37°C , 5% CO_2 .
13. Next day change medium for fresh mTeSR without ROCK inhibitor (10 ml/100 mm dish). iPSC after Versene II passaged requires culturing cells for about 3-4 days until the culture reaches 80% confluence. Change medium daily with fresh mTeSR. Maintain differentiation-free iPSC culture.

D. Monolayer plating

1. The day before plating monolayer mark 10 spots (views) at the bottom of each of the three T150 flasks using a marker resistant to 70% ethanol (Figure 3A). Coat three T150 flasks with Matrigel (20ml per flask). Place flasks in the incubator.
2. Prepare 140-150 ml of mTeSR medium containing 5 μM ROCK inhibitor.
3. Add 25 ml mTeSR medium containing 5 μM ROCK inhibitor to each of three T150 flasks coated overnight with matrigel. Place flasks in the incubator.
4. Remove 6-well plates from the incubator. When iPSC cells are at around 80% confluence (cells are ready for a passage), iPSC cells are ready for Monolayer. Mark all differentiated cells, which need to be removed (Figure 1).
5. Aspirate the spent medium. Remove all marked differentiated cells and wash cells with DMEM/F-12 (2 ml/well).
6. Aspirate DMEM/F-12 and add 5 ml of room temperature Accutase to each 100 mm dish. Incubate cell for 8 min at 37°C .

7. After 8 min of incubation, add 5 ml per well of mTeSR containing 5 μ M ROCK inhibitor and resuspend cells as single cells without scraping plate surface, by pipetting up and down using a P1000 pipette. Pipette cells 10-12 times, turn the dish by 180° (upside down) and pipette 5 more times. Collect cells from all wells in a 50 ml conical tube. You should not see any cell clumps.
Note: DO NOT aspirate the iPSC cells after incubation with Accutase. iPSC cells will be detached from the surface of the well.
8. Wash all wells twice with 5 ml of mTeSR containing 5 μ M ROCK Inhibitor. Collect all cells in the same conical tube.
9. Centrifuge the cells at 53 $\times g$ (500 RPM in a Sorvall 75006445 rotor with 75006441 K buckets) for 7 min at room temperature. Aspirate the supernatant and resuspend cells in 10 ml of mTeSR containing ROCK Inhibitor.
10. Mix the pooled cell suspension by inverting 20 times or more if necessary. Perform the live cell count using 0.4% Trypan Blue Solution.
Note: iPSC cell viability should be not lower than 80%.
11. Prepare required number of cells. Optimal cell number will vary depending on the scale of differentiation. iPSC-CVPCs differentiation protocol requires 3.66×10^4 live cells per cm^2 (5.5×10^6 per one 150 cm^2 flask). For three T150 flasks, prepare in a 50 ml conical tube 33 ml of cell suspension containing 1.815×10^7 cells. Mix cell suspension very well by inverting the tube 20 times.
12. Add 10 ml of cell suspension per T150 flask dropwise using a 10 ml pipette to each of the three flasks containing 25 ml of mTeSR with ROCK Inhibitor.
Note: It is critical to plate cells uniformly on the entire surface of the flask. To help distribute the cells uniformly plate one flask at the time, adding cells dropwise to the entire surface of the flask. This step may require practice.
13. Once 10ml of cells was added shake the newly plated flask in a cross shape (T-shape). Place the flask at a clean, leveled surface (table) and shake the flask again in a cross shape (T-shape).
Note: Start with stronger movement and continually decrease the shaking, finishing with a very gentle movement. This step may require practice.
14. Place flasks in the incubator without stacking them. Incubate the cells until next morning, at 37 °C, 5% CO₂.
15. Next day change medium for fresh mTeSR without ROCK inhibitor (35 ml/T150 flask). Monolayer for iPSC-CVPCs cell differentiation requires culturing cells for about 4-5 days until the monolayer reaches 80% confluence. Change medium with fresh mTeSR daily.

- E. Estimation of monolayer confluence and optimal time for initiation of iPSC-CVPCs differentiation using ccEstimate
- Variable growth rates across different iPSC lines results in them reaching optimal confluence at the monolayer stage at different time points (*i.e.*, faster growing lines will obtain the optimal confluence earlier) and hence impact differentiation outcome. To enable the differentiation of large number of

different iPSC lines we developed cell confluence Estimates (ccEstimate), an automatic pipeline, that analyzes images of monolayer-grown cells, determines their confluence at various timepoints and predicts when the cells will reach 80% confluence. We also used ccEstimate after the initiation of differentiation, *i.e.*, after addition of CHIR99021, to measure the actual cell confluency in an unbiased, *i.e.* operator independent, way.

ccEstimates are performed by first dividing each T150 flask into 10 sections (Figure 3) and acquiring images for each section every 24 h after cells are plated as a monolayer. The final images are acquired immediately after treatment with CHIR99021, which occurs when confluence is at 80% (Day 0). The time required for cells to reach 80% confluence is estimated on the basis of the confluence curve derived for each section in each flask. To digitally measure iPSC confluency, ccEstimate performs image analysis using the EBImage package in R (Pau *et al.*, 2010). Images are read using the readImage function. As lighting may be different between the center and the border of an image, only the central part of the image is retained. To separate cells from the background and calculate confluency (*i.e.*, the fraction of the surface of the flask that is covered by cells) the following operations are performed. Confluency measurement data is collected for at least the first three days after plating as monolayer to train a generalized linear model (GLM) using the function glm in R to estimate when cells must be treated with CHIR99021. Estimation is performed separately for each flask section and CHIR is added to all three flasks associated to a given line when at least 75% of sections have 80% confluency (Figure 3).

Using ccEstimate, allows one to start differentiation at the same confluency level for each iPSC line, thereby reducing or neutralizing the effects of different growth rates. Based on our data, on average, each line required 4.23 ± 1.12 days after plating a monolayer to reach 80% confluence. The correlation between the number of days required to reach 80% confluence and the %CM population was -0.05, suggesting that iPSC growth rate does not affect differentiation outcome (D'Antonio-Chronowska *et al.*, 2019b).

1. Mark the spots (views) at the bottom of the vessel. In case of using T150 flask mark 10 spots as indicated in Figure 3A.
2. Take images of the cells starting from 24 h after plating monolayer. Provide the following nomenclature to the file:

UDID_NNN SUBJECT_CLONE_PASSAGE_MONO_DAY_FLASK_VIEW_DATE_

Example: UDID_001_ipSCORE-2-3_C5_P22_MONO_D1_FL1_VIEW1_20150723_

Where:

UDID – Unique Differentiation Identifier

NNN – UDID number

SUBJECT – Subject ID from whom iPSC was derived (iPSC line name)

CLONE – iPSC clone number

PASSAGE – iPSC passage number

MONO – indicates Monolayer stage

DAY – number of the day of monolayer stage

FLASK – Indicates number of flask when performing differentiation in multiple flasks

VIEW – location in the flask (Please refer to the Figure 3A for details)

DATE – Date of the imaging in format YYYYMMDD

Note: Make sure that the markers are not present at the image. Make sure that the files names are unique and according to the abovementioned example. This will allow for an automatic analysis of the images and prediction of the time when monolayer reaches 80% confluency.

3. Every 24 h take images in the same spots

Optional: Use automatic imaging system to take images

Images (JPG, PNG or TIFF format) are analyzed according to following procedure:

1. The image is transformed to monochromatic by determining the intensity of each pixel as the average of the intensities of the red, green and blue channels.
2. Edges are sharpened using high-pass filter. The matrix used for this filter is 15 x 15 with values -1 on the diagonals and +28 in the center.
3. Contrasts are enhanced by multiplying the pixel intensities by 2.
4. Mean and standard deviation of the pixel intensities are calculated. The image is transformed from monochromatic to binary by setting all pixels with intensity more than two standard deviations higher than the mean to white (intensity = 1) and all other pixels to black (intensity = 0).
5. The resulting binary image is dilated using a disc-shaped structuring element with diameter 5 pixels.
6. 1,000 50 x 50 pixels sub-images are randomly selected. For each sub-image, the number of white pixels is calculated. Each sub-image is considered as “cell” if at least 50% of its pixels is white, “background” if at least 50% of its pixels is black. Confluence is calculated as the number of “cell” sub-images divided by 1,000.

ccEstimate is available on GitHub (<https://github.com/s041629/ccEstimate>) and requires minimal knowledge of R. After installing the package and its dependencies, it is sufficient for the user to run the function `ccEstimate(input_file.jpg)`, where `input_file.jpg` is the image obtained from the monolayer iPSC culture. This function returns a numeric value, included between 0 and 1, representing the confluency. For predicting the optimal moment for initiating differentiation, the user will have to save all images associated with the same sample in a single folder and then run `run_confluence_estimation(input_folder)`. The ccEstimate package includes an example dataset, which can be investigated using the `run_confluence_estimation(run_example = TRUE)` function.

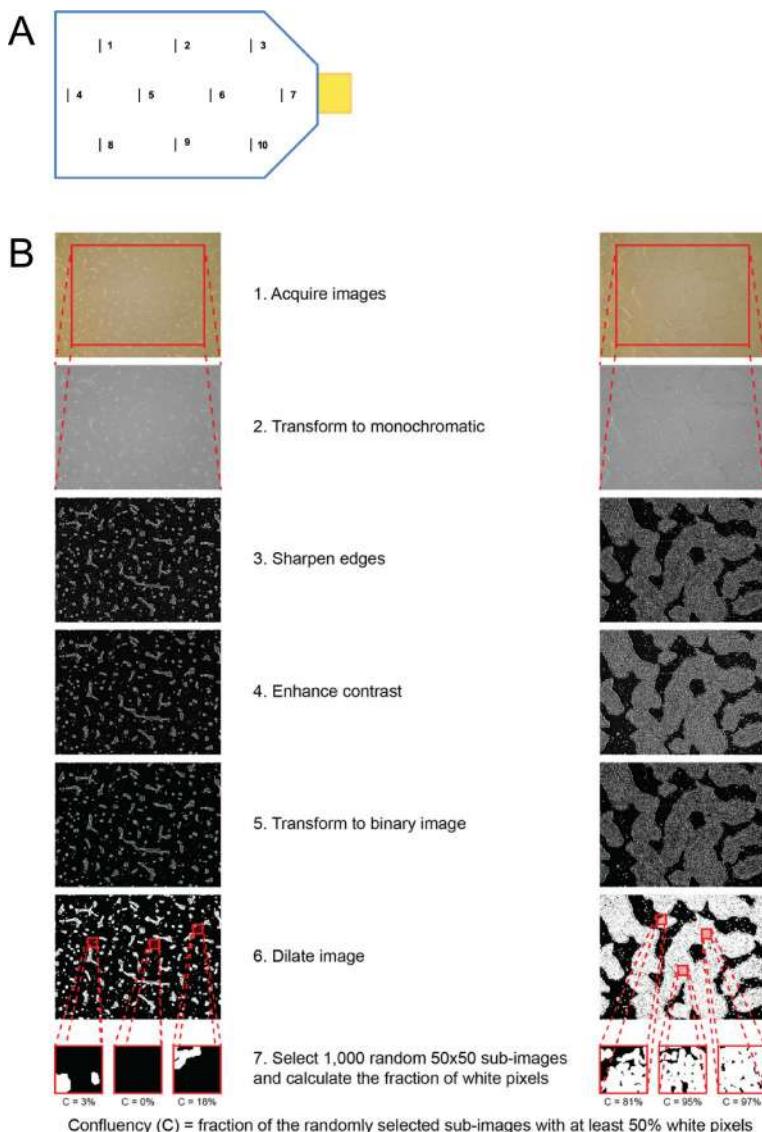


Figure 3. Schematic representation of the estimation of monolayer confluency and optimal time for initiation of iPSC-CVPCs differentiation using ccEstimate. A. Schematic representation of where to locate marks for taking images. Lines and numbers indicate 10 locations (views) where images will be taken daily. B. Schematic representation of image processing performed by ccEstimate. Adapted from D'Antonio-Chronowska *et al.* (2019b).

F. iPSC-CVPCs differentiation

Refer to Figure 4 for a schematic representation of the differentiation protocol and to Figure 5, Figure 6 and Figure 7 and Video 1 for representative images of iPSC-CVPCs.

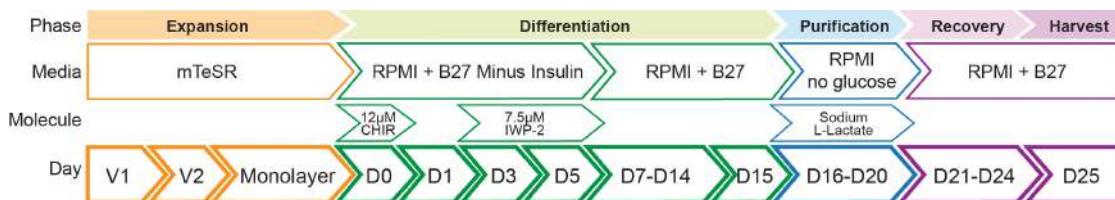


Figure 4. Schematic representation of the iPSC-CVPCs differentiation protocol. CHIR99021 (CHIR), Versene 1 (V1), Versene 2 (V2). Adapted from D'Antonio-Chronowska et al. (2019b).

Notes:

- a. It is critical to maintain 24h schedule of media change (media change should be performed at the exact same time, on the days indicated in Steps F1-F26), especially during first 10 days of differentiation. For example: if D0–initiation of the differentiation (Step F1) is performed at 9:15 AM, following media changes should be done at 9:15AM according the schedule of media changes indicated in Steps F2-26.
- b. Mix well all the supplements with media by inverting 20-30 times.
- c. All media changes are performed with 35 ml/T150 flask.

13. Day 0 (D0): When iPSC monolayer reaches about 80% confluence initiate the iPSC-CVPCs differentiation by replacing mTeSR medium with RPMI Minus medium (see Recipes: Table 6) supplemented with 12 μM CHIR99021 (see Recipes: Table 11).
14. D1: 24 h after initiation of the differentiation replace spent medium with fresh RPMI Minus medium.
15. D2: No media change.
16. D3: 72 h after initiation of the differentiation prepare a combined fresh and spent RPMI Minus medium by mixing 18 ml of fresh RPMI Minus medium and 18 ml of spent RPMI Minus medium. Mix media gently by inverting and add 7.5 μM IWP-2 (see Recipes: Table 12). Mix well by inverting. Replace spent medium with combined RPMI Minus medium supplemented with 7.5 μM IWP-2.
Note: Replace spent medium with the combined medium prepared with the medium from the same flask. The effect of replacing combined medium prepared with the medium from different flask has not been extensively tested.
17. D4: No media change.
18. D5: 48 h after addition of IWP-2 (120 h after initiation of the differentiation) replace spent medium with fresh RPMI Minus medium.
19. D6: No media change.
20. D7: 168 h after initiation of the differentiation replace spent RPMI Minus medium with fresh RPMI Plus medium (see Recipes: Table 7).
21. D8: No media change.
22. D9: 216 h after initiation of the differentiation replace spent medium with fresh RPMI Plus medium.

23. D10: No media change.
24. D11: Replace spent medium with fresh RPMI Plus medium.
25. D12: No media change.
26. D13: Replace spent medium with fresh RPMI Plus medium.
27. D14: No media change. Coat new T150 flasks with Matrigel (20 ml per flask).
28. D15: Passage the iPSC-CVPCs using Accutase.

Note: Mechanical separation of iPSC-CVPCs at D15 is critical for efficient metabolic cell purification using Lactate.

- a. (Optional) Take images and/or video recording of the cells. Refer to Figure 5A for a representative image of iPSC-CVPCs at D15.
- b. Aspirate spent medium and wash cells with PBS (20 ml/T150 flask).
- c. Aspirate PBS and add 10 ml of room temperature Accutase to each T150 flask. Incubate cells for 10 min at 37 °C.
- d. After 10 min of incubation add 10 ml per flask of CVPCs Harvest medium (see Recipes: Table 9).
- e. Gently scrape cells from the surface of the flask, using a cell scraper.
- f. Collect cells in a 125 ml sterile bottle.
- g. Wash plate three additional times, each with 10 ml of CVPCs Harvest medium. Collect all cells in the same bottle.

Note: Do not break the cells into single cells. Cells will re-attach better if they are in small clumps of few cells per clump.

- h. Mix all the cells by inverting the bottle and divide them into appropriate amount of 50ml conical tubes to centrifuge the cells.
- i. Centrifuge cells for 8 min at 136 x g (800 RPE in a Sorvall 75006445 rotor with 75006441 K buckets) at room temperature.
- j. After centrifugation, aspirate the supernatant and resuspend the cells in 20 ml of RPMI Plus medium supplemented with 5 µM ROCK Inhibitor.
- k. Mix the cell suspension by inverting 10-20 times. Perform the live cell count using 0.4% Trypan Blue Solution.

Note: Because the cells were passaged in small clumps (and not as single cells), the goal is to obtain an estimate and not a precise cell count. The estimated cell count will be used to determine the number of new T150 flasks onto which cells will be re-plated.

- l. Determine the number of flasks necessary to re-plate the cells. Calculate $1.5\text{--}2.0 \times 10^8$ cells per each fresh flask ($1\text{--}1.3 \times 10^6/\text{cm}^2$).
- m. Resuspend the cells into appropriate volume of RPMI Plus medium supplemented with 5 µM ROCK Inhibitor, necessary to plate fresh flasks (35 ml/T150 flask). Mix cells well by inverting.
- n. Aspirate the DMEM medium used to coat the fresh flasks with matrigel.
- o. Re-plate the cells onto fresh flasks coated overnight with matrigel (35 ml/T150 flask) at the

density $1\text{--}1.3 \times 10^6/\text{cm}^2$ (please see Step F16k). Distribute cells uniformly across the entire surface of the flask.

Note: To help distribute the cells uniformly plate one dish at the time and shake the newly plated dish in a cross shape (T-shape).

- p. Place plates in the incubator. Incubate the cells until next day, at 37°C , 5% CO₂ without stacking the plates.
29. D16: Aspirate spent medium and wash cells with PBS (20 ml/T150 flask). Replace RPMI Plus medium with RPMI Lactate medium (see Recipes: Table 8).
30. D17: Replace spent medium with fresh RPMI Lactate medium.
31. D18: No media change.
32. D19: Replace spent medium with fresh RPMI Lactate medium.
33. D20: No media change.
34. D21: Aspirate spent medium and wash cells with PBS (20 ml/T150 flask). Replace RPMI Lactate medium with RPMI Plus medium
35. D22: No media change.
36. D23: Replace spent medium with fresh RPMI Plus medium.
37. D24: No media change.
38. D25: Collect all cells.
 - a. (Optional) Take images and/ or video recording of the cells. Refer to Figure 5B for a representative image of iPSC-CVPCs at D25.
 - b. Aspirate spent medium and wash cells with PBS (20 ml/T150 flask).
 - c. Aspirate PBS and add 10 ml of room temperature Accutase to each T150 flask. Incubate cells for 10 min at 37°C .
 - d. After 10 min of incubation add 10 ml per flask of CVPCs Harvest medium.
 - e. Gently scrape cells from the surface of the flask, using a cell scraper.
 - f. Collect cells in a 125 ml sterile bottle.
 - g. Wash plate three additional times, each with 10 ml of CVPCs Harvest medium. Collect all cells in the same bottle.
 - h. Mix all the cells by inverting the bottle and divide them into appropriate amount of 50ml conical tubes to centrifuge the cells.
 - i. Centrifuge cells for 8 min at $136 \times g$ (800 RPE in a Sorvall 75006445 rotor with 75006441 K buckets) at room temperature.
 - j. After centrifugation, aspirate the supernatant and resuspend the cells by pipetting up and down in 10-20 ml of CVPCs Harvest medium (5 ml for each flask from which the cells were collected).
 - k. Mix the cell suspension by inverting 10-20 times. Perform the live cell count using 0.4% Trypan Blue Solution.
39. Estimate the number of cells to be cryopreserved or processed for molecular assays, flow cytometry and/or immunofluorescence.

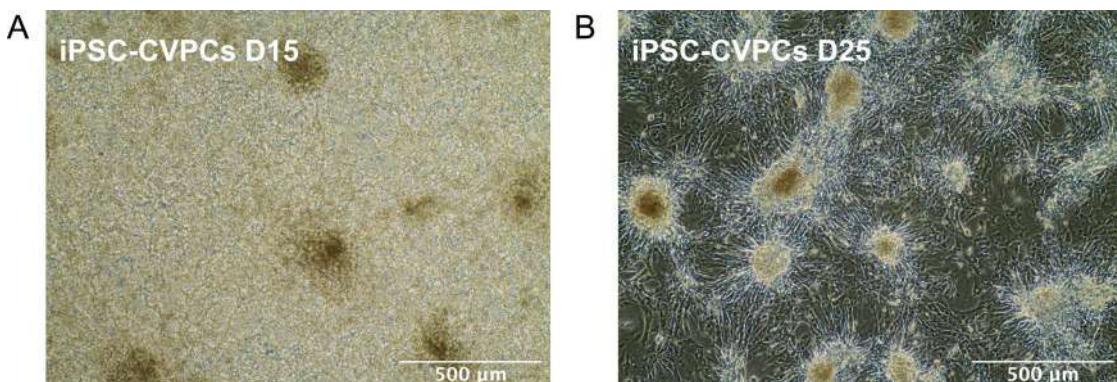


Figure 5. Representative images of iPSC-CVPCs. A. D15: Cells grow in multiple layers and form a thick sheet. B. D25: Most of the cells form a single cell layer, however some cells can form aggregates and clusters.



Video 1. Representative video of iPSC-CVPCs beating at D15

G. Cryopreservation of iPSC-CVPCs

11. Prepare 2x iPSC- CVPCs freezing medium by preparing 20% DMSO solution in FBS. Prepare 0.25 ml of 2x iPSC-RPE freezing medium per each cryovial intended to be cryopreserved. Freeze cells at a final density of $1.2 \times 10^7/\text{ml}$ (depending on the downstream experiments the volume and the concentration of the cryopreserved iPSC-CVPCs cells in a single cryovial can be modified).
Optional: If a serum free conditions are required, prepare the 2x iPSC-CVPCs freezing medium using KOSR instead of FBS.
12. Prepare and print the labels for cryovials. Prepare $n + 2$ number of labels ($n =$ number of cryovials to be cryopreserved). Prepare and affix the labels on all cryovials to be frozen, use one label for the Mr. Frosty and one label for record keeping (*i.e.*, lab book).
13. Transfer desired number of cells to be cryopreserved into a new 15 ml or 50 ml conical tube.
14. Centrifuge cells for 5-8 min at $136 \times g$ (800 RPM in a Sorvall 75006445 rotor with 75006441 K buckets) at room temperature (adjust the time of centrifugation depending on the volume of cells).

15. After centrifugation, aspirate the supernatant and resuspend the cells in 0.25 ml of FBS (or KOSR) per each cryovial to be frozen at the concentration of $2.4 \times 10^7/\text{ml}$ (*i.e.*, for 10 cryopreserved vials resuspend 6×10^7 cells in 2.5 ml of FBS or KOSR).
16. Open all pre-labeled cryovials and add 0.25 ml of cell suspension to each cryovial.
17. Add 0.25 ml of 2x iPSC-CVPCs freezing medium to each cryovial containing the iPSC-CVPCs cell suspension.
18. Close all cryovials and gently invert them 5-6 times to mix cell suspension and 2x iPSC-CVPCs freezing medium (see Recipes: Table 10). Transfer cryovials to Mr. Frosty freezing container.
19. Immediately transfer Mr. Frosty into a -80 °C freezer. When freezing large number of cryovials (*i.e.*, multiple Mr. Frosties) prepare individual batches, with each batch containing only the number of cryovials that will fit into one Mr. Frosty.
20. After 24-48 h, transfer the cells into a liquid nitrogen vapor tank. Update accordingly the records (*i.e.*, box maps).

Here, we provide detailed protocols for flow cytometry (FC) and immunofluorescence (IF) which can be applied to perform quantitative (FC) and qualitative (FC and IF) quality control of derived iPSC-CVPC cells.

H. Flow cytometry

24. After the live cell count (Step F26k), determine how many cells should be fixed for flow cytometry analysis and transfer desired number of cells into a 15 ml conical tube. Use at least $2-5 \times 10^6$ cells.
25. Centrifuge cells for 5 min at $136 \times g$ (800 RPM in a Sorvall 75006445 rotor with 75006441 K buckets) at room temperature.
26. After centrifugation, aspirate the supernatant and resuspend the cells in 10 ml of PBS.
Optional: If the volume of cell suspension used for flow cytometry is smaller than 0.5 ml then add directly to the cells 14 ml of PBS and centrifuge mix of cells and PBS for 8 min at $136 \times g$ (800 RPM in a Sorvall 75006445 rotor with 75006441 K buckets) at room temperature.
27. Fix the cells using cold 90% methanol:
 - a. During centrifugation prepare fresh 1% Formaldehyde in PBS. Mix well. Prepare 1ml of 1% Formaldehyde for each sample.
 - b. After centrifugation decant or aspirate supernatant.
 - c. Resuspend the cells using with P1000 pipette in 1ml of 1% Formaldehyde. Incubate cells for 20 min at room temperature.
 - d. After the incubation add 5 ml of PBS and centrifuge samples for 8 min at $216 \times g$ (1,000 RPM in a Sorvall 75006445 rotor with 75006441 K buckets) at room temperature.
 - e. After centrifugation, dispose the supernatant containing formaldehyde into an appropriate waste container.

- f. Resuspend cells in 5 ml of PBS and centrifuge samples again for 8 min at 216 x g at room temperature.
 - g. After centrifugation, aspirate the supernatant and using a P1000 pipette resuspend the cells in 1-2 ml of cold 90% Methanol
 - h. Fix cells for at least 30 min at +4 °C or store them in 90% Methanol for up to few weeks.
28. After cells were fixed proceed to permeabilization and blocking
- a. Remove fixed cells from +4 °C fridge and add 5-8 ml of PBS to dilute Methanol.
 - b. Centrifuge samples for 10-12min at 216 x g (1,000 RPM in a Sorvall 75006445 rotor with 75006441 K buckets) at room temperature.
 - c. After centrifugation, dispose the supernatant containing methanol into an appropriate waste container.
 - d. Add 5 ml of PBS.
 - e. Centrifuge samples for 10 min at 216 x g (1,000 RPM in a Sorvall 75006445 rotor with 75006441 K buckets) at room temperature.
 - f. After centrifugation decant or aspirate supernatant.
 - g. Using a P1000 pipette resuspend the cells in 2-3 ml of Permeabilizing/Blocking buffer (see Recipes: Table 15).
 - h. Incubate cells for 30 min at room temperature
 - i. After incubation add 5 ml of PBS
 - j. Centrifuge samples for 10 min at 216 x g (1,000 RPM in a Sorvall 75006445 rotor with 75006441 K buckets) at room temperature.
 - k. After centrifugation decant or aspirate supernatant, add 5 ml of PBS and centrifuge the cells again for 10 min at 216 x g at room temperature.
 - l. After centrifugation decant or aspirate supernatant.
 - m. Using a P1000 resuspend the cells in FACS buffer (see Recipes: Table 14). At the concentration of 1×10^7 / ml.
29. After cell permeabilization and blocking stain the cells
- a. Following Table 21 prepare the primary antibodies staining mix. Stain cells at the concentration of 1×10^7 / ml (i.e., 2.5×10^5 cells should be stained in 25 µl of staining mix).
 - b. Transfer 25 µl of fixed and permeabilized cells into 2 wells of a 96-well round bottom assay plate. In order to limit usage of the antibodies and cells when staining multiple lines, mix equal number of cells from each line and transfer 25 µl of the cell mix into the control well (Mouse IgG1 antibody).
- Note: Staining of 2.5×10^5 cells allows for an efficient cells and reagent usage, however it is also possible to use 5×10^5 or 1×10^6 cells per staining maintaining the same antibodies dilution ratios and scaling up the volume.*
- c. Centrifuge plate at 863 x g (2,000 RPM in a Sorvall 75006445 rotor with 75006441 K buckets) for 8-10 s counting from when the speed reaches 863 x g (2,000 RPM in a Sorvall 75006445 rotor with 75006441 K buckets) at room temperature.

In detail:

- i. Set the centrifuge for $863 \times g$ (2,000 RPM), 1 min, room temperature); start the centrifuge and wait until the speed reaches $863 \times g$ (2,000 RPM).
 - ii. Count to 8-10 s and stop the centrifuge. The pellet after the centrifugation should be clearly visible especially when using 1×10^6 cells.
 - d. After centrifugation, gently aspirate the supernatant, being very careful not to aspirate any cells. If using vacuum to aspirate cells, use a P20 tip without a filter (or a P200 + P20 tips without filters). Leave about 10-20 μl of liquid in each well to avoid aspirating the cells.
 - e. Add 25 μl (or appropriate amount) of staining mix. Using a multichannel pipette set for 20 μl mix cells and antibodies gently by pipetting up and down 20 times.
 - f. Incubate cells with primary antibodies for 45 min in a +4 °C fridge.
 - g. During last 5 min of incubation, prepare the secondary staining mix following Table 21. Keep the secondary staining mix on ice, protected from light until use.
 - h. After 45 min, add 150 μl of FACS buffer (see Recipes: Table 14).
 - i. Centrifuge plate at $863 \times g$ for 8-10 s as described above (Step H6c).
 - j. After centrifugation, gently aspirate the supernatant, being very careful not to aspirate any cells as described above (H6d).
 - k. Using a multichannel pipette add 200 μl of FACS buffer and mix cells gently 5-6 times.
 - l. Centrifuge plate like in Step H6c.
 - m. Repeat Steps H6j-F6l to wash the cells for total of two washes.
 - n. Resuspend cells in 25 μl (or appropriate amount) of secondary staining mix. Using a multichannel pipette set for 20 μl mix cells and antibodies gently by pipetting up and down 20 times.
 - o. Incubate cells with secondary antibodies for 45 min in a +4 °C fridge, protected from light.
 - p. After the incubation with secondary antibodies follow the Steps H6h-F6m to wash the cells for total of two washes.
 - q. After the last centrifugation aspirate gently the supernatant and using a multichannel pipette resuspend the cells in 200 μl of FACS-FIX buffer (see Recipes: Table 16). Gently pipette the cells 5-6 times.
 - r. Using a P1000 pipette transfer each sample, one at a time, into a Corning™ Falcon™ Test Tubes with Cell Strainer Snap Cap passing the cells through the strainer in a cap.
Note: Straining cells is critical to ensure single cell acquisition and to prevent clogging flow cytometer.
 - s. With an additional 250 μl wash each well and transfer to the appropriate tube passing the cells through the strainer in a cap. Depending on the number of cells used for staining dilute the cells to an appropriate concentration to avoid clogging the flow cytometer.
 - t. Place all the tubes in an appropriate rack and wrap them in an aluminum foil to protect from light.
30. Proceed with acquisition using a flow cytometer FACS Canto II (or an alternative flow cytometer).

31. Perform the flow cytometry analysis using FlowJo software V 10.4. Refer to the Figure 6 for an example of iPSC-CVPCs flow cytometry staining results.

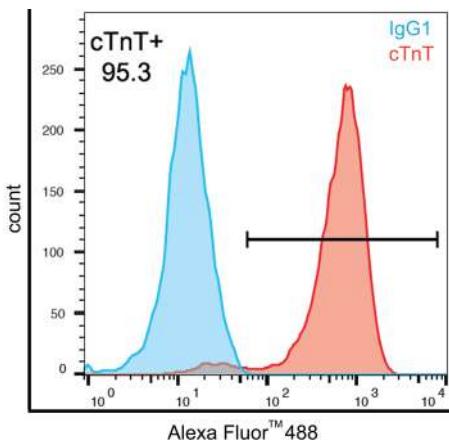


Figure 6. Flow-cytometry analysis of iPSC-CVPCs (iPSCORE_28_2) at Day 25 showing high staining of cardiac troponin T (cTnT, TNNT2) in red and immunoglobulin class control IgG1 in blue. Percentage of live single cardiac troponin T positive cells (cTNT+).

I. Immunofluorescence

18. Coat Millicell EZ SLIDE 8-well glass slides for at least 15 min with 0.1% Gelatin solution (see Recipes: Table 2).
19. Plate fresh or cryopreserved iPSC-CVPCs cells on the Matrigel coated Millicell EZ SLIDE 8-well glass slides. Plate at least 6 wells per line at the density of $1.6\text{--}2.1 \times 10^5/\text{cm}^2$.
20. Culture cells for at least 3-5 days until cells recover from passage or cryopreservation.
21. Aspirate the medium and wash cells twice with PBS. Aspirate the PBS.
22. Fix cells with 4% PFA for 20 min at room temperature.
23. Remove the PFA solution disposing it into an appropriate waste container.
24. Wash cells twice with PBS.
25. Permeabilize the cells using IF Perm Buffer II (see Recipes: Table 17). Incubate the cells for 8 min at room temperature.
26. Aspirate the IF Perm Buffer II.
27. Block the cells with IF Blocking Buffer II (see Recipes: Table 18) for 30 min at room temperature.
28. In the last 5 min of the blocking prepare the primary antibody solutions in IF Staining Buffer (see Recipes: Table 19 and Table 21) for the appropriate concentrations of the antibodies. Store antibodies solutions on ice until use.
29. After blocking aspirate all the buffer, wash cells twice with PBS and add antibodies solutions to the appropriate wells.
30. Incubate cells with the antibodies solution overnight at 4°C .
31. Next day (morning) prepare the secondary antibody solutions in IF staining Buffer. Refer to the Table 21 for the appropriate concentrations of the antibodies. Keep antibodies solutions on ice

until use, protected from light.

32. Aspirate the primary antibodies solutions and wash cells three times with PBS. After last wash aspirate all PBS.
33. Immediately add the secondary antibodies solutions to the appropriate wells. Incubate cells for 45–60 min at room temperature in darkness.
34. Aspirate the secondary antibodies solutions and wash cells three times with PBS. After last wash aspirate all PBS.
35. Detach the walls of the Millicell EZ SLIDE 8-well glass slides.
36. Add ProLong® Gold Antifade Reagent with DAPI following manufacturer's recommendations and gently mount the cover glass slide avoiding bubbles. Use a pencil rubber to gently remove any bubbles. Store the slide(s) at room temperature for several hours (best until next day) in darkness to allow proper mounting.
37. Acquire images using an appropriate immunofluorescence microscope (best is a confocal laser scanning fluorescence microscope). Refer to Figure 7 for an example of iPSC-CVPCs immunofluorescence staining.

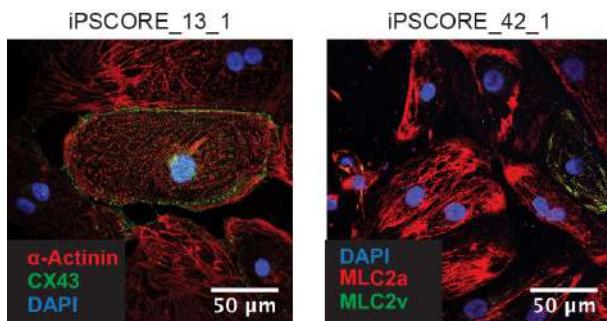


Figure 7. Immunofluorescence analysis of gap junction connexin 43 (CX43), anti-Myosin Light Chain 2 (MLC2v) antibody (Mouse monoclonal anti-Myosin Atrial Light Chain 7 (MLC2a), α -Actinin of iPSC-CVPCs at D30. Adapted from D'Antonio-Chronowska *et al.* (2019b).

Recipes

- D. Cell culture reagents and media preparation
 1. Matrigel solution (Table 1)

Table 1. Preparation of Matrigel solution

Reagent	Matrigel solution (referred to as Matrigel)
Components and preparation	1 mg Corning® Matrigel® Growth Factor Reduced (GFR) Basement Membrane Matrix 24 ml of ice cold DMEM/F-12 medium Mix well by pipetting and inverting
Method of sterilization	None
Notes	<ul style="list-style-type: none">• All plasticware used for preparation of Matrigel should be cold. Best if stored in a -20 °C freezer and removed immediately prior to use• Prepare the Matrigel solution on ice• Volumes of Matrigel solution used for coating:• 2 ml–1 well of a 6-well plate• 10 ml–100 mm tissue culture dish• 20 ml–T 150 tissue culture flask• When using other vessel adjust the volume of Matrigel to 210 µl/cm²
Storage	<ul style="list-style-type: none">• Store aliquots of Matrigel at -80 °C• Store coated plates in an incubator with humidity and gas control set to maintain 37 °C and 95% humidity• Plates coated with Matrigel should be used within two weeks; best if prepared one day before use

2. Gelatin solution (Table 2)

Table 2. Preparation of 0.1% Gelatin solution

Reagent	0.1% Gelatin solution
Components and preparation	50 ml PBS 0.05 g Gelatin from porcine skin Mix well inverting
Method of sterilization	None
Storage	4 °C

3. 1.10 mM ROCK inhibitor, Y-27632 dihydrochloride (Table 3)

Table 3. Preparation of 1.10 mM ROCK inhibitor, Y-27632 dihydrochloride

Reagent	10 mM ROCK inhibitor, Y-27632 dihydrochloride (referred to as ROCK Inhibitor)
Components and preparation	5 mg ROCK inhibitor, Y-27632 dihydrochloride 1,561 µl UltraPure™ DNase/RNase-Free Distilled Water Mix well by pipetting
Method of sterilization	Sterile filter with a 0.2 µm filter
Notes	<ul style="list-style-type: none">• Prepare 50-100 µl aliquots.• Protect from light• We recommend 10 µM ROCK Inhibitor for thawing and 5 or 10 µM for single cell iPSC passages
Storage	1 month at -20 °C

4. 10x Dispase Solution (Table 4)

Table 4. Preparation of 10x Dispase Solution

Reagent	10x Dispase
Components and preparation	200 mg Dispase II, powder 10 ml DMEM/F-12 medium Mix well by pipetting and inverting
Method of sterilization	Sterile filter with a 0.2 µm filter
Notes	<ul style="list-style-type: none">• Prepare 1 ml aliquots in a 15 ml conical tubes• To prepare 1x Dispase add 9 ml of DMEM/F-12
Storage	10x: one month at -20 °C 1x: two weeks at 4 °C

5. mTeSR™ 1 complete medium (Table 5)

Table 5. Preparation of mTeSR™ 1 complete medium

Medium	mTeSR™ 1 complete medium (referred to as mTeSR)
Components and preparation	100 ml of 5x Supplement (part of mTeSR™ 1 kit) 400 ml of Basal medium (part of mTeSR™ 1 kit) Mix well by inverting
Method of sterilization	None
Notes	Prepare aliquots if needed
Storage	4 °C

6. RPMI Minus (-) medium (Table 6)

Table 6. Preparation of RPMI Minus (-) medium

Medium	RPE RPMI Minus (-) medium
Components and preparation	500 ml RPMI 1640 medium 10 ml B-27™ Supplement, minus insulin 5 ml Penicillin-Streptomycin (10,000 U/ml) Mix well by inverting
Method of sterilization	None or sterile filter with a 0.2 µm filter if needed
Notes	Prepare aliquots if needed
Storage	1 week at 4 °C

7. RPMI Plus (+) medium (Table 7)

Table 7. Preparation of RPMI Plus (+) medium

Medium	RPMI Plus (+) medium
Components and preparation	500 ml RPMI 1640 medium 10 ml B-27™ Supplement (50x), serum free 5 ml Penicillin-Streptomycin (10,000 U/ml) Mix well by inverting
Method of sterilization	None or sterile filter with a 0.2 µm filter if needed
Notes	Prepare aliquots if needed
Storage	1 week at 4 °C

8. RPMI Plus (+) medium (Table 8)

Table 8. Preparation of RPMI Lactate medium (4mM Sodium L-lactate)

Medium	RPMI Lactate medium
Components and preparation	500 ml RPMI 1640 medium, no glucose 2,049.18 µl 1M Sodium L-lactate solution (final concentration - 4mM) (see Recipes: Table 11) 5.1 ml MEM Non-Essential Amino Acids Solution (100x) 5.1 ml Penicillin-Streptomycin (10,000 U/ml) Mix well by inverting
Method of sterilization	None or sterile filter with a 0.2 µm filter if needed
Notes	Prepare aliquots if needed
Storage	4 °C

9. RPMI Lactate medium (4 mM Sodium L-Lactate) (Table 9)

Table 9. Preparation of iPSC-CVPCs Harvest Medium

Medium	iPSC-CVPCs Harvest Medium
Components and preparation	500 ml RPMI 1640 medium 125 ml FBS (final concentration 20%) Mix well by inverting
Method of sterilization	None or sterile filter with a 0.2 µm filter if needed
Notes	Prepare aliquots if needed
Storage	4 °C

10. iPSC-CVPCs 2x freezing medium (Table 10)

Table 10. Preparation of iPSC-CVPCs 2X freezing medium

Reagent	iPSC-CVPCs 2x freezing medium
Components and preparation	4 ml FBS 1ml DMSO Mix well by pipetting
Method of sterilization	None
Notes	Prepare fresh
Storage	Do not store

11. 10 mM CHIR-992021 solution (Table 11)

Table 11. Preparation of 10 mM CHIR-992021 solution

Reagent	10 mM CHIR-992021 solution
Components and preparation	5 mg CHIR-992021 996.4 µl DMSO Mix well by pipetting and inverting
Method of sterilization	None
Notes	Prepare single use aliquots (for three T150 flasks prepare aliquots of 135 µl)
Storage	-80 °C: 6 months

12. 5 mM IWP-2 solution (Table 12)

Table 12. Preparation of 5 mM IWP-2 solution

Reagent	5 mM IWP-2 solution
Components and preparation	10 mg IWP-2 4.29 ml DMSO Dissolve at 37 °C vortexing and inverting every 5-7 min until the solution is clear
Method of sterilization	None
Notes	Prepare single use aliquots (for three T150 flasks prepare aliquots of 165-170 µl)
Storage	-20 °C: 1 month

13. 1 M Sodium L-lactate solution (Table 13)

Table 13. Preparation of 1 M Sodium L-lactate solution

Reagent	1 M Sodium L-lactate solution
Components and preparation	10 g Sodium L-lactate 89.2379 ml 1M HEPES sodium salt solution Mix well by pipetting and inverting until the solution is clear
Method of sterilization	None or sterile filter with a 0.2 µm filter if needed
Notes	Prepare single use aliquots (for one bottle of RPMI Lactate medium prepare aliquots of 2.1 ml)
Storage	-20 °C

E. Buffer preparation

1. FACS Buffer (Table 14)

Table 14. Preparation of FACS Buffer

Buffer	FACS Buffer
Components and preparation	500 ml PBS 1% BSA 0.05% NaN ₃ (Optional) Mix well by inverting
Method of sterilization	Sterile filter with a 0.2 µm filter
Notes	Prepare aliquots if needed
Storage	4 °C for several months

2. Permeabilizing/Blocking Buffer (Table 15)

Table 15. Preparation of Permeabilizing/Blocking Buffer

Buffer	Permeabilizing/Blocking Buffer
Components and preparation	500 ml PBS 5% Goat serum 0.5% BSA 0.2% Triton™ X-100 Mix well by inverting
Method of sterilization	Sterile filter with a 0.2 µm filter
Notes	Prepare aliquots if needed
Storage	4 °C for several months

3. FACS-FIX Buffer (Table 16)

Table 16. Preparation of FACS-FIX Buffer

Buffer	FACS-FIX Buffer
Components and preparation	FACS Buffer 1% Formaldehyde Mix well by inverting
Method of sterilization	None
Notes	Prepare aliquots if needed
Storage	4 °C for several months

4. IF Perm Buffer II (Table 17)

Table 17. Preparation of IF Perm Buffer II

Buffer	IF Perm Buffer II
Components and preparation	PBS 5% BSA Mix well by inverting
Method of sterilization	None
Notes	Prepare fresh
Storage	Do not store

5. IF Blocking Buffer II (Table 18)

Table 18. Preparation of IF Blocking Buffer II

Buffer	IF Blocking Buffer II
Components and preparation	PBS 0.1% Tween® 20 Mix well by inverting
Method of sterilization	None
Notes	Prepare fresh
Storage	Do not store

6. IF Staining Buffer (Table 19)

Table 19. Preparation of IF Staining Buffer

Buffer	IF Staining Buffer
Components and preparation	PBS 1% BSA Mix well by inverting
Method of sterilization	None
Notes	<ul style="list-style-type: none">• Prepare fresh• Add antibodies few min before adding to the fixed and permeabilized cells
Storage	Do not store. Keep on ice and protected from light, when necessary, prior to use

7. 70% ethanol (Table 20)

Table 20. Preparation of 70% ethanol

Reagent	70% ethanol
Components and preparation	200 proof Ethanol denatured (<i>i.e.</i> , VWR, catalog number 71002-398) Deionized water For Every 1 L of 70% ethanol mix 700 ml of 200 proof Ethanol denatured with 300ml of deionized water Mix well by inverting
Method of sterilization	None
Notes	<ul style="list-style-type: none">• This alcohol is used for sterilization of surfaces, gloves etc.; therefore, usage of a non-denatured, pure molecular grade ethanol is unnecessary
Storage	<ul style="list-style-type: none">• Store tightly closed according to local regulations

F. Antibodies

Table 21. Antibodies concentrations

Assay	Antibody	Cat number	Dilution
Flow cytometry	cTNT; TNNT2	MS-295-P0	1:100
	IgG1	MG100	1:50
	Goat-anti-Mouse Alexa Fluor™ 488	A-11001	1:200
	α-Actinin	A7811	1:200
Immunofluorescence	CX43/GJA1	71-0700	1:1,000
	MYL2; MLC2v	10906-1-AP	1:100
	MYL7; MLC2a	311 011	1:200
	Donkey-anti-Rabbit Alexa Fluor™ 488	A21206	1:800
	Goat anti-Mouse Alexa Fluor™ 568	A-11004	1:800

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Competing interests

Authors are co-founders and shareholders in ATGCures, Inc. Otherwise, authors declare no conflicts or competing interests.

Ethics

iPSC lines were generated from 181 individuals (108 female and 73 male) recruited as part of the iPSCORE project (Panopoulos *et al.*, 2017). These individuals included seven monozygotic (MZ) twin pairs, members of 32 families (2-10 members/family) and 71 singletons (*i.e.*, not related with any other individual in this study) and were of diverse ancestries: European (118), Asian (27), Hispanic (12), African American (4), Indian (3), Middle Eastern (2) and mix ethnicity (15). The age of subject at recruitment ranged from 9 to 88 years old. Informed consent was obtained from all subjects in the study. Recruitment was approved by the Institutional Review Boards of the University of California, San Diego and The Salk Institute (Project no. 110776ZF).

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Derivation of Induced Pluripotent Stem Cells from Human Fibroblasts Using a Non-integrative System in Feeder-free Conditions

Alvaro A. Beltran¹, Sarahi G. Molina² and Adriana S. Beltran^{2, 3, *}

¹Department of Neuroscience, University of North Carolina, Chapel Hill, NC 27599, USA; ²Human Pluripotent Stem Cell Core, University of North Carolina, Chapel Hill, NC 27599, USA; ³Department of Pharmacology, University of North Carolina, Chapel Hill, NC 27599, USA

*For correspondence: beltran@med.unc.edu

[Abstract] Induced pluripotent stem cells (iPSCs) are genetically reprogrammed somatic cells that exhibit features identical to those of embryonic stem cells (ESCs). Multiple approaches are available to derive iPSCs, among which the Sendai virus is the most effective at reprogramming different cell types. Here we describe a rapid, efficient, safe, and reliable approach to reprogram human fibroblasts into iPSCs that are compatible with future iPSCs uses such as genome editing and differentiation to a transplantable cell type.

Keywords: Feeder-free, Fibroblasts, Sendai virus, Induced pluripotent stem cells, Transgene-free

[Background] Induced pluripotent stem cells (iPSCs) are genetically reprogrammed adult cells that exhibit morphological and functional qualities remarkably similar to those of embryonic stem cells (ESCs) (Takahashi and Yamanaka, 2006; Yu et al., 2007). They offer a great opportunity not only for disease modeling, but for the development of therapeutic strategies for pathologies that involve tissue degeneration. Furthermore, the iPSCs promise relies on a safe replenishable cell source derived in chemically defined media and free of random DNA integration.

Reprogramming somatic cells into iPSCs requires the forced expression of transcription factors that support the pluripotent state, including OCT4, SOX2, KLF4, c-MYC, NANOG, and LIN-28 (Takahashi and Yamanaka, 2006; Takahashi et al., 2007; Yu et al., 2007). Multiple approaches are available to deliver the transcription factors into the cells, including those that require integration into the host chromosomes (Takahashi and Yamanaka, 2006; Kane et al., 2010). Exogenous DNA integration can lead to unpredictable effects on the quality of the cells and safety after transplantation. Other approaches include DNA based vectors that exist episomally (Yu et al., 2011; Weltner et al., 2012) and thus, decrease the possibility of integration, and finally those that do not integrate into the host genome and are known as transgene-free. The transgene-free methods include mRNA (Warren and Wang, 2013), recombinant protein (Zhou et al., 2009) and Sendai virus (Fusaki et al., 2009). Delivering the pluripotency transcription factors as mRNA or recombinant protein is poorly effective and costly. In contrast, Sendai virus, is a highly effective RNA virus that efficiently reprogram different types of somatic cells.

Sendai is a cytoplasmic RNA replication incompetent virus (SeV) that safely and effectively delivers the reprogramming factors into somatic cells, and does not integrate into the genome or alter the genetic

information of the cell (Li *et al.*, 2000; Fusaki *et al.*, 2009). Furthermore, the virus is cleared out from the cells after a few passages assuring zero footprint of both the vectors and transgenes. The Sendai reprogramming system is commercially available as CytoTune iPS 2.0 for research purposes and as CTS CytoTune iPS 2.1 for clinical use. Both systems contain vectors encoding the four Yamanaka factors (OCT4, SOX2, KLF4, and c-MYC) optimized for generating iPSCs from human somatic cells, making Sendai virus the most rapid, efficient and cost-efficient method to generate transgene-free iPSCs.

Reliable and safe derivation of human iPSCs relies on the use of defined and qualified reagents that allow a smooth transition to downstream technologies and is compatible with GMP (Good Manufacture Practice) quality standards for large-scale cell production. Here we describe a reprogramming approach for human fibroblasts that uses a non-integrative system, chemically defined culture medium in feeder-free conditions. This approach enables rapid, efficient, safe and reliable derivation of iPSCs compatible with future uses including genome editing and differentiation to a transplantable cell type.

Materials and Reagents

1. Falcon® 6-Well Flat-Bottom Plate, Tissue Culture-Treated (Falcon, catalog number: 38016)
2. Serological pipettes multiple sizes (Corning)
3. Falcon 15 ml conical centrifuge tubes (Falcon, catalog number: 352097)
4. Falcon 50 ml conical centrifuge tubes (Falcon, catalog number: 352070)
5. Cells: Human fibroblast expanded from skin biopsies preferentially at passage 1 to passage 5. Early passage fibroblast can be obtained from a commercial source (*i.e.*, ATCC), a repository (*i.e.*, Coriell Institute) or expanded in the lab from skin biopsies (Vangipuram *et al.*, 2013 describes the procedure in detail)
6. Corning® Matrigel® hESC-Qualified Matrix (Corning, catalog number: 354277)
7. CytoTune™-iPS 2.0 Sendai Reprogramming Kit (Thermo Scientific, catalog number: A16518)
8. DMEM/F-12 (Gibco, catalog number: 11320033)
9. Fetal Bovine Serum (Gibco, catalog number: 16000044)
10. MEM NEAA (Gibco, catalog number: 11140050)
11. Pen Strep (Gibco, catalog number: 15-140-122)
12. mTeSR™ 1 (STEMCELL Technologies, catalog number: 85850)
13. TrypLE™ Express Enzyme (1x) (Gibco, catalog number: 12605036)
14. Anti-Human TRA-1-60, Mouse monoclonal (Thermo Fisher Scientific, catalog number: 41-1000)
15. Anti-Human OCT4, Rabbit polyclonal, IgG1 (Abcam, catalog number: ab19857)
16. Anti-Human SOX2 Antibody, Rabbit polyclonal IgG1 (Abcam, catalog number: ab97959)
17. STEMdiff Trilineage Differentiation kit, STEMCELL Technologies, catalog number: 05230)
18. Universal Mycoplasma Detection Kit (ATCC, catalog number: 30-1012K)
19. Dulbecco's PBS (DPBS) without Calcium and Magnesium (Gibco, catalog number: 14190136)
20. Polybrene Hexadimethrine Bromide (Sigma-Aldrich, catalog number: H9268)
21. 0.5 M EDTA (Gibco, catalog number: 15575020)

22. Fibroblast culture medium (see Recipes)
23. 5 mM EDTA solution (see Recipes)
24. 10 mM Y27632 stock solution (see Recipes)

Equipment

1. Tissue culture incubator Heracell™ 150i (Thermo Fisher Scientific, catalog number: 51026283)
2. Beckman Avanti J-30I Refrigerated with plate adapters (Beckman Coulter, catalog number: 17039)
3. Tissue culture laminar flow hood (NuAire Class II Type A2 NU-540)
4. Evos XL core microscope (Thermo Scientific, catalog number: AMEX1100)
5. Countess II FL Automated Cell Counter (Thermo Fisher Scientific, catalog number: AMQAF1000)
6. Water bath Isotemp (Fisher Scientific, catalog number: S35936)
7. Portable Pipet-aid (Drummond, model: DP-101, catalog number: 4-000-101)
8. Micro pipettes (Eppendorf)

Procedure

Day -1: Plate dermal fibroblast for reprogramming

Use low-passage (passage 1 to 5) human fibroblasts for reprogramming experiments. The reprogramming efficiency decreases with each passage. Hence, we don't recommend reprogramming fibroblast after passage 6. A schematic timeline and representative pictures can be seen in Figure 1.

1. Coat two wells of a 6 well plate with 2 ml of 5 mg/ml Matrigel and make sure it covers the entire well. Incubate at 37 °C for 30 min.
2. Inspect the fibroblast culture for the desired confluence (more than 70%), aspirate the culture medium and rinse twice with 2 ml DPBS.
3. Add 500 µl TrypLE™ Express enzyme and incubate at 37 °C and 5% CO₂ for 2 to 5 min or until fibroblasts have detached. TrypLE™ is used because it has lower cell toxicity than standard Trypsin and it is an animal-free product.
4. Add 1 ml fibroblast culture medium and transfer cell suspension to a 15-ml conical tube.
5. Centrifuge at 200 x g for 5 min. Remove and discard supernatant.
6. Resuspend cells in 1 ml of fresh fibroblast culture medium. Recipe available in the recipe section
7. Count fibroblasts using the desired method (e.g., Countess Automated Cell Counter), and plate 1 x 10⁵ cells/well (10,000 cells/cm²) in the Matrigel-coated 6-well dish in 2 ml of fibroblast culture medium.
8. Incubate 24 h at 37 °C with a humidified atmosphere and 5% CO₂.

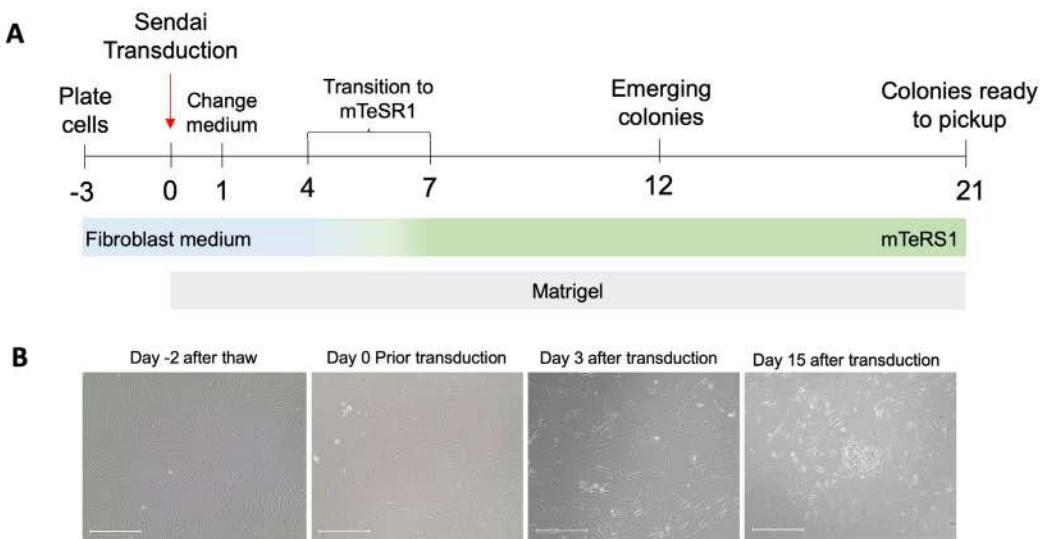


Figure 1. Derivation of induced pluripotent stem cells from fibroblast using Sendai virus and chemically defined medium. A. Schematic view of general reprogramming procedure including culture medium used. B. Images of fibroblast morphology changes during the early stages of reprogramming. Scale bars: 650 μ m.

Day 0: Transduction

1. Inspect the fibroblast culture, proceed with transduction if cell density is approximately 40-50% confluent, and cells have fully adhered and extended. If those parameters are not met, wait another 24 h.
2. Warm 1 ml of fibroblast culture medium in a water bath for each well to be transduced calculate the volume of each premade virus needed to reach a multiplicity of infection of (MOI) of 5:5:3 (KOS MOI = 5, hc-Myc MOI = 5, hKlf4 MOI = 3) using the live cell count from the day of the seeding, and the titer information on the CoA (calculate based on the lot number, the CoA can be download from thermofisher.com/cytotune). Calculate the volume of virus using the formula:

$$\text{Volume of virus } (\mu\text{l}) = \frac{\text{MOI } \left(\frac{\text{CIU}}{\text{cell}}\right) \times \text{cell number}}{\text{titer of virus } \left(\frac{\text{CIU}}{\text{ml}}\right) \times 10^{-3} \left(\frac{\text{ml}}{\mu\text{l}}\right)}$$

CIU = Cell infectious units

For example, to calculate the volume of hKOS virus with a titter of 1.1×10^8 CIU;

$$\text{Volume of virus } (\mu\text{l}) = \frac{5 \left(\frac{\text{CIU}}{\text{cell}}\right) \times 100,000}{1.1 \times 10^8 \left(\frac{\text{CIU}}{\text{cell}}\right) \times 10^{-3} \left(\frac{\text{ml}}{\mu\text{l}}\right)} = 4.5 \mu\text{l}$$

3. Thaw one set of CytoTune™ 2.0 Sendai aliquots from -80 °C and briefly centrifuge the tube. Place it immediately on ice until ready to use.

4. Add the calculated volumes of each of the three CytoTune™ 2.0 Sendai virus to 1 ml of pre-warmed fibroblast culture medium.
5. OPTIONAL: Add 4 µg/ml of polybrene to the medium. Ensure that the solution of virus and polybrene is thoroughly mixed by pipetting the mixture gently up and down. Complete the next step within 5 min. Polybrene increases the transduction efficiency of the virus, however, it can be toxic for some cell types. It neutralizes the charge repulsion between the virus and the cell surface increasing the overall transduction efficiency.
6. Aspirate the fibroblast culture medium from the cells, and add the reprogramming virus and polybrene mixture to the well containing the cells.
7. Close the 6-well dish, securely place on the plate adaptor, and centrifuge at 1,200 x g for 45 min at room temperature. Once the centrifugation is complete, add an additional 1 ml of prewarm fibroblast culture medium.
8. Incubate the plate overnight in a 37 °C incubator with a humidified atmosphere of 5% CO₂.

Day 1: Remove virus

After 24 h, aspirate medium with viruses and add 2 ml fresh fibroblast culture medium. Expect to see cytotoxicity 24-48 h post-transduction, this is an indication of high uptake of the virus. See Figure 2.

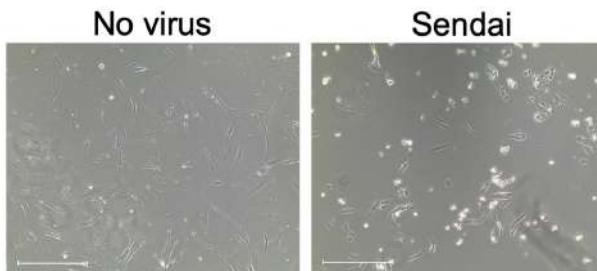


Figure 2. Images of morphological changes seventy-two hours after transduction with Sendai virus. Scale bars: 650 µm.

Days 2 to 3

Remove 2 ml of used medium and add 2 ml of fibroblast culture medium. Expect to see changes in cell morphology.

Day 4: Transition to defined medium

There are many different chemically defined culture medium commercially available for deriving and maintaining iPSCs. We have used mTeSR1 medium (Stem Cell Technologies), STEMFLEX (Life technologies) and mTeSR plus (Stem Cell Technologies) agnostically.

1. Remove 500 µl of fibroblast culture medium and add 500 µl of fresh mTeSR1.

Day 5

1. Remove 1 ml of culture medium and add 1 ml of fresh mTeSR1.

Day 6

1. Remove 1.5 ml of culture medium and add 1.5 ml of fresh mTeSR1.

Day 7

Remove 2 ml of medium culture medium and add the same volume of fresh mTeSR1.

Days 8-20

Feed cells daily with 2 ml until colonies are ready to be passaged. Remove partially reprogrammed and differentiated colonies by scraping them before medium change. Fully reprogrammed colonies have round shape with well-defined borders, cells display identical morphology with a high ratio of nucleus to cytoplasm and prominent nucleoli. In contrast, partially reprogrammed colonies have undefined borders, an amorphous shape, and are composed of different types of cells.

Day 21: First passage

1. When colonies are ready to pick, prepare one Matrigel coated dish per colony to be passage, and incubate at 37 °C for at least 30 min.
2. Aspirate Matrigel from plates and add 2 ml mTeSR1 + 10 µM ROCK inhibitor.
3. Use a 22 gauge needle or a pulled glass pipette to cut colonies in a grid-like pattern into small fragments. See Figure 3.
4. Use a 100 µl pipettor to scrape fragments and collect them. Immediately transfer the fragments to the Matrigel coated plates with mTeSR1 + 10 µM ROCK inhibitor.
5. Rock plate back-and-forth and side-to-side to evenly distribute the cell fragments and incubate overnight at 37 °C with a humidified atmosphere of 5% CO₂.
6. Feed cells daily until ready to passage (usually every 6 to 7 days).
7. Repeat Steps 1 to 10 for 7 passages. We found that > 95% of the iPSCs clones at passage 7, are transgene free.

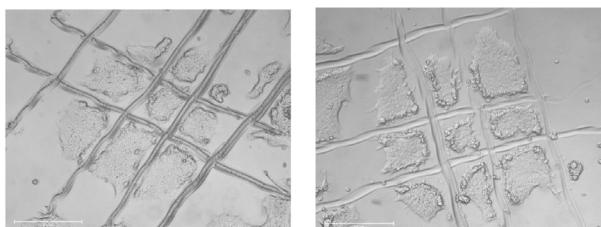


Figure 3. Images of colonies cut in a grid-like pattern into small fragments. Scale bars: 650 µm.

IPSCs cloning and expansion

After passage 7, iPSCs are passage with EDTA buffer, as follow:

1. Coat 6 wells of a 6-well tissue culture plate with Matrigel and place in incubate at 37 °C for 30 min.
2. Prior to use, allow the Matrigel-coated plate to equilibrate to room temperature for at least 1 h.
3. Just before dissociating cells for passaging, aspirate the liquid Matrigel solution from the wells and replace with 1 ml of mTeSR1 + 10mM Y27632 cell culture media per well. Set aside.
4. Wash the cells with 2 ml 0.5 mM EDTA, aspirate.
5. Add 2 ml of room temperature 0.5 mM EDTA solution to the cells.
6. Incubate the culture at 37 °C for 3 to 5 min, or until cells begin to separate uniformly throughout the entire colony. Do not allow the cultures detach in the EDTA solution.
7. As soon as the cells appear rounded and uniform separation is seen throughout the colonies, carefully aspirate the EDTA solution from the well. Do not rinse.
8. Immediately add 1 ml of mTeSR1 + 10mM Y27632. With a 5 ml pipet, take up the 1 ml of media from the well, and very gently dispense it against the culture surface to dissociate the cells from the dish. Repeat 1 to 2 more times, if needed.
9. Be careful not to over-pipet the cell suspension.
10. Dispense the cells gently into the 15 ml conical tube containing an additional 3 ml of pre-warmed media.
11. Pipet the solution very gently 1 time to mix, and dispense 1 ml of the cell suspension drop-wise into each of the 6 new Matrigel-coated wells, and immediately rock plate back-and-forth and side-to-side to evenly distribute the colony pieces across the well.
12. Incubate undisturbed at 37 °C and 5% CO₂ overnight.
13. Replace cell culture media every day with 2.5 ml of fresh mTeSR1, warmed to room temperature.
14. Monitor cells daily and passage as needed.

Characterization and cryopreservation

Cells are expanded for cryopreservation at passage 10. Then, iPSCs are characterized (Figure 4) and prepare for downstream usage in genome engineering experiments or differentiation into specific cell types. Characterization includes:

1. Immunofluorescence staining of cell surface markers such Anti-Human TRA-1-60, Mouse monoclonal and transcription factors such as anti-Human OCT4, Rabbit polyclonal, IgG1 and anti-Human SOX2 Antibody, Rabbit polyclonal IgG1.
2. Pluripotency and trilineage differentiation capabilities (using the STEMdiff Trilineage Differentiation kit, and the Taqman hPSC Score card Panel).
3. Test for mycoplasma contamination using commercially available kits such as The Universal Mycoplasma Detection Kit.
4. G-banding Karyotype.

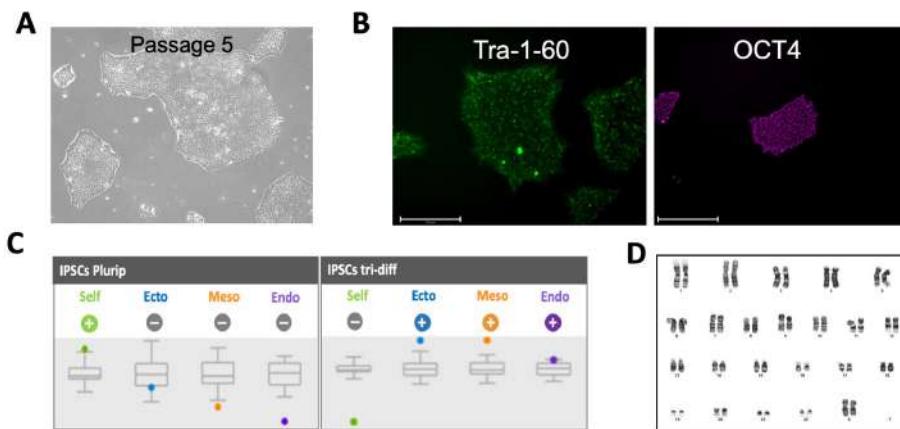


Figure 4. Characterization of iPSC derived from fibroblast. A. Bright field images of iPSCs, B. Immunofluorescence staining for pluripotency markers Tra-1-60 and OCT4. C. Taqman ScoreCard assay for pluripotent iPSCs (left panel) and iPSCs differentiated into endoderm, mesoderm and ectoderm (right panel). D. G-banding karyotype.

Recipes

1. Fibroblast culture medium

The following recipe is to prepare 500 ml of fibroblast culture medium. Aseptically mix the following:

445 ml high glucose DMEM

50 ml heat-inactivated fetal bovine serum (FBS)

5 ml non-essential amino acids

2. 5 mM EDTA solution

Dilute 0.5 ml of 0.5 M EDTA in 500 ml DPBS without Calcium and Magnesium. Store at room temperature

3. 10 mM Y27632 stock solution

Aseptically add 3.122 ml of sterile water to 10 mg of Y27632

Mix thoroughly by pipetting, aliquot in Eppendorf's and store at -80 °C

Avoid repeated freezing and thawing

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Competing interests

The authors do not have any financial or non-financial interest in the subject matter or materials discussed in this manuscript.

Ethics

This research was carried out in accordance with the UNC-CH institutional guidelines.

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Protocol for Isolation, Stimulation and Functional Profiling of Primary and iPSC-derived Human NK Cells

Janine E. Melsen^{1,*}, Maria Themeli², Monique M. van Ostaijen-ten Dam¹, Els van Beelen³, Gertjan Lughart¹, Rob C. Hoeben⁴, Marco W. Schilham¹ and Harald M. Mikkers^{4,*}

¹Department of Pediatrics, Leiden University Medical Center, Leiden, The Netherlands; ²Department of Hematology, Amsterdam UMC, Amsterdam, The Netherlands; ³Department of Immunohematology & Blood Transfusion, Leiden University Medical Center, Leiden, The Netherlands; ⁴Department of Cell & Chemical Biology, Leiden University Medical Center, Leiden, The Netherlands

*For correspondence: J.E.Melsen@lumc.nl; H.Mikkers@lumc.nl

[Abstract] Natural killer (NK) cells are innate immune cells, characterized by their cytotoxic capacity, and chemokine and cytokine secretion upon activation. Human NK cells are identified by CD56 expression. Circulating NK cells can be further subdivided into the CD56^{bright} (~10%) and CD56^{dim} NK cell subsets (~90%). NK cell-like cells can also be derived from human induced pluripotent stem cells (iPSC). To study the chemokine and cytokine secretion profile of the distinct heterogenous NK cell subsets, intracellular flow cytometry staining can be performed. However, this assay is challenging when the starting material is limited. Alternatively, NK cell subsets can be enriched, sorted, stimulated, and functionally profiled by measuring secreted effector molecules in the supernatant by Luminex. Here, we provide a rapid and straightforward protocol for the isolation and stimulation of primary NK cells or iPSC-derived NK cell-like cells, and subsequent detection of secreted cytokines and chemokines, which is also applicable for a low number of cells.

Keywords: CD56, Natural killer cells, Induced pluripotent stem cells, Cytokines, Chemokines, Peripheral blood, Luminex

[Background] Natural killer (NK) cells are part of the innate immune system and provide the first line defense against viral infections and malformations. In the human blood, two distinct NK cell populations can be identified based on CD56 and CD16 expression: CD56^{bright}CD16⁺/− and CD56^{dim}CD16⁺ NK cells (Melsen *et al.*, 2016). CD56^{bright} NK cells represent the minor subset (~10% of NK cells) and are known for their cytokine and chemokine secretion, but low cytotoxicity. In contrast, the CD56^{dim} NK cells have high cytotoxic capacity. To test functional responses of NK cells in the absence of other cells, NK cells first need be enriched from peripheral blood mononuclear cells (PBMC) by negative enrichment. To study the production of effector molecules by the distinct NK cell populations upon stimulation, intra- and extracellular flow cytometry can be performed (Eberlein *et al.*, 2010). However, this technique is limited to the number of effector molecule-specific antibodies available. Moreover, multiple samples are required to analyze the major effector molecules making this technique less suitable for a low number of cells. As an alternative, the distinct NK cell subsets can be sorted and stimulated. Cytokines and chemokines can be subsequently measured in the supernatant by Luminex (Lughart *et al.*, 2016). The

advantages are: 1) distinct NK cell subsets cannot influence each other, 2) the supernatant can be harvested at multiple timepoints, which allows studying kinetics of the same cells, 3) > 25 effector molecules can be studied at once. Moreover, since no cell harvesting and fixation is required, cells could be stored or used for further experiments.

As an alternative for primary NK cells, NK cells can be derived from human iPSC. The different protocols used are based on the stepwise differentiation of human iPSC into mesoderm, hemogenic or hematopoietic progenitor cells, and subsequently into CD56⁺ NK cells. CD34⁺CD45⁺ hematopoietic progenitors or CD34⁺CD31⁺ hemogenic progenitors are generated using either stromal cells like OP9 or embryoid bodies in the presence of hematopoietic and vascular growth factors (Knorr *et al.*, 2013). The CD34⁺ progenitors are further differentiated towards NK cells using a cytokine cocktail in either the presence or absence of OP9-DL1 stroma cells (Knorr *et al.*, 2013; Zeng *et al.*, 2017). Cytokine cocktails used contain SCF, FLT3L, IL-3, IL-15 and IL-7 in the absence of OP9-DL1 (Knorr *et al.*, 2013), or SCF, FLT3L, and IL-7 when co-culturing on OP9-DL1 cells (Zeng *et al.*, 2017). The latter conditions simultaneously generate T cells (Themeli *et al.*, 2013 and 2020), however, substitution of IL-7 by IL-15, or the addition of IL-15 results in much purer (> 99%) CD56⁺ NK cell populations (Zeng *et al.*, 2017). In our hands, CD56⁺ NK cells generated from iPSC best resemble primary CD56^{bright} NK cells (Themeli *et al.*, 2020). If the generation of T cells is hampered, by example in the case of RAG2 deficiency, previously undescribed small populations with NK cell-specific cytokine secretion profiles can be found (Themeli *et al.*, 2020). To functionally profile rare NK cell-like populations a sensitive and easy-to-use protocol is paramount. Here we describe such a protocol that allows a rapid assessment of NK cell-specific secretion profiles using as few as 10,000 cells.

Materials and Reagents

NK cell isolation

General

43. Laboratory disposables:

Pipettes

15 ml tubes

50 ml tubes (Greiner, Cellstar, catalog number: 227261)

Pasteur pipettes

Eppendorf tubes (Eppendorf, catalog number: 0030121023)

44. 50 ml Syringe (Becton Dickinson Medical, catalog number: BD300865), 10 ml Syringe (Becton Dickinson Medical, catalog number: BD 307736)

45. 0.22 µm syringe filter (Whatman FP30 CA-S, catalog number: 10462300)

46. Syringe needle (Becton Dickinson MICROLANCE 3, 19 G x 40 MM, catalog number: 301500)

47. 96-wells round bottom plate non-sterile (Corning, catalog number: 3799)

48. Micronic tubes (NBS scientific, catalog number: MP32022)

49. 5 ml round-bottom polystyrene tubes (Corning, Falcon, catalog number: 352052)

50. PBS (Fresenius Kabi, catalog number: 8717973380153), 4°C
51. Bürker-Türk counting chamber (VWR, catalog number: HECH40444702)
52. Türk's solution (Merck, Sigma-Aldrich, catalog number: 1092770100)
53. Bovine serum albumin (BSA) (Merck,Sigma-Aldrich, catalog number: A9576), 4 °C
54. EDTA (Merck, Calbiochem, catalog number: 324503, Molecular weight 372.24), RT
55. Distilled water (Aqua BBraun, B Braun, catalog number: 0082479E)
56. NaOH (Merck, catalog number: 106498)
57. Fetal Calf Serum (FCS, heat inactivated for 30 min at 56 °C to inactivate complement) (Merck, Sigma-Aldrich, catalog number: F7524), -20°C
58. EDTA solution 0.5 M (see Recipes), RT
59. AIMV medium (Thermo Fisher Scientific, Gibco, catalog number: 31035025), 4 °C
60. Collection medium (see Recipes), 4 °C
61. Antibody for cell sorting (see Recipes), 4 °C
CD56 clone N901 (ECD) (Beckman Coulter, for instance, catalog number: A82943), 4 °C or
clone 5.1H11 (Biolegend, for instance, catalog number: 362550), 4°C or clone B159 (Becton Dickinson, for instance, catalog number: 560361), 4 °C

Only required for primary NK cell isolation

1. Pre-separation filters 30 µm (Miltenyi, catalog number: 130-041-407), RT
2. MACS columns MS or LS (Miltenyi, catalog number: 130-042-201 or 130-042-401), RT
3. Ficoll Paque Plus (Merck, Sigma, catalog number: GE17-1440-02), RT, dark
4. RPMI 1640 Medium (Thermo Fisher Scientific, Gibco, catalog number: 72400054), 4 °C
5. Human serum albumin 200 g/L (Sanquin, Albuman, catalog number: 8717185830897) 4 °C
6. Penicillin-Streptomycin 100x (Merck, Sigma-Aldrich, catalog number: P0781), -20 °C
7. NK cell isolation kit human (Miltenyi, catalog number: 130-092-657), 4 °C
8. CD33 clone P67.6 (PE) (Becton Dickinson, for instance, catalog number: 345799), 4 °C
9. CD14 clone M5E2 (PE-Cy7) (Becton Dickinson for instance, catalog number: 557742), 4 °C
10. CD3 clone UCHT1 (BV421) (Becton Dickinson, for instance, catalog number: 562426), 4 °C
11. CD19 clone SJ25C1 (BV510) (Becton Dickinson, for instance, catalog number: 562947), 4 °C
12. MACS buffer (see Recipes), 4 °C
13. Dilution medium (see Recipes), 4 °C
14. Wash medium (see Recipes), 4 °C

Only required for iPSC-derived NK cell isolation

1. 30 µm CellTrics filter (Sysmex, catalog number: 04-004-2326)
2. CD7 clone 124-1D1 (eBioscience, for instance, catalog number: 25-0079-41), 4 °C or clone 4H9 (Becton Dickinson, for instance, catalog number: 347483), 4 °C or clone M-T701 (Becton Dickinson, catalog number: 561934), 4 °C

Stimulation

1. Laboratory disposables:
 - Pipettes
 - 15 ml tubes
 - 50 ml tubes
 - Eppendorf tubes
2. 96-well round bottom plate (Greiner, catalog number: 650185)
3. AIMV medium (Thermo Fisher Scientific, Gibco, catalog number: 31035025)
4. Fetal Calf Serum (FCS, heat inactivated for 30 min at 56 °C to inactivate complement) (Merck, Sigma-Aldrich, catalog number: F7524), -20 °C
5. Recombinant human IL-12 (Peprotech, catalog number: 200-12), -20 °C
6. Recombinant human IL-15 (Peprotech, catalog number: 200-15), -20 °C
7. Recombinant human IL-18 (MBL International, catalog number: B001-5), -20 °C
8. Interleukin mix (see Recipes)

Functional profiling

1. Laboratory disposables:
 - Pipettes
 - 15 ml polypropylene tubes
 - 0.5 ml polypropylene tube
 - Reagent reservoirs
2. Aluminium foil
3. Sealing tape (for instance, Merck, Greiner, catalog number: A5596-100EA)
4. Ice
5. Paper towels
6. Bio-Plex Pro Human Cytokine 27-plex Immunoassay (Bio-Rad, catalog number: M50-0KCAF0Y), 4 °C

Equipment

5. Laminar flow cabinet for sterile work (biosafety level II) (Euroflow EF4, CleanAir by Baker)
6. Tube rack to hold 15, 50 ml tubes (for instance, VWR, catalog numbers: 89215-778 and 89215-778)
7. Micropipettes (P10, P100, P1000) (for instance, Gilson, catalog number: F167380)
8. 12 channel multichannel pipette (for instance, Eppendorf, catalog number: 3125000060)
9. Table top centrifuge with adapters for plates and for 15 ml and 1.5 ml tubes (Eppendorf, model: 5810 R)
10. CO₂ Incubator (at 5% CO₂ and 37 °C) (for instance, Panasonic, model: MCO 170-AIC)
11. Magnetic stirrer (for instance, VWR, catalog number: 89215-778)

12. FACS Aria cell sorter (Becton Dickinson, Aria I, II, III) but any equivalent fluorescence-activated cell sorter should work
13. MiniMACS or MidiMACS separator (Miltenyi, catalog number: 130-042-102 or 130-042-302, respectively)
14. MACS MultiStand (Miltenyi, catalog number: 130-042-303, RT)
15. Autoclave (for instance, VWR, Ward's, catalog number: 470230-598)
16. Vortex (for instance Scientific Industries, model: Vortex Genie 2, catalog number: 200-SI-0236)
17. Bio-Plex 200 system (Bio-Rad, for instance, catalog number: 171000201)
18. Bio-Plex handheld magnetic washer (Bio-Rad, catalog number: 171020100)
19. Plate shaker (for instance, Biosan, model: PSU-2T)

Software

1. Diva software (Becton Dickinson, v6.0 or later, 2007 or later)
2. Bio-Plex Manager software (Bio-Rad, v6.2, 2018)

Procedure

Primary NK cell isolation

A. Peripheral blood mononuclear cell (PBMC) isolation

Note: At room temperature (RT) unless stated otherwise.

1. Dilute at least 15 ml blood 2x in dilution medium and mix.
Note: From 15 ml blood typically ~1.5 x10⁶ NK cells can be isolated. This number will yield ~7.5 x 10⁴ CD56^{bright} NK cells.
2. Add 4 ml Ficoll to a 15 ml tube.
3. Carefully layer the diluted blood sample (10 ml) onto the Ficoll (do not mix).
4. Repeat Steps 2 and 3 for the remaining diluted blood.
5. Centrifuge 15 min at 1,000 x g without brake.
6. Harvest the PBMC by carefully pipetting (using a Pasteur pipette) the white layer of cells between plasma and ficoll.
7. Transfer the PBMC to a new 50 ml tube (pool per sample max 25 ml).
8. Add wash medium up to 50 ml.
9. Centrifuge 10 min at 800 x g.
10. Remove the supernatant by aspiration and resuspend the cells.
11. If applicable, pool the cells in 150 ml tube.
12. Add wash medium up to 10 ml.
13. Centrifuge 10 min at 540 x g.
14. Remove supernatant and resuspend cells in the recommended volume of MACS buffer (~1 ml MACS buffer for every 10 ml of undiluted blood).

15. Count the cells with Türk's solution (dilute 10 µl cell suspension with 90 µl Türk's solution). Keep the remaining cells at 4 °C.
16. Keep 2 x 10⁶ PBMC aside for single stain controls in sorting procedure.

B. NK cell isolation from PBMC using MACS

Note: Keep cells and buffers at 4 °C during the NK cell isolation. Check the manufacturer's instructions for any updates in the protocol.

1. Transfer cells to 15 ml tube and add up to 14 ml MACS buffer.
2. Centrifuge 5 min 540 x g.

Note: Volumes for magnetic labeling are minimum volumes, when working with less than 1 x 10⁷ cells, use this volume, when working with more than 1 x 10⁷ cells, scale up the volumes accordingly.

3. Remove supernatant by aspiration. Resuspend cells in 40 µl of MACS buffer per 1 x 10⁷ cells.
Example: For 1.5 x 10⁷ cells, add 60 µl of MACS buffer
4. Add 10 µl of NK cell Biotin-Antibody Cocktail per 1 x 10⁷ cells.
5. Mix well and incubate for 5 min at 4 °C.
6. Add 30 µl of MACS buffer per 1 x 10⁷ cells.
7. Add 20 µl of NK cell MicroBead Cocktail per 1 x 10⁷ cells.
8. Mix well and incubate for 10 min at 4 °C.
9. Place the column (MS for ≤ 1 x 10⁷ cells, LS for > 1 x 10⁷ PBMC) in the magnetic field of the MACS separator, which is placed on the MACS MultiStand. A maximum of 1 x 10⁸ PBMC can be loaded per LS column.

10. Put the pre-separation filter on top of the column.
11. Position the column into a 15 ml tube.
12. Rinse the column by pipetting MACS buffer in the filter: 500 µl (MS column), 3 ml (LS column)
13. Replace the 15 ml tube filled with buffer with a new 15 ml tube (the flow-through with NK cells will be collected in this tube).

Important: Only pipet new volumes when the column reservoir is empty.

14. Pipette the labeled PBMC in the filter. A minimum volume of 500 µl is required for magnetic separation. If necessary, add MACS buffer to the cell suspension.
15. Wash column by pipetting MACS buffer in the filter: 500 µl (MS column), 3 ml (LS column)
16. Repeat Step 15 twice.
17. Centrifuge the 15 ml tube containing the unlabeled NK cells and centrifuge 5 min at 540 x g.
18. Resuspend in MACS buffer (~500-1,000 µl) and count.

C. FACS of NK cell subsets

Note: Keep cells and buffers at 4 °C.

1. Prepare antibody mixes (30 µl per 1 x 10⁶ NK cells). Prevent bleaching by keeping the antibody mixes in the dark! See Recipes for example calculation. Important: use titrated antibodies.

Note: Titration is performed on 10⁶ PBMC using the recommended dilution as the median of 5 concentrations. A 2-fold dilution factor is typically applied. Recommended dilutions should be saturating but at the same not alter the mean fluorescence intensity of the antigen negative cells.

2. Prepare single stain control mixes (1 antibody + MACS buffer), 25 µl per single stain. Use same dilution as used for complete antibody mix. Do not forget to include 1 unstained control.
3. Transfer 2 x 10⁵ PBMC into a well of a 96 wells plate (1 well/single stain).
4. Pellet cells by centrifugation for 5 min, 540 x g at 4 °C (for tube), or 2 min, 540 x g at 4 °C (for plate).
5. For tube: remove supernatant from the 15 ml tube by aspiration and resuspend in the correct amount of antibody mix. Incubate in the dark for 30 min at 4 °C.
For plate: remove supernatant from the wells by flicking the plate once above the sink. Remove any access liquid by gently tapping the plate once onto a paper towel. Add 25 µl of single stain mix. Incubate in the dark for 30 min at 4 °C.
6. Wash cells by adding 10 ml (for tube) and 200 µl (for plate) MACS buffer to the cells.
7. Pellet cells by centrifugation as before: 5 min, 540 x g at 4 °C (for tube), 2 min, 540 x g at 4 °C (for plate).
8. Remove supernatant as described before (Step 5).
9. For tube: resuspend NK cells in MACS buffer, to a concentration of 1 x 10⁷ NK cells per ml.
For plate: resuspend the PBMC in 70 µl MACS buffer, and collect them in micronic tubes.
10. Prepare round bottom polystyrene tubes with 3 ml collection medium (max. 6 x 10⁵ cells can be collected in 1 tube).
11. Sort the cells at low pressure (100 µm nozzle, 20 PSI, this is important for the functioning of the NK cells after the sorting). Use the gating strategy as depicted in Figure 1. If more subsets are desired, the CD56^{bright} can be further subdivided in a CD16⁻ and CD16⁺ subset. Sorting of 1.5 x 10⁶ NK cells will yield (when taking into account cell loss) ~7.5 x 10⁴ CD56^{bright} NK cells and 1 x 10⁶ CD56^{dim} NK cells.

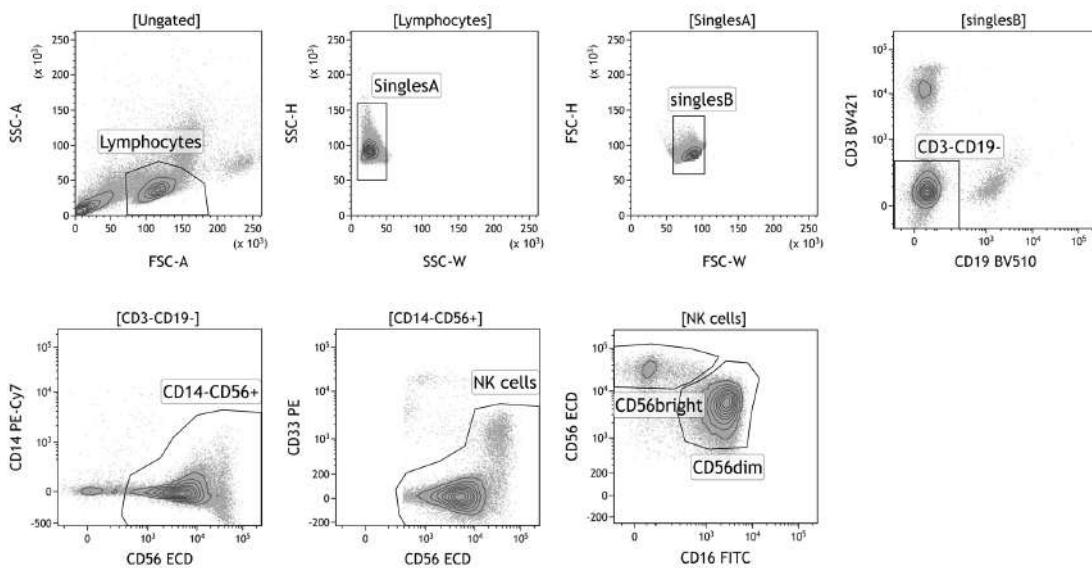


Figure 1. Gating strategy for sorting of primary NK cell subsets. First, lymphocytes are gated based on forward- and side scatter. Next, doublets, CD3⁺ T cells, CD19⁺ B cells, CD14⁺ monocytes, and CD33⁺CD56⁻ myeloid cells are excluded. NK cells can be further subdivided based on intensity expression of CD56 and CD16: CD56^{bright}CD16^{+/-} and CD56^{dim}CD16⁺ NK cells.

iPSC-derived NK cell isolation by FACS

Note: Keep cells and buffers at 4 °C during the NK cell isolation.

1. Collect the floating cells in the iPSC differentiation culture (Figure 2A) into a 15 ml or 50 ml conically shaped tube. The tube size depends on the scale of differentiation (typical format: a few wells from a 6-well plate).
- Note: ~1 x 10⁶ floating cells are typically harvested from one 6 well plate. Depending on the success of differentiation 20-70% of the floating cells are CD7⁺CD56⁺ NK cells.*
2. Pass the cell suspension through a 30 µm CellTrics filter (Sysmex) and collect the cells in a 15 ml or 50 ml conically shaped tube.
3. Centrifuge the collected cell suspension at 400 x g for 5 min at 4 °C.
4. Remove the supernatant by aspiration and resuspend the pelleted cells in 500 µl ice-cold PBS (wash Step 1).
5. Centrifuge the cells at 400 x g for 5 min at 4 °C.
6. Remove the supernatant by aspiration and resuspend the pelleted cells in 500 µl ice-cold MACS buffer (wash Step 2).
7. Centrifuge the cells at 400 x g for 5 min at 4 °C.
8. Remove the supernatant by aspiration and resuspend the pelleted cells in 500 µl ice-cold MACS buffer (wash Step 3).
9. Take a small aliquot (10 µl) and count the cells.

10. Transfer the cells into a 0.5 ml Eppendorf tube or 96 wells plate (round-bottom or V-shaped bottom well). Required number of tubes/wells: unstained control, single stain controls, complete stain. Use 2×10^4 - 5×10^4 cells for the single stain or unstained controls. The rest is used for the combi-stain of the cells that will be sorted. Never go beyond 1×10^6 cells/ 25 μ l staining solution.
11. Centrifuge the cells at $400 \times g$ for 5 min at 4 °C (for tube) or 2 min, $540 \times g$ at 4 °C (for plate).
12. Prepare antibody mixes: single stain control mixes (1 antibody + MACS buffer), 25 μ l per single stain, and a complete antibody mix containing at least anti-CD7 and anti-CD56 to identify NK cell-like cells.
Note: Use identical dilutions for the same antibodies and shield the antibodies from light, particularly, when tandem dyes are used as conjugates. Do not forget to include an unstained control and preferentially also a positive control consisting of mononuclear blood cells, by example from peripheral blood.
13. Remove the supernatant by aspiration (fortube) or by flicking the plate once above the sink (for plate). Remove any access liquid by gently tapping the plate once onto a paper towel (for plate).
14. Resuspend the cells in the antibody mix.
15. Incubate in the dark for 30 min at 4 °C (typically in the fridge).
16. Centrifuge the cells at $400 \times g$ for 5 min at 4 °C (for tube) or 2 min, $540 \times g$ at 4 °C (for plate).
17. Remove the supernatant as described before (Step 13) and resuspend the cells in 200 μ l ice-cold MACS buffer.
18. Centrifuge the cells at $400 \times g$ for 5 min at 4 °C (for tube) or 2 min, $540 \times g$ at 4 °C (for plate).
19. Remove the supernatant as described before (Step 13) and resuspend the single stain, unstained cells, and positive control cells in 70 μ l MACS buffer and transfer them into Micronic or 5 ml round-bottom polystyrene tubes. The cells to be sorted are resuspended at a concentration of 10×10^6 cells per ml.
20. Prepare 5 ml round-bottom polystyrene tubes with 3 ml collection medium (max 6×10^5 cells can be collected in 1 tube).
21. Sort the CD7 $^+$ CD56 $^+$ cells at low pressure (100 μ m nozzle, 20 PSI). Use the gating strategy as depicted in Figure 2B.

Note: Dependent on the question to be addressed and required purity antibodies against additional NK cell markers such as CD16 may be used.

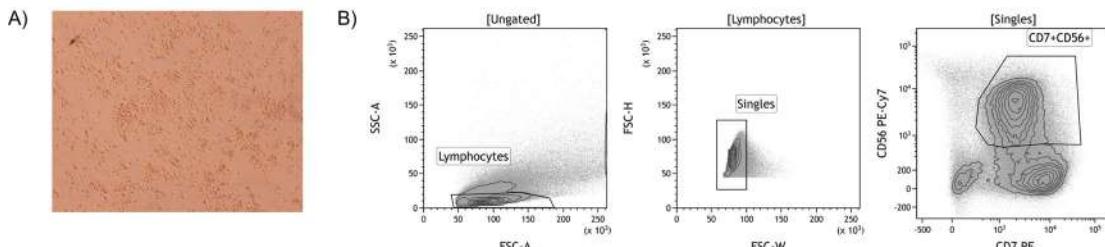


Figure 2. Isolation of iPSC-derived NK cells by FACS. A. An example of the differentiation of iPSC towards NK cells after co-culturing with OP9-DL1 cells for 3 weeks (100x magnification).

Non-adherent cells are harvested and CD56⁺ NK cells are isolated using FACS. B. Gating strategy to isolate iPSC-derived CD7⁺CD56⁺ NK cells.

NK cell stimulation

1. Spin down the sorted NK cell subsets and resuspend at a concentration of 5.6×10^4 cells/ml in AIMV medium with 1% Penicillin-Streptomycin (the final FCS concentration will be ~5%).
2. Add 180 µl /well NK cells in a sterile 96-wells round-bottom plate.
3. Add 20 µl of IL-12, IL-15 and IL-18 mix and medium without cytokines (AIMV + 1% Penicillin-Streptomycin) to the different wells.
Note: Cells from healthy donors that are cultured in IL-12, IL-15 and IL-18 are the positive control for IFN-γ production. Cells cultured in medium without cytokines act as negative control. Preferentially, stimulations should be performed in triplicate. If more cells were sorted than needed stimulations with single or double cytokine mixtures should be included.
4. Include 1 well with 200 µl medium (AIMV + 1% Penicillin-Streptomycin + 5% FCS) and 1 well with 200 µl medium supplemented with interleukins (as background for the Luminex).
5. Wrap plastic foil around plate to prevent evaporation.
6. Culture cells for 20 h in incubator at 37 °C, 5% CO₂.
Note: When studying kinetics, take a sample of 10 µl at multiple time points.
7. Spin down plate, 540 x g for 2 min.
8. Harvest supernatant and store in sealed 96 wells plate or Micronic tubes at -20 °C.

Luminex

Note: The protocol below has been optimized and therefore differs from the manufacture's instruction.

1. Spin down the standard vial and reconstitute a single vial of standard in 500 µl of diluent (= AIMV medium + 5% FCS), gently vortex for 5 s and incubate on ice for 30 min.
2. Label 9 0.5 ml polypropylene tubes Std1-Std8 and Blank.
3. Add the specified volume of diluent to each tube (Table 1).
4. Vortex the reconstituted standard gently for 5 sec, take 128 µl and add to tube Std1.
5. Vortex Std1 and transfer 50 µl from tube Std1 to tube Std2 and vortex. Important: use new pipet tips for every volume transfer.
6. Continue with serial dilutions form tube S2 to S8 by transferring 50 µl each time.

Table 1. Serial dilutions to generate a standard curve

Tube	Diluent (=AIMV+5% FCS)	Transfer volume
Std1	72 µl	128 µl Standard
Std2	150 µl	50 µl Std1
Std3	150 µl	50 µl Std2
Std4	150 µl	50 µl Std3
Std5	150 µl	50 µl Std4
Std6	150 µl	50 µl Std5
Std7	150 µl	50 µl Std6
Std8	150 µl	50 µl Std7
Blank	150 µl	-

7. Thaw supernatant, and centrifuge at 1,000 $\times g$ for 4 min at RT.
8. Transfer supernatant to clean polypropylene tube.
9. Dilute samples 1:6 in diluent (once thawed keep samples on ice) and equilibrate to RT before use.
10. Make a plate lay-out (for example Figure 3) to check the number of wells required. Use duplicates for the standards and blanks. Fill the plate vertically.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Std1	Std1	blank									
B	Std2	Std2	blank									
C	Std3	Std3	Sample1									
D	Std4	Std4	Sample2									
E	Std5	Std5	Sample3									
F	Std6	Std6	Sample4									
G	Std7	Std7	Sample5									
H	Std8	Std8	Sample6									

Figure 3. Plate lay-out. An example of a plate lay-out with 8 standards and 1 blank in duplicate, and 6 samples.

11. Add the required volume of assay buffer to a 15 ml tube (Table 2).
12. Vortex the stock beads for 30 s at medium speed. Carefully open the cap and pipet any liquid trapped in the cap back into the vial. Important: do not centrifuge the stock beads, since the beads will be spun down.
13. Dilute the beads by pipetting the required volume of stock beads into the 15 ml tube (Table 2).
Important: protect the beads from light with aluminium foil.

Table 2. Bead dilutions

Wells	10x stock beads	Assay buffer	Total volume
96	288 µl	5,712 µl	6,000 µl
48	144 µl	2,856 µl	3,000 µl

14. Bring standards and samples to RT before use.
15. Vortex the diluted beads at medium speed for 30 s and pour the beads into a reagent reservoir. Transfer 50 µg of beads to each well of the flat bottom plate.
16. Add 100 µl wash buffer per well. Position the plate for at least 60 s on the handheld magnet and quickly decant the waste solution. Remove any access liquid by tapping the plate onto a paper towel. Remove plate from the magnet, carefully resuspend the beads by tapping the plate, and repeat this washing step.
17. Gently vortex the diluted standards, blanks and samples for 5 s. Transfer 50 µl to each well. Important: use a new pipette tip for each volume transfer.
18. Cover the plate with a new sheet of sealing tape and protect from light with aluminum foil. Incubate on shaker at 450 rpm for 45 min at RT.
19. Prepare the dilution of detection antibodies 10 min before use. Add the required volume of detection antibody diluent to a new 15 ml tube (Table 3).
20. Vortex the stock detection antibodies at medium speed for 15-20 s. Spin down the stock for 30 s.
21. Dilute the 10x detection antibodies by pipetting the required volume into the 15 ml tube (Table 3).
22. After the 45 min incubation, remove the sealing tape and wash 3x with 100 µl washing buffer.
23. Vortex the diluted detection antibodies for 5 s, pour into a reagent reservoir and transfer 12.5 µl to each well.
24. Cover plate with a new sealing tape and protect from light with aluminum foil. Incubate on shaker at 450 rpm for 30 min at RT.

Table 3. Dilution of detection antibodies

Wells	10x detection antibodies	Detection diluent	antibody	Total volume
96	150 µl	1,350 µl		1,500 µl
48	75 µl	675 µl		750 µl

25. Prepare the streptavidin-PE dilution 10 min before use. Add the required volume of assay buffer to a new 15 ml tube (Table 4).

Table 4. Dilution streptavidin-PE

Wells	100x SA-PE	Assay buffer	Total Volume
96	30 µl	2,970 µl	3,000 µl
48	15 µl	1,480 µl	1,500 µl

26. Vortex the 100x SA-PE for 5 s at medium speed and spin down for 30 s.
27. Pipette the required volume of SA-PE to the 15 ml tube (Table 4). Vortex and protect from light.
28. Power-up the Bio-plex system (30 min in advance).
29. After the 30 min incubation of the detection antibody, remove the sealing tape and wash the plate 3x with 100 µl washing buffer.
30. Vortex the diluted SA-PE for 5 s, pour into a reagent reservoir, and transfer 25 µl to each well.
31. Cover the plate with sealing tape and aluminum foil. Incubate on shaker at 450 rpm for 10 min at RT.
32. Wash the plate 3x with 100 µl wash buffer.
33. To resuspend beads for plate reading, add 80 µl of assay buffer to each well and cover the plate with sealing tape. Incubate on shaker at 850 ± 50 rpm for 30 s at RT.
34. Remove the tape and read the plate (using low PMT settings). In case you expect low concentrations, use high PMT settings.
35. Data analysis: For a detailed handbook we refer to online tutorials by Bio-Rad (Reference 1 for instance).

Note: It is important to subtract the background values (the wells with only medium or medium supplemented with interleukins) from the other values, which are automatically generated by the software based on the standard curve. In addition, check whether the experiment was successful by verifying that the negative control (cells without stimulation) is truly negative, and that the positive control (cells stimulated with IL-12, IL-15 and IL-18) shows IFN-γ production. An example of cytokine production by a variety of NK cells is shown in Figure 4.

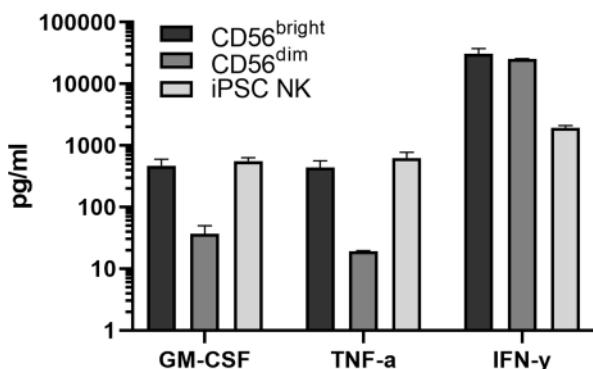


Figure 4. Example of Luminex measurements of stimulated primary and iPSC-derived NK cells. The Bio-Plex Pro Human Cytokine 27-plex Immunoassay allows detection of 27 cytokines/chemokines. NK cells are producers of predominantly GM-CSF, TNF-α and IFN-γ and various chemokines. GM-CSF, TNF-α and IFN-γ production is shown upon the culture of primary

NK cell subsets ($CD56^{\text{bright}}$ and $CD56^{\text{dim}}$) and iPSC-derived NK cells ($CD7^+CD56^+$) in the presence of IL-12, IL-15 and IL-18 for 20 h. The data is a representative of at least 2 independent experiments. The means and standard deviations are shown.

Recipes

1. Dilution medium
500 ml RPMI 1640 medium
5 ml penicillin-streptomycin 100x
Add Penicillin-Streptomycin to RPMI 1640 medium and shake
2. Wash medium
500 ml RPMI 1640 medium
5 ml Penicillin-Streptomycin 100x
2 ml Human serum albumin 200 g/L (final = 0.8 g/L)
Add Penicillin-Streptomycin and human serum albumin to RPMI 1640 medium and shake
3. EDTA solution 0.5 M
1,000 ml distilled water
186.12 g EDTA
Add EDTA to a bottle with distilled water, add magnetic stirring bar, and put the bottle on a magnetic stirrer. Add pellets NaOH until EDTA dissolves (around pH 7.5-8). Autoclave and store de bottle at RT. Use a syringe filter 0.22 μm to filter the solution before use
4. MACS buffer
500 ml PBS
8.3 ml 30% BSA (final = 0.5%)
2 ml 0.5 M EDTA solution (final = 2 mM)
Add BSA and filtered EDTA solution to the PBS and mix
5. Antibody mix for primary NK cells
MACS buffer
1:20 CD33 PE
1:200 CD16 FITC
1:100 CD56 ECD
1:100 CD14 PE-Cy7
1:200 CD3 BV421
1:100 CD19 BV510
Add the antibodies to the MACS buffer (in an Eppendorf tube) and vortex. Keep the mix in the dark at 4 °C
6. Collection medium
AIMV medium
30% FCS

1% penicillin-streptomycin

Add the FCS and Penicillin-Streptomycin to the AIMV medium and mix

7. Interleukin mix

AIMV medium

1% Penicillin-Streptomycin

10 ng/ml IL-12

10 ng/ml IL-15

20 ng/ml IL-18

Add the Penicillin-Streptomycin and interleukins to the AIMV medium and vortex

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Competing interests

All authors have declared to have no competing interests.

Ethics

Primary NK cells were isolated from buffy coats of healthy adult donors (Sanquin Blood bank, Region Southwest, Rotterdam, The Netherlands). Human materials for iPSC were collected according to the approval by the “Medical Ethics Committees” of the Erasmus MC (MEC-2016-606) or the LUMC (P13-080). The experiments involving human materials were done in accordance with the principles outlined in the “Declaration of Helsinki”.

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Differentiation of Human Induced Pluripotent Stem Cells (hiPSCs) into Osteoclasts

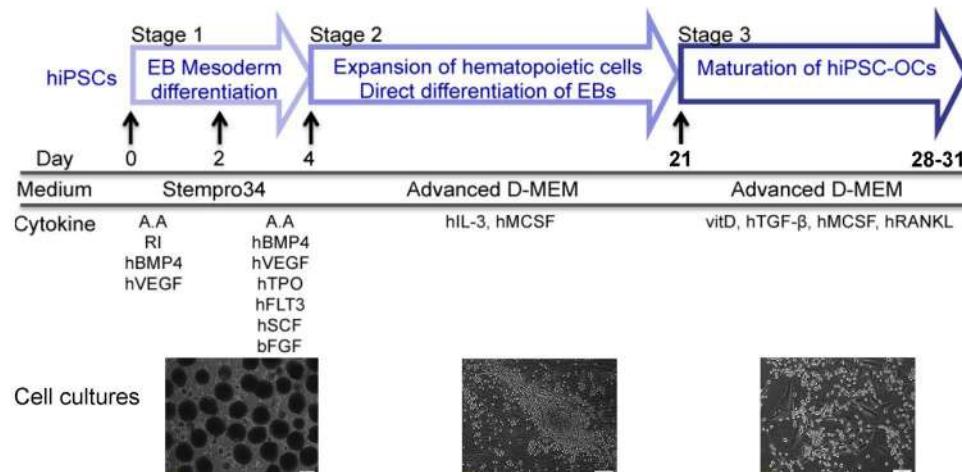
I-Ping Chen*

Department of Oral Health and Diagnostic Sciences, School of Dental Medicine, University of Connecticut Health, Farmington, CT, USA

*For correspondence: ipchen@uchc.edu

[Abstract] Defects in bone resorption by osteoclasts result in numerous rare genetic bone disorders as well as in some common diseases such as osteoporosis or osteopetrosis. The use of hiPSC-differentiated osteoclasts opens new avenues in this research field by providing an unlimited cell source and overcoming obstacles such as unavailability of human specimens and suitable animal models. Generation of hiPSCs is well established but efficient differentiation of hiPSCs into osteoclasts has been challenging. Published hiPSC-osteoclast differentiation protocols use a hiPSC-OP9 co-culture system or hiPSC-derived embryoid bodies (EBs) with multiple cytokines. Our three-stage protocol consists of 1) EB mesoderm differentiation, 2) expansion of myelomonocytic cells and 3) maturation of hiPSC-osteoclasts. We generate uniformly-sized EBs by culturing Accutase-dissociated hiPSCs on Nunclon Sphera microplates and promote EB mesoderm differentiation in a cytokine cocktail for 4 days. For Stage 2, EBs are transferred to gelatin-coated plates and cultured with hM-CSF and hIL-3 to expand the myelomonocytic population. By supplementing with vitamin D, hTGF β , hM-CSF and hRANKL, cells collected at the end of Stage 2 are differentiated into mature osteoclasts (Stage 3). Compared to other techniques, our protocol does not require a co-culture system; induces EBs into mesoderm differentiation in a homogenous manner; uses less cytokines for differentiation; requires only a short time for osteoclast maturation and produces sufficient numbers of osteoclasts for subsequent molecular analyses.

Graphic abstract:



Keywords: hiPSCs, Osteoclast differentiation, Embryoid bodies, Cytokines, Mesoderm differentiation

[Background] The technology for generating patient-specific hiPSCs, which theoretically can be differentiated into any cell type, opens new avenues for medical research in disease modeling, including bone disorders (Deyle *et al.*, 2012; Quarto *et al.*, 2012a and 2012b; Cherry and Daley, 2013; Ding *et al.*, 2013; Matsumoto *et al.*, 2013; Chen *et al.*, 2017). Research focusing on rare genetic bone disorders not only has the potential to find treatment for patients, but also contributes to a better understanding of skeletal biology. Many skeletal diseases involve dysfunctional osteoclasts, osteoblasts and/or osteocytes (Chen, 2014). Osteoclasts are bone resorbing cells and osteoblasts are bone forming cells. Osteocytes are derived from mature osteoblasts and are entrapped in the bone matrix that they produce (Bonewald, 2011). Bone marrow stromal cells (mesenchymal osteoblast-like cells) can be cultured from bone marrow, bone biopsies or bone excised during surgical procedures. Mesenchymal cells are proliferative and can be differentiated and passaged. The myelomonocytic population in bone marrow and peripheral blood can be differentiated into osteoclasts by culturing with human macrophage stimulating factor (M-CSF) and receptor activator of nuclear factor- κ B ligand (RANKL) (Chen *et al.*, 2011). However, once differentiated, these cells are terminally differentiated and can be used for experiments only once.

While reliable and consistent methods for reprogramming somatic cells into hiPSCs are well-established (Takahashi *et al.*, 2007; Yu *et al.*, 2007; Park *et al.*, 2008; Stadtfeld *et al.*, 2008; Sommer *et al.*, 2009; Yu *et al.*, 2009; Warren *et al.*, 2010), differentiation of hiPSCs into bone cells is still more challenging. Several studies describe protocols for hiPSC differentiation into osteoblasts (Kanke *et al.*, 2014; Kuhn *et al.*, 2014; Ochiai-Shino *et al.*, 2014; Kang *et al.*, 2016), but there are relatively few protocols described for hiPSC differentiation into osteoclasts (Choi *et al.*, 2009; Grigoriadis *et al.*, 2010; Jeon *et al.*, 2016). Choi *et al.* and Grigoriadis *et al.* differentiated hiPSC into osteoclasts via a hiPSC-OP9 co-culture system and through EB formation steps, respectively (Choi *et al.*, 2009; Grigoriadis *et al.*, 2010). A critical step for co-culture systems is to match cell densities of undifferentiated hiPSCs and OP9 cells, which can otherwise contribute to inconsistent outcomes. Generating EBs by conventional methods in cell culture dishes may cause variable EB sizes and can affect the differentiation efficiency. In addition, the use of complex cytokine cocktails is less economical. Jeon *et al.* (2016) reported a hiPSC-osteoblast and hiPSC-osteoclast co-culture system, which is less useful for investigating cell-autonomous osteoblast and osteoclast phenotypes in disease models.

Our protocol described here generates uniformly-sized EBs by dissociating hiPSC colonies into single cells and plating a fixed number of cells onto Nunclon Sphera microplates. EBs are stimulated to enter the mesoderm lineage in the first 4 days of differentiation and are transferred to gelatin-coated plates to expand the myelomonocytic population, which contains the osteoclast progenitors. Differentiation into osteoclasts is achieved by culturing these cells in the presence of vitamin D, hTGF β , hM-CSF and hRANKL. The resulting mature and functional osteoclasts are tartrate-resistant acid phosphatase (TRAP)-positive multinucleated cells and are able to resorb bone.

Materials and Reagents

Materials

1. Pipet tips (200 µl, 1 ml)
2. Cell strainer 70 µm (Corning, catalog number: 352350)
3. Nunclon Sphera 96-well plates, U-bottom (Thermo Fisher Scientific, catalog number: 174929)
4. Tissue culture 96-well plate, polystyrene (Greiner Bio-One, catalog number: 655180)
5. Tissue culture 6-well plate, polystyrene (BD, Falcon, catalog number: 08-772-1B)
6. Serological pipet, 5 ml (Fisher Scientific, catalog number: 13-678-11D)
7. Serological pipet, 10 ml (Fisher Scientific, catalog number: 13-678-11E)
8. 15 ml polypropylene conical tubes (Thermo Scientific, catalog number: 339650)
9. 50 ml polypropylene conical tubes (Thermo Scientific, catalog number: 339652)
10. Nalgene filter (0.2 µm PES membrane) 150 ml (Thermo Scientific, catalog number: 5650020)
11. Nalgene filter (0.2 µm PES membrane) 250 ml (Thermo Scientific, catalog number: 5680020)
12. Microcentrifuge tube (Fisher Scientific, catalog number: 05-408-129)

Reagents

62. hiPSCs generated from healthy donors (Chen *et al.*, 2013 and 2017)
63. Matrigel (Corning, catalog number: 354234)
64. PeproGrow embryonic stem cell (hESC) medium (Peprotech, catalog number: BM-hESC)
65. Accutase (Millipore Sigma, catalog number: SCR005)
66. Phosphate buffered saline, no calcium, no magnesium (Thermo Fisher Scientific, catalog number: 10010049)
67. DMEM/F12 medium (Thermo Fisher Scientific, catalog number: 11330032)
68. Stempro-34 medium (Thermo Fisher Scientific, catalog number: 10639011)
69. L-Glutamine (200 mM) (Thermo Fisher Scientific, catalog number: 25030081)
70. 1-Thioglycerol (MTG) (Sigma-Aldrich, catalog number: M6145)
71. Ascorbic acid (Sigma-Aldrich, catalog number: A4034)
72. MEM Non-essential amino acids (NEAA 100x) (Thermo Fisher Scientific, catalog number: 11140-050)
73. Rock inhibitor Y-27632 (Selleck Chemicals, catalog number: S1049)
74. Recombinant human BMP4 (Peprotech, catalog number: 120-05ET)
75. Recombinant human VEGF (Peprotech, catalog number: 100-20)
76. Recombinant human TPO (Peprotech, catalog number: 300-18)
77. Recombinant human Flt3-ligand (Peprotech, catalog number: 300-19)
78. Recombinant human IL-3 (Peprotech, catalog number: 200-03)
79. Recombinant human MCSF (Peprotech, catalog number: 300-25)
80. Recombinant human RANKL (Peprotech, catalog number: 310-01)
81. Recombinant human TGF-β1 (Peprotech, catalog number: 100-21C)

82. Recombinant human SCF (Peprotech, catalog number: 300-07)
83. Recombinant human FGF basic (Peprotech, catalog number: 100-18B)
84. Gelatin 0.1% solution (EMD Millipore, catalog number: ES-006-B)
85. Advanced DMEM medium (Thermo Fisher Scientific, catalog number: 12491023)
86. Alpha MEM medium (Thermo Fisher Scientific, catalog number: 12571063)
87. Fetal bovine serum (Gibco, catalog number:10437020)
88. 1 α , 25-dihydroxyvitamin D₃ (Sigma Aldrich, catalog number: D1530)
89. CD14 antibody (FITC-conjugated; Biolegend, catalog number: 325604)
90. CD43 antibody (APC-conjugated, Miltenyi Biotec, catalog number: 560198)
91. CD45 antibody (APC-conjugated, Biolegend, catalog number:304012)
92. EDTA 0.5 M, pH 8.0 (Thermo Fisher Scientific, catalog number: 15575-038)
93. TRIzol (Thermo Fisher Scientific, catalog number: 10296028)
94. Direct-zol RNA (Zymo Research, catalog number: R2052)
95. DNase I (Invitrogen, catalog number: 18068015)
96. Superscript II reverse transcriptase (Invitrogen, catalog number: 18064071)
97. SsoAdvanced Universal SYBR Green Supermix (Bio-Rad, catalog number: 1725271)
98. Glutaraldehyde 25% solution (Alfa Aesar, catalog number: A17876)
99. Acid phosphatase, leukocyte (TRAP) kit (Sigma-Aldrich, catalog number: 387A)
100. Primers for detection of mesoderm differentiation and osteoclast maturation by qPCR (Table 1)
101. Matrigel coating solution (see Recipes)
102. hiPSC culture medium (see Recipes)
103. EB mesoderm differentiation medium-1 (see Recipes)
104. EB mesoderm differentiation medium-2 (see Recipes)
105. Myelomonocytic expansion medium (see Recipes)
106. FACS buffer (see Recipes)
107. Osteoclast differentiation medium (see Recipes)
108. TRAP staining solution (see Recipes)
109. Reconstitution of hBMP4 (see Recipes)
110. Reconstitution of other cytokines (see Recipes)

Table 1. Sequences of qPCR primers to detect marker gene expression for mesoderm differentiation and osteoclastogenesis

Gene	Forward primer	Reverse primer
CDX2	5'-CCCTAGGAAGCCAAGTGAAAACC-3'	5'-CTCCTTGGCTCTGCGGTTCTG-3'
CD34	5'-AAATCCTCTTCCTCTGAGGCTGGA-3'	5'-AAGAGGCAGCTGGTATAAGGGTT-3'
<i>Brachyury (T)</i>	5'-TGTCCCAGGTGGCTTACAGATGAA-3'	5'-GGTGTGCCAAAGTTGCCAATACAC-3'
HAND1	5'-GAAAGCAAGCGGAAAAGGGAG-3'	5'-GGTGCGCCCTTAATCCTCTT-3'
SCL	5'-AAGGGCACAGCATCTGTAGTCA-3'	5'-AAGTCTTCAGCAGAGGGTCACGTA-3'
CTSK	5'-CAGTGAAGAGGTGGTCAGA-3'	5'-AGAGTCTTGGGGCTTACCTT -3'
<i>Calcitonin Receptor</i>	5'-TCTCAGGAGTGAAAGCATTGCACATA-3'	5'-AATGCTATGACCGAATGCAGCAGTTA-3'
NFATc1	5'-AGAATTCGGCTTGCACAGG -3'	5'- CTCTGGTGGAGAACAGAGC -3'
TRAP (APC5)	5'-ACCTAGTTGTTCTGTGATGCCT-3'	5'-GGGATCTGTAATCTGACTCTGTCTT-3'
HPRT	5'-ACTTGTCGCAGAACATC-3'	5'-GTGGCGAACAGTGTAGAA-3'

Equipment

20. Pipettes
21. Water bath
22. AirClean 600 PCR workstation (AirClean Systems, model: AC648A)
23. Stereomicroscope (Carl Zeiss, model: Stemi 508)
24. Cell culture incubator (Thermo Fisher Scientific, model: HeraCell 240i, catalog number: 51026332)
25. Eppendorf refrigerated centrifuge (Eppendorf, model: 5810R)
26. CFX96 Touch Real-time PCR Detection System (Bio-Rad, catalog number: 1855196)
27. MACSQuant Analyzer 10 (Miltenyi Biotech, catalog number: 130-096-343)
28. Upright microscope (Carl Zeiss, model: Axio Imager.D2m)
29. Tabletop scanning electron microscope (Hitachi, model: TM1000)

Software

3. CFX manager software (Bio-Rad, 18450000)
4. FlowJo™ software (BD Bioscience, <https://www.flowjo.com/>)
5. GraphPad Prism (GraphPad, <https://www.graphpad.com/>)
6. Fiji ImageJ (NIH, image.nih.gov)
7. ZEN microscope software (Zeiss, <https://www.zeiss.com/microscopy/us/products/microscope-software/zen-lite.html>)

Procedure

D. Maintenance of hiPSCs cultures

- a) Prepare Matrigel-coated 6-well plate by adding 1 ml of coating solution (see recipe Table 4) to each well and leave the plate at room temperature for 1 h before use.
- b) Maintain hiPSCs in PeproGrow hESC medium (see recipe Table 5) and change medium every other day.
- c) Passage undifferentiated hiPSCs every 4-5 days.
 - i. Aspirate old medium and add 2 ml fresh PeproGrow hESC medium to each well.
 - ii. Scrape undifferentiated hiPSC colonies into small pieces using 200 µl pipet tips under a stereomicroscope within an AirClean PCR Workstation. Depending on the size, a hiPSC colony can be broken up into 15-40 pieces.
 - iii. Aspirate the Matrigel coating solution from the freshly prepared plates and transfer the lifted hiPSC fragments to new Matrigel-coated plates.

E. Mesoderm differentiation of hiPSC-derived embryoid bodies (EBs) (Stage 1)

- a) Examine hiPSC cultures and ensure hiPSCs are undifferentiated. Lift and remove the differentiated hiPSCs, if any, using a 200 µl pipet tip under a microscope in the PCR Workstation.
- b) Prewarm an aliquot of Accutase solution in 37 °C water bath for 10 min.
- c) Aspirate hiPSC culture medium. Wash hiPSCs with pre-warmed PBS (2 ml per well) twice. Add 1 ml of Accutase solution to each well and incubate culture plates in 37 °C incubator for 10 min.
- d) Add 2 ml of DMEM/F12 medium to each well. Pipette the cell suspension with 10 ml pipette gently up and down to dislodge the hiPSCs. Transfer the contents to 50 mL conical tubes.
- e) Centrifuge the cells at 340 $\times g$ for 7 min at 4 °C. Resuspend cells in EB basal medium-1 (see recipe Table 6). Filter the cells using a 70 µm strainer. Count cells.
- f) Plate 15,000 cells to each well in 150 µl EB mesoderm differentiation medium-1 (see recipe Table 6) on a Nunclon Sphera 96-well plate. Keep the plates at 37 °C in a 5% CO₂ and 5% O₂ incubator for 2 days. Each well forms one EB.
- g) Change half medium by pipetting out 75 µl of EB mesoderm differentiation medium-1 and adding 75 µl of EB mesoderm differentiation medium-2 (see recipe Table 7).
- h) Keep the plates at 37 °C in a 5% CO₂ and 5% O₂ incubator for 2 more days.
- i) Analyze mesoderm differentiation by examining the expression levels of mesoderm marker genes in EBs cultured for 1, 2, 3, and 4 days by qPCR (Figure 1).

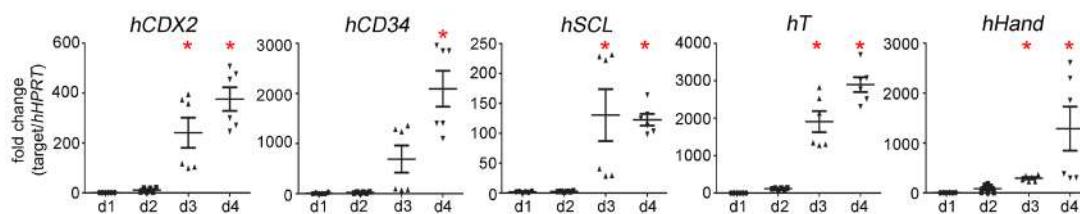


Figure 1. Expression levels of mesoderm marker genes *hCDX2*, *hCD34*, *hSCL*, *hT*, *hHand*, in EBs cultured for 1, 2, 3, and 4 days (d1, d2, d3 and d4). * $P < 0.05$ by one-way ANOVA compared to day 1 samples. Data was relative quantification and presented as mean \pm SEM.

F. Expansion of myelomonocytic population released from EBs (Stage 2)

- Prepare gelatin-coated plate by adding 1 ml of 0.1% gelatin solution to each well of a 6-well plate and leave at room temperature for 1 h before use.
- Collect EBs from Nunclon Sphera 96-well plate by suctioning out the medium with EBs using a 1 ml pipet tip and transfer these EBs to a 50 ml conical tube.
- Let EBs sink down by gravity for 3-5 min.
- Aspirate medium with a glass Pasteur pipette carefully without removing EBs in the bottom.
- Resuspend EBs in myelomonocytic expansion medium (see recipe Table 8) according to numbers of EBs. Seed 40-50 EBs in one well of 6-well plate with 4 ml of myelomonocytic expansion medium. For example: EBs collected from a full 96-well plate can be resuspended in 8 ml medium and plated in 2 wells of a gelatin-coated 6-well plate.
- Keep the plates at 37 °C in a 5% CO₂ and 5% O₂ incubator.
- EBs attach to the plates. Some cells are released from EBs. The floating non-adherent cells are the myelomonocytic population.
- Change expansion medium after 4 days with the same medium but increase hMCSF concentration from 50 ng/ml to 100 ng/ml.
- Continue culture for another 13 days (medium changes at days 4, 8, 12, and 16).
- Floating cells released from EBs at days 10, 13, 17, 21 are collected and stained with primary antibodies against CD14 (1:20), CD43 (1:50), and CD45 (1:50) in FACS buffer (see recipe Table 9) for 30 min on ice. Protect from light. Table 2 shows FACS analysis data.

Table 2. CD14, CD43, and CD45 expression in cells collected at day 10, 13, 17, and 21 days in Stage 2 analyzed by flow cytometry

	CD14+ (%)	CD43+ (%)	CD45+ (%)
Day 10	37.78 \pm 15.36	41.80 \pm 26.94	64.76 \pm 19.83
Day 13	59.13 \pm 24.56	76.28 \pm 14.56	75.01 \pm 15.96
Day 17	79.53 \pm 18.02	83.18 \pm 11.03	86.76 \pm 6.74
Day 21	84.77 \pm 5.05	59.50 \pm 13.38	72.18 \pm 11.09

Data presented: mean \pm SD. Data collected from 3 hiPSC lines. Each line had 3-4 technical repeats.

G. Promotion of osteoclast maturation (Stage 3)

- a) Collect floating cells (17 days of Stage 2) to a 15 ml or 50 ml conical tube. Typically, 0.2-0.45 million cells can be expected from EBs grown on one well of a gelatin-coated 6-well plate.
- b) Centrifuge the cells at 340 $\times g$ for 7 min at 4 °C. Count cells.
- c) Plate cells at a density of 10,000 cells per well in a 96-well plate and on bone chips.
- d) Culture osteoclast progenitors in osteoclast differentiation medium (see recipe Table 10). Change medium every 2-3 days.
- e) Multinucleated mature osteoclasts form in 7-10 days in osteoclast differentiation medium (Figure 2A).
- f) Fix hiPSC-OCs at day 10-12 for TRAP staining (Figures 2B-2C).
 - i. Remove culture medium. Wash gently with PBS once.
 - ii. Fix cells in 2.5% glutaraldehyde (150 μl per well) at room temperature for 10 min.
 - iii. Prepare TRAP staining solution (see recipe Table 11).
 - iv. Add 100 μl of TRAP solution in each well of 96-well plate.
 - v. Incubate culture plate at 37 °C for 45 min to 1 h.
 - vi. Remove the TRAP solution. Rinse plates with distilled water.
 - vii. Dry the plate before taking images.
- g) Fix TRAP stained cells on bone chips at day 14. Take images of resorption pits with TM1000 tabletop scanning electron microscope (Figure 2D).
- h) Confirm differentiation status of osteoclasts by examining expression levels of osteoclast marker genes by qPCR (Table 3).

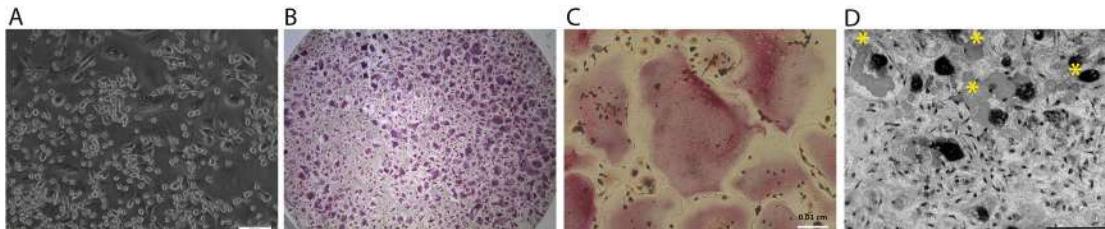


Figure 2. hiPSC-OC cultures. A. Bright field of hiPSC-OC culture at day 10 in OC differentiation medium. Scale bar = 100 μm ; B. TRAP staining of one well of 96-well plate. Purplish large sized cells are TRAP positive multinucleated osteoclasts; C. High magnification of TRAP-positive multinucleated cells. Scale bar = 100 μm ; D. Resorption pits (indicated by yellow asterisks) on bone chip. Large black spots are TRAP positive osteoclasts. Scale bar = 200 μm .

Table 3. qPCR data of expression levels of osteoclast marker genes in hiPSC-osteoclasts

	<i>hTRAP</i>	<i>hNFATc1</i>	<i>hCATHEPSIN K (CTSK)</i>	<i>hCALCITONIN R</i>
OC progenitors	0.95 ± 0.09	1.10 ± 0.09	1.05 ± 0.08	1.60 ± 1.22
Mature OCs	7.09 ± 1.90*	7.66 ± 1.99*	22.82 ± 3.25*	39.22 ± 10.08*

Data presented: mean ± SD. Data collected from 3 hiPSC lines. Each line had 3 technical repeats. * $P < 0.05$ by Student's *t*-test. OC progenitors are the floating cells collected at day 17

of stage 2 and cultured in osteoclast differentiation medium without 1 α ,25-dihydroxyvitamin D₃, hTGF β -1, and hRANKL.

Data analysis

1. qPCR data were analyzed using CFX manager software. The significant difference was determined by Student's *t*-test or one-way ANOVA followed by Tukey's post-hoc test using GraphPad Prism software.
2. Flow cytometry data were analyzed using FlowJo software.

Notes

1. Differentiation efficiency of stem cells can vary based on the size of EBs (Moon *et al.*, 2014, Ng *et al.*, 2015). The step of forming uniform-sized EBs is important to achieve consistent efficiency of hiPSC-OCs differentiation.
2. We determined the duration of Stage 2 as 17 days based on flow cytometry data for the expression levels of CD14, CD43, and CD45, which are expressed highest during these 17 days with little variability in comparison to other time points.
3. The non-specific/spontaneous differentiation of hiPSCs can be identified by their heterogeneous morphology within a hiPSC colony. Differentiation usually starts in the center of a large hiPSC colony and appears as a dark field under a brightfield microscope.
4. Isogenic hiPSCs have identical genetic background except for a genetic variant that has been introduced. Thus isogenic hiPSCs are the best controls to use when researchers aim to determine the impact of a specific disease mutation.

Recipes

1. Matrigel coating solution (Table 4)
 - i. Thaw frozen Matrigel in refrigerator overnight and aliquot Matrigel in ice-cold microcentrifuge tubes (60 μ l for one 6-well plate)
 - ii. Thaw Matrigel aliquots on ice and transfer Matrigel to ice-cold DMEM/F12 medium. For one 6-well plate, add 60 μ l Matrigel to 6 ml DMEM/F12 medium

Table 4. Matrigel coating solution

Composition	Volume (for one 6-well plate)
Matrigel	60 μ l
DMEM/F12 medium	6 ml

2. hiPSC culture medium (Table 5)
 - a. Centrifuge the vial with lyophilized Peprotech growth factor prior to opening and reconstitute with sterile water. Use 500 μ l water for each 500 ml PeproGrow hESC basal medium kit
 - b. Add the reconstituted growth factor to the basal medium aseptically and mix well by swirling or pipetting. Store at 2-8 °C and use within 2 weeks

Table 5. hiPSC medium

Composition	Volume
PeproGrow hESC medium	500 ml
Peprotech growth factor	500 μ l

3. EB mesoderm differentiation medium-1 (Table 6)
 - a. Prepare 100 ml EB mesoderm differentiation medium as shown in Table 6
 - b. Sterilize EB basal medium (including Stempro-34, L-glutamine, MTG) by filtering through a 0.2 μ m PES membrane filter and keep at 2-8 °C for up to 1 month
 - c. Add hBMP4, hVEGF, Y-27632, and ascorbic acid in EB basal medium just before use

Table 6. EB mesoderm differentiation medium-1

Composition	Volume	Final concentration
Stempro-34 basal medium	99 ml	
L-glutamine (200 mM)	1 ml	2 mM
MTG (11.5 M)	3.5 μ l	0.4 mM
hBMP4 (100 ng/ μ l)	25 μ l	25 ng/ml
hVEGF (100 ng/ μ l)	50 μ l	50 ng/ml
Rock inhibitor Y-27632 (10 mM)	100 μ l	10 μ M
Ascorbic acid (25 mg/ml)	200 μ l	50 μ g/ml

4. EB mesoderm differentiation medium-2 (Table 7)
 - a. Prepare 100 ml EB mesoderm differentiation medium as shown in Table 7
 - b. Sterilize EB basal medium (including Stempro-34, L-glutamine, MTG) by filtering through a 0.2 μ m PES membrane filter and keep at 2-8 °C for up to 1 month
 - c. Add 2x concentration of hBMP4, hVEGF, Y-27632, and ascorbic acid in EB basal medium just before use

Table 7. EB mesoderm differentiation medium-2

Composition	Volume	Final concentration
Stempro-34 basal medium	99 ml	
L-glutamine (200 mM)	1 ml	2 mM
MTG (11.5 M)	3.5 µl	0.4 mM
hBMP4 (100 ng/µl)	50 µl	50 ng/ml
hVEGF (100 ng/µl)	100 µl	100 ng/ml
hTPO (100 ng/µl)	100 µl	100 ng/ml
hFLT3-ligand (100 ng/µl)	100 µl	100 ng/ml
hSCF (100 ng/µl)	100 µl	100 ng/ml
hFGF basic (40 ng/µl)	100 µl	40 ng/ml
Ascorbic acid (25 mg/ml)	400 µl	100 µg/ml

5. Myelomonocytic expansion medium (Table 8)

- Prepare 100 ml myelomonocytic expansion medium as shown in Table 8
- Sterilize myelomonocytic expansion medium (including advanced DMEM, FBS, L-glutamine, β-mercaptoethanol) by filtering through a 0.2 µm PES membrane filter and keep at 2-8 °C for up to 1 month
- Add hIL-3 and hM-CSF aseptically only before use. Increase hM-CSF concentration from 50 to 100 ng/ml starting from day 4 medium change

Table 8. Myelomonocytic expansion medium

Composition	Volume	Final concentration
Advanced DMEM medium	88.5 ml	
FBS	10 ml	10%
L-glutamine (200 mM)	0.5 ml	1 mM
NEAA (100x)	1 ml	1x
β-mercaptoethanol	50 µl	0.1 mM
hIL-3 (100 ng/µl)	25 µl	25 ng/ml
hM-CSF (100 ng/µl)	50 or 100 µl	50 or 100 ng/ml

6. FACS buffer (Table 9)

- Prepare 500 ml of FACS buffer as shown in Table 9
- Store at 2-8 °C for up to 6 months

Table 9. FACS buffer

Composition	Volume	Final concentration
PBS	488 ml	
FBS	10 ml	2%
EDTA (0.5 M)	2 ml	2 mM

7. Osteoclast differentiation medium (Table 10)

- Prepare 100 ml osteoclast differentiation medium as shown in Table 10
- Sterilize osteoclast differentiation basal medium (including alpha-MEM, FBS) by filtering through a 0.2 µm PES membrane filter and keep at 2-8 °C for up to 1 month
- Add vitamin D, hTGFβ, hM-CSF, and hRANKL aseptically only before use

Table 10. Osteoclast differentiation medium

Composition	Volume	Final concentration
Alpha MEM	90 ml	
FBS	10 ml	10%
1α,25-dihydroxyvitamin D ₃	2 × 10 ⁻⁴ M	2 × 10 ⁻⁷ M
hTGFβ-1(20 ng/µl)	25 µl	5 ng/ml
hM-CSF (100 ng/µl)	30 µl	30 ng/ml
hRANKL (100 ng/µl)	50 µl	50 ng/ml

8. TRAP staining solution (Table 11)

- All reagents are included in the acid phosphatase, leukocyte (TRAP) kit
- Premix Fast Garnet GBC and sodium nitrate
- Add the rest of reagents after 2 min

Table 11. TRAP staining solution

Composition	Volume
Distilled water	11.25 ml
Fast Garnet GBC	125 µl
Sodium nitrate	125 µl
Naphthol AS-BI phospho solution	125 µl
Acetate solution	500 µl
Tartrate solution	250 µl

9. Reconstitution of hBMP4

- Centrifuge the vial prior to opening
- Reconstitute in 5 mM HCl, pH 3.0 to a concentration of 0.1-1.0 mg/ml
- Do not vortex

- d. Further dilute in a buffer containing 0.1% BSA as carrier protein and store in working aliquots at -80 °C
10. Reconstitution of other cytokines
 - a. Centrifuge the vial prior to opening
 - b. Reconstitute in water to a concentration of 0.1-1.0 mg/ml
 - c. Do not vortex
 - d. Further dilute in a buffer containing 0.1% BSA as carrier protein and store in working aliquots at -80 °C

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Competing interests

The author declares no conflict of interest.

Ethics

The hiPSC lines from peripheral blood were generated as previously described (Chen *et al.*, 2013; Chen *et al.*, 2017). This work was in accordance with guidelines of the Institutional Review Board of the University of Connecticut Health (IRB protocol 09-199).

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Isolation of Extracellular Vesicles Derived from Mesenchymal Stromal Cells by Ultracentrifugation

María José Ramírez-Bajo^{1, 2, \$,*}, Elisenda Banon-Maneus^{2, 3},
Jordi Rovira^{1, 2}, Josep M. Campistol^{1, 2, 3, 4, #} and Fritz Diekmann^{1, 2, 3, 4, \$, #,*}

¹Laboratori Experimental de Nefrologia i Trasplantament (LENIT), Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS), Barcelona, Spain; ²Red de Investigación Renal (REDINREN), Madrid, Spain; ³Laboratori Experimental de Nefrologia i Trasplantament (LENIT), Fundació Clínic per la Recerca Biomèdica (FCRB), Barcelona, Spain; ⁴Departament de Nefrologia i Trasplantament Renal, ICRN, Hospital Clínic, Barcelona, Spain; ^{\$}Current address: Departament de Nefrologia i Trasplantament Renal, ICRN, Hospital Clínic, Villarroel 170, 08036 Barcelona, Spain

*For correspondence: fdiekman@clinic.cat; mramire1@clinic.cat

#Contributed equally to this work

[Abstract] Extracellular vesicles (EVs) are a heterogeneous group of membranous vesicles that differ on their biogenesis and release pathways, such as exosomes, microvesicles and apoptotic bodies. They are involved in cell-to-cell communication delivering signal molecules (proteins, nucleic acids, lipids, etc.) that can regulate different physiological processes, as well as the development and progression of several diseases. There are different methods and commercial kits to isolate EVs and depending on the methodology one could obtain EVs with different degrees of efficiency, purity and it can be more or less time-consuming. Then, the choice has to be according to the different advantages and disadvantages, and their use for downstream applications. Here, we describe the EVs isolation method from mesenchymal stromal cells by ultracentrifugation. This EVs isolation can be performed using common media and buffers, and only with the requirement of an analytical ultracentrifuge. Moreover, this method can be used to obtain large quantity of EVs with a good reproducibility for developing *in vitro* and *in vivo* experiments and studying their biological actions.

Keywords: Mesenchymal stromal cells, Stem cells, Extracellular vesicles, Ultracentrifugation, Nanoparticle tracking analysis and Electronic microscopy

[Background] Mesenchymal stem cells (MSCs) have a protective effect on the progression of different diseases contributing to the immunomodulation and the inflammatory state (Bartholomew *et al.*, 2002; Togel *et al.*, 2005; Azmi *et al.*, 2013; Ebrahimi *et al.*, 2013; Ben-Ami *et al.*, 2014). Their protective action is not only due to their transdifferentiation, but also their paracrine mechanisms such as release of extracellular vesicles (EVs) showing an immunomodulatory, anti-inflammatory, anti-apoptotic and proangiogenic function (Bruno *et al.*, 2009; Camussi *et al.*, 2010a; Grange *et al.*, 2020). Moreover, the application of EVs from MSCs in clinical practice has the advantage of being a safe therapy without the disadvantages related to other MSCs therapies, including the possibility to be rejected, gene stability, poor long-term differentiation and probability of viral transfer (Kunter *et al.*, 2007; Wang *et al.*, 2012).

In the last years, there is an increasing interest in the application of EVs in the transplant area, as an immunomodulatory and anti-inflammatory therapy on transplant recipients, and their addition on perfusion solution to pre-condition the graft before transplantation (Koch *et al.*, 2015; Gregorini *et al.*, 2017; Stone *et al.*, 2017; Rigo *et al.*, 2018; Ramírez-Bajo *et al.*, 2020b). In particular, EVs therapy in the context of a hypothermic or normothermic perfusion machine could increase the long-term survival and increase the number of available organs. This improvement could be related to a decrease of inflammation, the preservation of energy metabolism and microvascular permeability avoiding the formation of edema. These properties show the great therapeutic potential of EVs in the transplantation process.

Cell-to-cell communication by EVs is a mechanism that has been preserved throughout the evolution in both prokaryotic and eukaryotic cells (Ratajczak *et al.*, 2006). Since its discovery 30 years ago, it has been demonstrated that EVs are produced by an enormous variety of cell types such as blood cells, dendritic, endothelial and epithelial cells, nervous system cells, adult and embryonic stem cells and even tumor cells (Harding *et al.*, 1984). EVs are a heterogeneous group of membranous vesicles that differ on their biogenesis and release pathways such as exosomes, microvesicles, microparticles, ectosomes, oncosomes and apoptotic bodies (Thery *et al.*, 2002 and 2018). EVs act as paracrine/endocrine effectors that transport bioactive molecules among the cells (cytosolic proteins, lipids, mRNA, miRNA, DNA, mitochondrial DNA and genomic DNA) of the surrounding microenvironment, or being carried remotely in biological fluids (Fevrier and Raposo, 2004; Camussi *et al.*, 2010b; Mittelbrunn *et al.*, 2011). These regulatory biological signals can regulate various physiological processes, as well as the development and progression of disorders (Valadi *et al.*, 2007; Guescini *et al.*, 2010; Balaj *et al.*, 2011).

A large number of studies are focused on EV's field and their isolation from different biofluids, developing different methods of isolation such as ultracentrifugation, density gradient, size exclusion chromatography, filtration, microfluidics, precipitation kits and magnetic beads technologies. The choice of isolation methods should depend on the type of starting material, volume, available equipment, scientific inquiry and subsequent analysis or use (Momen-Heravi *et al.*, 2013). There are described different protocols associated with difference in purity and time of isolation. Moreover, EVs composition is dependent on the isolation protocol used, obtaining different subpopulations with different soluble proteins and nucleic acids. For these reasons, it is important to follow a series of criteria based on current best practice that represents the minimal experimental requirements for definition of EVs that allow the interpretation and their replication by other researchers (Lotvall *et al.*, 2014).

The most commonly used protocol for isolation is ultracentrifugation which has advantages such as the applicability for EVs isolation from large volumes as urine or cell culture conditioned media, the use of common media and buffers, easy to handle, and absence of impact on EVs except gravitational force (Konoshenko *et al.*, 2018). This method can be used to obtain large quantity of EVs with a good reproducibility for developing studies about their biological actions. However, it needs expensive equipment and the isolated EVs could present co-isolating contaminants such as soluble proteins and nucleic acids. In our previous publications, we showed a comparison of the therapeutic effect of MSC and their EVs in an *in vivo* model of chronic kidney allograft rejection (Ramírez-Bajo *et al.*, 2020a) and

chronic cyclosporine nephrotoxicity (Ramírez-Bajo *et al.*, 2020b). The ultracentrifugation protocol allows to produce a great quantity of EVs from high volumes of MSC-conditioned medium required for a periodic administration of therapies to different animal groups. We observed a good reproducibility between EVs batches by this methodology.

Materials and Reagents

1. 22 G needle
2. 15 ml conical tubes (Sarstedt, catalog number: 62.554.502)
3. 50 ml conical tubes (Sarstedt, catalog number: 62.548.004)
4. Serological pipettes 10 ml (Sarstedt, catalog number: 86.1254.001)
5. Serological pipettes 25 ml (Sarstedt, catalog number: 86.1685.001)
6. Tissue Culture Flask, 150 cm² filter (TPP, catalog number: 90151)
7. Tissue Culture Flask, 75 cm² filter (TPP, catalog number: 90076)
8. 0.22 µm sterile syringe filters (Sarstedt, catalog number: 831.826.001)
9. 0.1 µm sterile syringe filters (Pall Life Sciences, catalog number: 4668)
10. 20 ml Sterile syringes (BD Plastipak, catalog number: 10569215)
11. Glass Pasteur pipettes (Deltalab, catalog number: 702)
12. Countess cell counting chamber slides (Thermo Fisher Scientific, Invitrogen, catalog number: C10228)
13. Open-Top Thinwall Ultra-Clear Tube, 38.5 ml capacity (Beckman Coulter, catalog number: 344058)
14. Lewis and Fisher rats (200 g)
15. Trypan blue (Thermo Fisher Scientific, Invitrogen, catalog number: C10228)
16. Alpha MEM (Lonza Bioscience, catalog number: BE02-002F)
17. RPMI 1640 Medium with L-Glutamine (Lonza Bioscience, catalog number: BE12-702F)
18. Fetal bovine serum (FBS) (EuroClone, catalog number: ECS0180L)
19. Penicillin-Streptomycin (10,000 U/ml) (Thermo Fisher Scientific, Invitrogen, catalog number: 15140122)
20. Trypsin-EDTA (0.25%), phenol red (Thermo Fisher Scientific, Invitrogen, catalog number: 25200072)
21. Dulbecco's phosphate buffered saline (PBS) 10x, Without Ca++ and Mg++ (Cultek, catalog number: BE17-517Q)
22. Sterile water for irrigation, Serrason solution (Serra Pamies, catalog number: 374561)
23. Dimethyl sulfoxide (DMSO) (Sigma-Aldrich, catalog number: 276855)
24. L-glutamine solution (Sigma-Aldrich, catalog number: G7513)
25. Complete alpha-MEM medium (see Recipes)
26. Freezing medium (see Recipes)

Equipment

1. -80 °C freezer
2. Water bath set to 37 °C (Thermo Fisher Scientific, model: JP Selecta™ Precisterm 6000141)
3. Refrigerated benchtop centrifuge (Thermo Fisher Scientific, model: Heraeus™ Multifuge™ X1)
4. Countess automated cell counter (Thermo Fisher Scientific, Invitrogen, catalog number: C10281)
5. DHD Air-Jacketed Automatic CO₂ Incubator (NuAire, catalog number: NU-5510E)
6. Inverted Modulation Contrast Microscope (Leica, model: DMIRB)
7. C2200 CBC Complete Balance (Cobos, 200-20018)
8. Preparative ultracentrifuge (Beckman Coulter, model: Optima L100XP)
9. SW32 Ti Swinging-Bucket Rotor Package (Beckman Coulter, catalog number: 369694)
10. NanoDrop Microvolume Spectrophotometers (Thermo Fisher Scientific, model: ND1000)
11. NanoSight instrument equipped with a 638 nm laser and CCD camera (model F-033) (Malvern, model: LM10)
12. Cryo-electronmicroscope (Jeol, model: JEM 2011)
13. EMR Carbon support film on copper 400 square mesh (EMR, catalog number: 22-1MC040-100)
14. Flow cytometer (Becton Dickinson, Biosciences, model: FACS Canto II)

Software

1. FlowJo v10 software (BD Bioscience, <https://www.flowjo.com/solutions/flowjo/downloads>).
2. Nanosight NTA Software version 3.1 (build 3.1.46) (Malvern Panalytical, <https://www.malvernpanalytical.com/en/learn/events-and-training/webinars/W150326NanoSightSoftwareRelease>)

Procedure

A. Mesenchymal stem cell preparation

Bone marrow-MSCs (BM-MSCs) were isolated from femurs of Lewis and Fisher rats (200 g). In brief, the bone shaft was extracted inserting a 22 G needle and flushed out with alpha-MEM supplemented with 10% FBS and 2 mM EDTA and cell clumps desegregated. The cell suspension was centrifuged at 400 × g for 20 min at room temperature. Rat BM-MSCs were seeded and expanded in alpha-MEM supplemented with 10% FBS (Ramírez-Bajo *et al.*, 2020a). Therefore, BM-MSCs were characterized according to standardized criteria defined by International Society for Cellular Therapy (ISCT) (see Note 1; Figure 1).

1. Culture MSCs in T75 cm² flask using 7 ml of complete alpha-MEM medium. Trypsinization is performed at approximately 80% confluence.
2. Wash cell culture with pre-warmed PBS, add 3 ml trypsin and incubate at 37 °C for 4 min.

3. Stop the reaction with 9 ml of complete medium and spin down at $600 \times g$ for 5 min at room temperature to count cells and check viability of culture.
4. Count MSC's number and their viability with an automated cell counter following manufacturer's instructions (see Note 2).
5. Plate the cells at a density of 5×10^5 cells in a flasks T150 cm² with 15 ml of complete alpha-MEM medium.
6. Incubate at 37 °C in 5% CO₂ environment.
7. Replace with fresh complete alpha-MEM medium (pre-warmed to 37 °C) every two days thereafter.

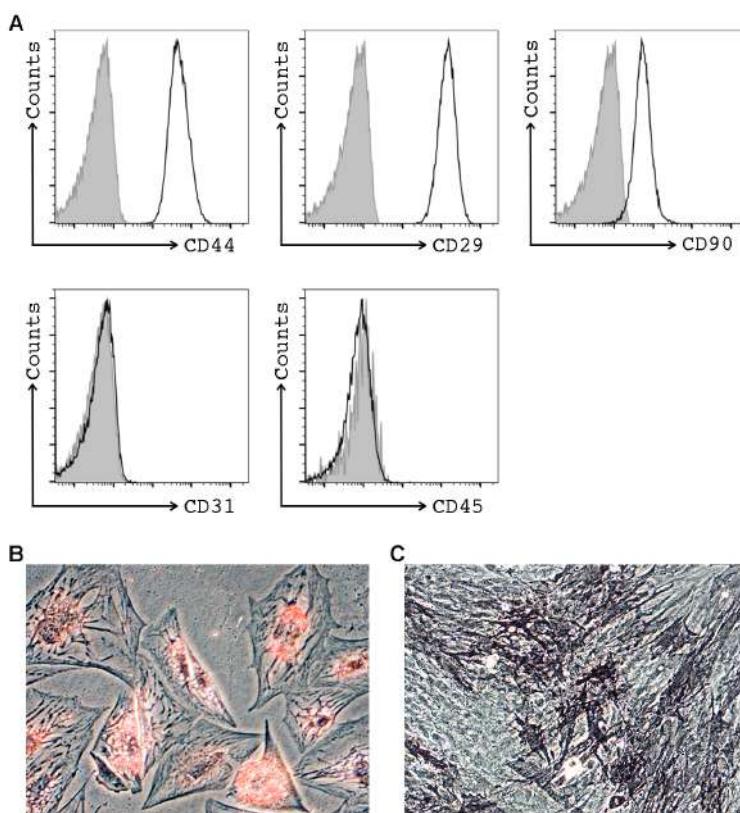


Figure 1. Representative images of MSCs characterization by flow cytometry and multi-lineage differentiation potential. A. Flow cytometry analysis of MSC. Cells were positive for surface stem cell markers (CD44, CD29 and CD90), and negatives for endothelial (CD31), and hematopoietic (CD45) markers. B. Adipogenic differentiation ($\times 200$ magnification) as demonstrated showing positivity for oil red staining. C. Osteogenic differentiation ($\times 100$ magnification) as demonstrated showing positivity for alkaline phosphatase activity, and was revealed by SigmaFast BCIP/NBT chromogen staining.

B. EVs isolation by ultracentrifugation

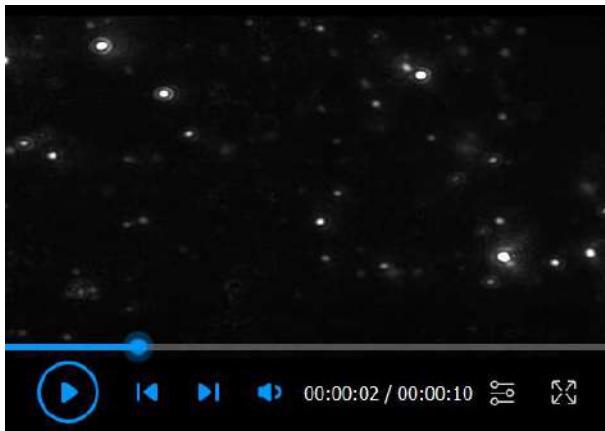
1. When the cells are approximately 80% confluence, aspirate the supernatant and add 6 ml of PBS (pre-warmed to 37 °C) to wash the residual FBS. Repeat washing step one more time.

2. Replace PBS with 15 ml of RPMI1640 deprived of FBS (pre-warmed to 37 °C) and incubate for 16 h.
3. Harvest the MSC conditioned serum free medium and transfer to 50 ml conical tube and keep at 4 °C.
4. Trypsinize MSCs and determine cell number and viability in an automated cell counter following manufacturer's instructions (repeat Steps A1-A3).
Our estimation at time of collection was $\pm 1.5 \times 10^6$ MSCs and $\pm 95\%$ of live cells.
5. Centrifuge MSC conditioned serum free medium at $3,000 \times g$ for 20 min at 4 °C and micro filtrate with 0.22 µm pore filter membranes to remove cell debris and apoptotic bodies.
6. Transfer cell-free supernatants into a 38.5 ml capacity open-top thin wall ultra-clear tube. Balance opposite tubes within 10 mg of each other (see Notes 3-4).
7. Ultracentrifuge at $100,000 \times g$ for 1 h, 4 °C, using SW32 Ti rotor.
8. Carefully remove tubes from the rotor.
9. Remove supernatant by aspiring carefully with a Pasteur pipette.
10. Resuspend EVs pellets with a 200 µl of freezing medium and froze them at -80 °C for later use (see Notes 5-6).

C. EVs characterization

See Note 7.

1. EVs quantification:
 - a. Nanosight Tracking Analysis (NTA)
Particle size distribution and concentration measurement:
 - i. Take 1 ml of EVs solution (dilution factor 1:40 in PBS 0.1 µm filtered) and vortex.
 - ii. With a syringe without needle, inject EVs solution slowly (avoiding bubbles) into the chamber of particle size analyzer for visualization. It is important to have approximately 20-60 particles in the field of view, to obtain an accurate concentration and size values (Video 1).



Video1. EVs moving under Brownian motion recorded by NTA. This video visualizes the expected Brownian motion of the EVs in liquid suspension illuminated by a laser light source. The EVs size distribution and concentration are measured using the combination of the light scattering properties with Brownian motion. The real time monitoring helps to determine changes in the characteristics of vesicle populations.

- iii. Take triplicate readings during 60 s at 30 frames per second (fps), camera level at 16 and manual monitoring of temperature. Figure 2A shows representative results obtained by NanoSight from EVs produced by MSCs.
- b. Protein quantification:
Determination of protein concentration ($A_{280\text{nm}}$) was checked by NanoDrop using 2 μl of EVs solution following the manufacturer's instructions.
2. Assessment of absence of contaminants by Electron Microscopy
 - a. Use a holey carbon support film on a 400-mesh copper grid. Glow discharge the grid and place 3 μl of the EVs sample (from Step B10) on a plunger (Leica EM GP) and blot it with Whatman No. 1 filter paper.
 - b. Vitrify the suspension by rapid immersion in liquid ethane (-179 °C) and mount the grid on a Gatan 626 cryo-transfer system and insert it into the microscope.
 - c. Take images using cryo-electron microscope operates at 200 kV, record on a GatanUltrascan US1000 CCD camera and analyze with a Digital Micrograph 1.8 ($n = 3$ per group) (Figures 2B-2C).

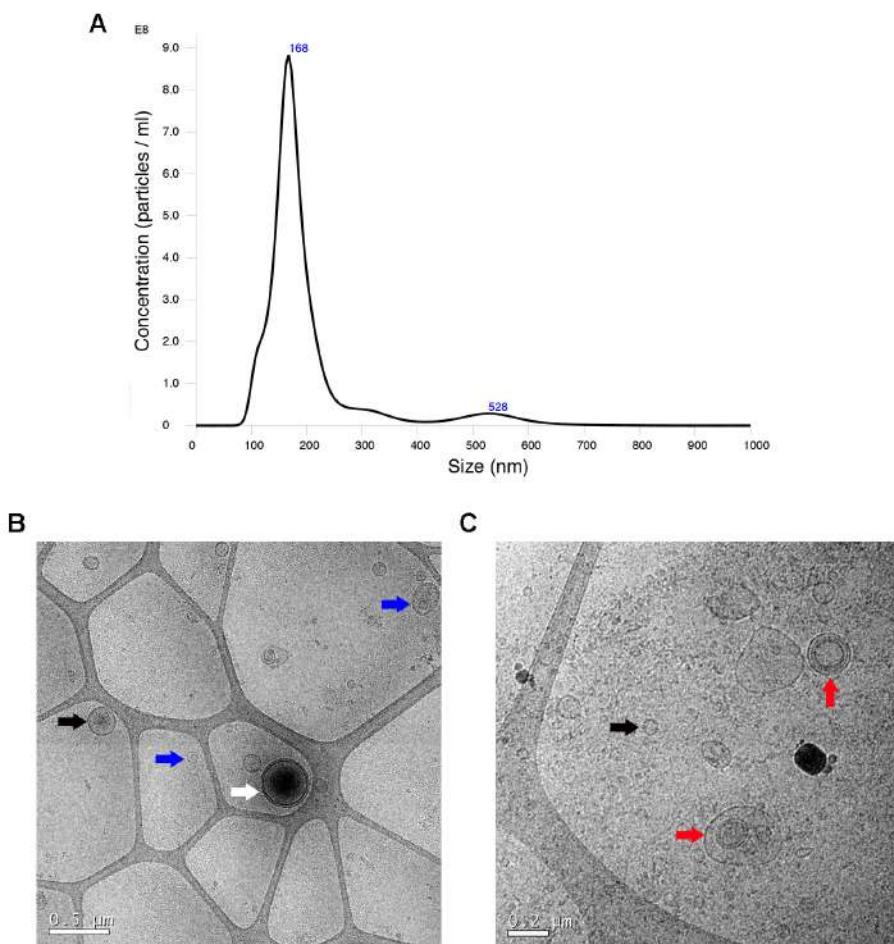


Figure 2. Characterization of EVs by electron microscopy and Nanoparticle Tracking Analysis (NTA). A. NTA measurement shows the concentration and size distribution. The mean EVs size was 201.9 ± 101.4 nm. B-C. Representative cryo-electron microscopy images of EVs (scale bars 0.5 and 0.2 μ m, respectively). Cryo-electron microscopy allows to visualize the grid with an irregular distribution of hole sizes and shapes, and inside of them EVs of various sizes with lipid bilayers. The white arrow points a vesicle with a double membrane (white arrow). Most vesicles have an intact membrane and a round shape. Some vesicles are single, double and multilayer, in the case that contains inside another smaller or two and more (black, blue and red arrow, respectively).

Data analysis

1. MSCs characterization by flow cytometry: Flow cytometric data obtained from BD FACS Canto II cytometer were analyzed with FlowJo software following the gating strategy described by Ramírez-Bajo et al., 2020b.
2. Nanosight tracking analysis: Data were analyzed with the Nanosight NTA Software version 3.1 (build 3.1.46), with detection threshold set to 5, and blur, min track length, and max jump distance set to auto.

Notes

1. According to standardized criteria defined by ISCT's guidelines, MSCs must a) be plastic adherent when kept under standard culture conditions, b) be positively expressed MSC markers (CD29, CD44, and CD90) and negatively endothelial (CD31) and hematopoietic lineage (CD45) markers, and c) retain a multipotent phenotype with the ability to differentiate in adipogenic and osteogenic lineage under the standard differentiation conditions (Figures 1B-1C).
2. In 2018, the International Society for Extracellular Vesicles (ISEV) published and update of the MISEV2014 guidelines with different recommendations to improve the reproducibility of published EVs results. The ISEV checklist summarizes the major aspects to follow in EV science (Lotvall *et al.*, 2014).

The estimation of number of cells/ml or /surface area and % of live/dead cells at time of collection (or at time of seeding with estimation at time of collection) is a mandatory requirement of ISEV Checklist.

3. To maintain sterility of EVs: Firstly, buckets must be cleaned with ethanol and let dry in the hood. Secondly, insertion or removal of the tubes from buckets has to be done also in the hood.
4. Balance the weight within the buckets. Thinwall tube needs to be full to provide tube wall support in the bucket. If the volume is too low it may collapse. For this reason, it is important to fill the tube with supernatant or adding PBS 0.1 µm sterile filtered.

Using SW32 Ti requires balancing the weight of the tubes with the buckets following Beckman's recommendation's: <https://www.beckman.com/resources/fundamentals/principles-of-centrifugation/balancing-your-rotor>

5. In order to resuspend the EVs pellets, you have to pipette them up and down with a 200 µl pipette but not too roughly, because it is better to avoid producing air bubbles.
6. EVs can be stored for 2 days at 4 °C. Storing samples at -80 °C is recommended for extended period.
7. Following ISEV recommendations it requires quantification by at least 2 methods. In our case, we determined particle number by Nanosight and protein count by NanoDrop Spectrophotometer. These results have to be expressed per volume of initial fluid or number of producing cells.

Recipes

1. Complete alpha-MEM medium
Alpha-MEM
10% FBS
1x L-Glutamine
1x P/S
Filter sterilize the solution and store at 4 °C

2. Freezing medium
RPMI 1640
1% DMSO

Filter sterilize the solution and store at 4 °C

Note: Filter all the solutions using a 20 ml syringe and a 0.22 µm filter into a new sterile tube.

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Competing interests

The authors declare no conflicts of interests.

Ethics

Bone marrow-MSCs were isolated from femurs of Lewis and Fisher rats (200g) (Ramírez-Bajo *et al.*, 2020b). The study was approved by and conducted according to the guidelines of the local animal ethics committee (Comitè Ètic d’Experimentació Animal, CEEA, Decret 214/97, Catalonia, Spain).

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