FACS-Mediated Isolation of Neuronal Cell Populations From Virus-Infected Human Embryonic Stem Cell–Derived Cerebral Organoid Cultures

Sylvie Janssens,[1,5,6] Michael Schotsaert,[2,5] Lara Manganaro,[2,3] Marion Dejosez,[1] Viviana Simon,[2] Adolfo García-Sastre,[2,4] and Thomas P. Zwaka[1]

[1]Huffington Center for Cell-Based Research, Black Family Stem Cell Institute, Department of Cell, Developmental and Regenerative Biology, Icahn School of Medicine at Mount Sinai, New York, NY
[2]Department of Microbiology, Global Health and Emerging Pathogens Institute, Icahn School of Medicine at Mount Sinai, New York, NY
[3]Present Address: INGM, National Institute of Molecular Genetics, Romeo ed Enrica Invernizzi, Milan, Italy
[4]Department of Medicine, Division of Infectious Diseases, Icahn School of Medicine at Mount Sinai, New York, NY
[5]These authors contributed equally to this work.
[6]Corresponding author: *sylvie.g.janssens@gmail.com*

Organoids—or pluripotent stem cell–derived in vitro-grown simplified mini organs—have become a tremendously important model to study human organ development and disease. To restrict the noise inherent to the heterogeneous cell mixtures derived from organoid cultures, we developed a new technique of fluorescence-assisted cell sorting (FACS) of virus-infected cerebral organoid cultures. This method still includes the advantage of growing cells in a more natural environment than traditional cell culture, but now renders samples suitable for downstream cell type-specific multi-omics analyses. The protocol starts from stem cell-derived mature brain organoids and includes steps for: preparing the culture for viral infection, production of the viral stocks, FACS sample preparation, and gating and sorting implementation. The protocol has been developed for Zika virus infection, but can be extrapolated to other viruses or fluorescent marker expression as illustrated in an alternate protocol using a single-cycle lentivirus expressing a fluorescent reporter protein. © 2018 by John Wiley & Sons, Inc.

Keywords: cerebral organoids • FACS • HIV • human ESC • lentiviral vector • virus • Zika

How to cite this article:
Janssens, S., Schotsaert, M., Manganaro, L., Dejosez, M., Simon, V., García-Sastre, A., & Zwaka, T. P. (2019). FACS-mediated isolation of neuronal cell populations from virus-infected human embryonic stem cell–derived cerebral organoid cultures. *Current Protocols in Stem Cell Biology*, *48,* e65. doi: 10.1002/cpsc.65

INTRODUCTION

In vitro–generated, stem cell–derived cerebral organoids closely mimic characteristic human fetal neurodevelopment *in vivo* while providing unlimited brain tissue comprising specific cell populations such as neuronal progenitors, neurons, and astrocytes. This

CURRENT PROTOCOLS
A Wiley Brand
Current Protocols in Stem Cell Biology e65, Volume 48
Published in Wiley Online Library (wileyonlinelibrary.com).
doi: 10.1002/cpsc.65

three-dimensional tissue allows for direct testing of brain development and modeling of complex human neurological disorders such as hereditary microcephaly, autism spectrum disorders, and Alzheimer's disease (Choi et al., 2014; Lancaster et al., 2013; Mariani et al., 2015). Brain organoids have also proven useful for dissecting the general impact of viral infections on brain development. For example, Zika virus infection of cerebral organoids confirmed the neurotropic nature of the virus and provided rapid confirmation of the causal link between prenatal Zika virus infection and congenital microcephaly (Garcez et al., 2016).

However, with the creation of more-complex multilayered tissue models comes the challenge of developing assays that can translate complex experimental starting conditions into useful data outputs. Possibilities to reduce this complexity include the use of organoids confined to specific brain regions (Qian et al., 2016) or microdissection of certain cerebral organoid anatomy combined with single-cell RNA sequencing (Camp et al., 2015). Nevertheless, a satisfying approach to study the effects of virus infection or other treatments in distinct cell populations of organoids has been lacking. We describe a protocol to isolate neural progenitors, astrocytes, and neurons from virus-infected cerebral organoids, validated for downstream epigenetic and gene expression assays (Janssens et al., 2018). This article outlines in detail how to prepare Zika and single-cycle lentiviral reporter viruses, prepare cerebral organoid cultures for viral infection, identify neuronal populations, and subsequently isolate infected and non-infected neural progenitor cells, neurons, and astrocytes suitable for downstream manipulations such as RNA and DNA extraction.

CAUTION: Manipulations of virus-containing cultures must be carried out in biological safety cabinets with appropriate personal protective equipment (PPE). Infectious waste must be collected and disposed of according to the correct biosafety level (BSL) guidelines.

BASIC PROTOCOL 1

GENERATION OF 2D CEREBRAL ORGANOID CULTURES FOR OPTIMAL VIRAL INFECTION

This protocol describes the generation of a 2D culture that is optimal for ample viral infection while maintaining the original cell types and cell interactions typical of cerebral organoids (Fig. 1). The successful isolation of virus-infected cells from cerebral organoids strongly correlates with the initial infection rate. Fluorescence-activated cell sorting (FACS) requires that a large enough portion of cells be separated from background noise, and downstream applications are limited by minimal material input. While developing this protocol, low infection rates were experienced when infecting mature cerebral organoids as a whole. Vibratome slicing and culturing of the cerebral organoid as 500-µm-thick slices elevated infection rates to some extent by increasing surface exposure to the virus (Fig. 1C). Nevertheless, switching to two-dimensional culture conditions was deemed necessary to reach satisfactory infection rates.

This protocol has been optimized for cerebral organoids generated according to Lancaster et al. (2013), but is expected to be adaptable to alternative neural organoid growing methods. A step-by-step protocol for generation of cerebral organoids (Lancaster & Knoblich, 2014) describes in detail the consecutive phases of making embryonic bodies, inducing neural fate, and establishing a three-dimensional culture. In our experience, H9 (WAO9) human embryonic stem cells (hESCs) are most suitable for generation of cerebral organoids containing high levels of brain tissues, while satisfactory results have been obtained when using H1 (WA01) cells.

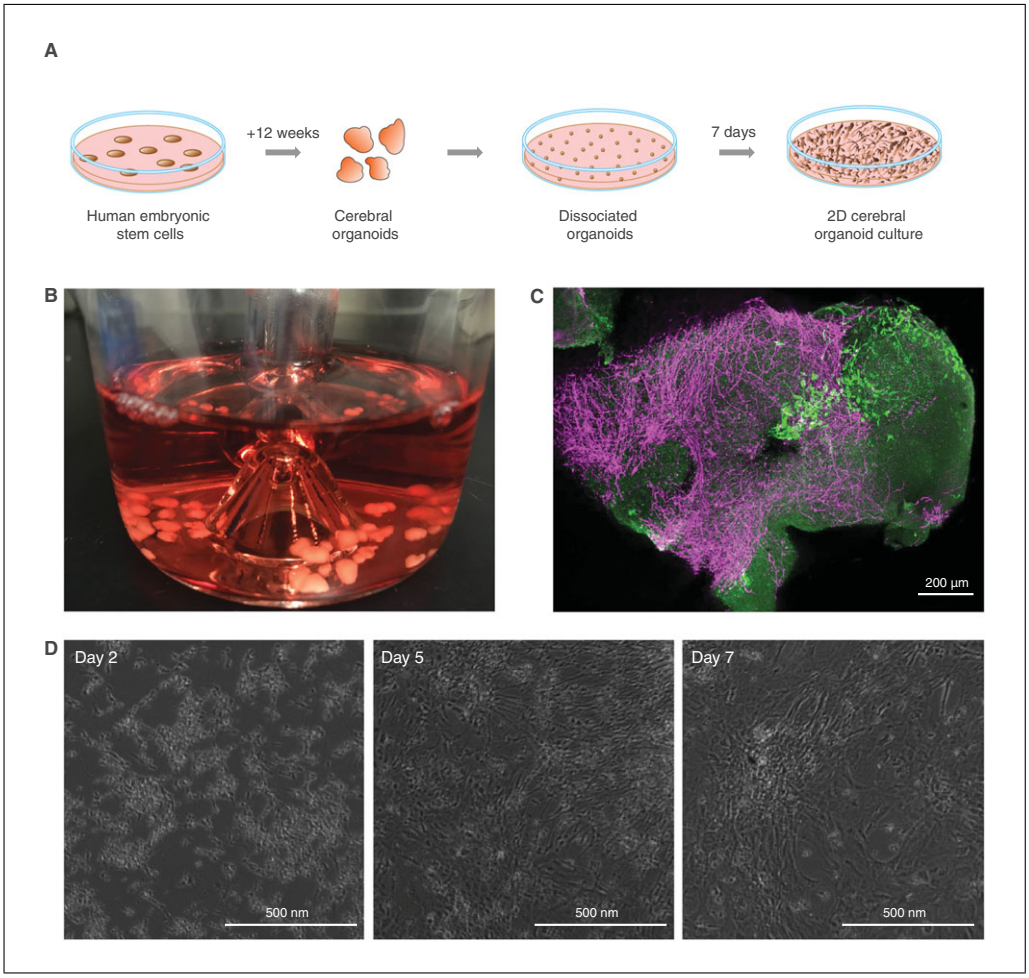

Figure 1 Generation of 2D cerebral organoid cultures. (**A**) Schematic overview of steps and time needed to create 2D cerebral organoid cultures suitable for viral infection. (**B**) Example of 3-month cerebral organoids (average size, 5 mm). (**C**) Confocal immunofluorescence maximum image projection of a 500-µm-thick ZIKV-infected cerebral organoid slice showing ZIKV-positive cells (E-antigen, green) and astrocytes (GFAP, purple). (**D**) Phase-contrast images of dissociated cerebral organoids at different time points after dissociation.

Materials

Growth factor–reduced Matrigel (Corning, cat. no. 356230)
DMEM/F12 (Gibco, cat. no. 11330-032)
hESC- or hiPSC-derived cerebral organoids
Accutase (Innovative Cell Technologies, cat. no. AT104)
Cerebral organoid medium (see recipe)

15- and 50-ml polypropylene centrifuge tubes
6- and 24-well flat-bottom cell culture plates
37°C, 5% CO_2 incubator
25-ml serological pipets
Sterile low-binding 1000-µl filter tips

Prepare Matrigel-coated 6-well plates

1. Thaw Matrigel in ice water overnight at 4°C and make 2-mg aliquots while keeping constantly on ice. Store at −80°C.

2. Add one Matrigel aliquot to 12 ml ice-cold DMEM/F12 in a 15-ml polypropylene centrifuge tube, dissolve, and immediately add 1 ml per well to two 6-well flat-bottom plates.
3. Incubate plates for 1 hr at room temperature.
4. Add 1 extra ml DMEM/F12 per well and store in a 37°C incubator for up to 1 week.

Collect and dissociate organoids

5. Pipet 2–3 mature organoids (Fig. 1B) per well into a 24-well plate using 25-ml serological pipets.
6. Remove excess medium that was transferred with the organoids, then add 0.5 ml Accutase per well and place in the 37°C incubator for 3–5 min.
7. Initiate dissociation by pipetting the organoid/Accutase mixture up and down several times using low-binding 1000-µl filter tips and incubate for another 3–5 min at 37°C. Do not exceed 10 min of exposure to undiluted Accutase.
8. Further dissociate the organoids into little chunks by pipetting up and down several more times and immediately transfer the solution to a 50-ml tube containing at least 5 ml cerebral organoid medium per 0.5 ml Accutase.

   It is important that dissociation be carried out gently to minimize damage to the cells. It is preferred to dissociate organoids into cell clusters rather than single cells to promote better cell survival.

9. Centrifuge at 100 × *g* for 5 min at room temperature. Transfer supernatant to a new 50-ml tube while keeping the pellet.
10. Centrifuge the supernatant at 300 × *g* for 3 min at room temperature. Transfer supernatant to a new 50-ml tube while keeping the second pellet.
11. Centrifuge the supernatant at 1000 × *g* for 3 min at room temperature. Discard supernatant and keep the third pellet.
12. Combine the three pellets and resuspend in cerebral organoid medium (1 ml per organoid transferred in step 5).

Plate cerebral organoids

13. Aspirate excess medium from the Matrigel-coated plates and add 2 ml organoid medium to each well.
14. Using a 1-ml pipet, slowly spread 0.5 ml organoid suspension into each well and place plates in a 37°C incubator.
15. After 12–18 hr, replace medium by carefully removing half of the medium with a 1-ml pipet and slowly adding the same amount of fresh medium dropwise into each well.
16. Culture for 6 more days, changing half of the medium every 2–3 days.

    During this time, the cells will regrow their processes and reestablish cellular interactions (Fig. 1D).

BASIC PROTOCOL 2

VIRAL INFECTION AND SAMPLE PREPARATION FOR FACS

This protocol describes how to prepare viral stocks, infect cerebral organoid cultures, and subsequently prepare infected cultures for FACS (Fig. 2). The main protocol describes infection with a replicating virus (Zika). Infection with a single-cycle lentiviral reporter virus encoding GFP under the control of a CMV promoter in position of its envelope

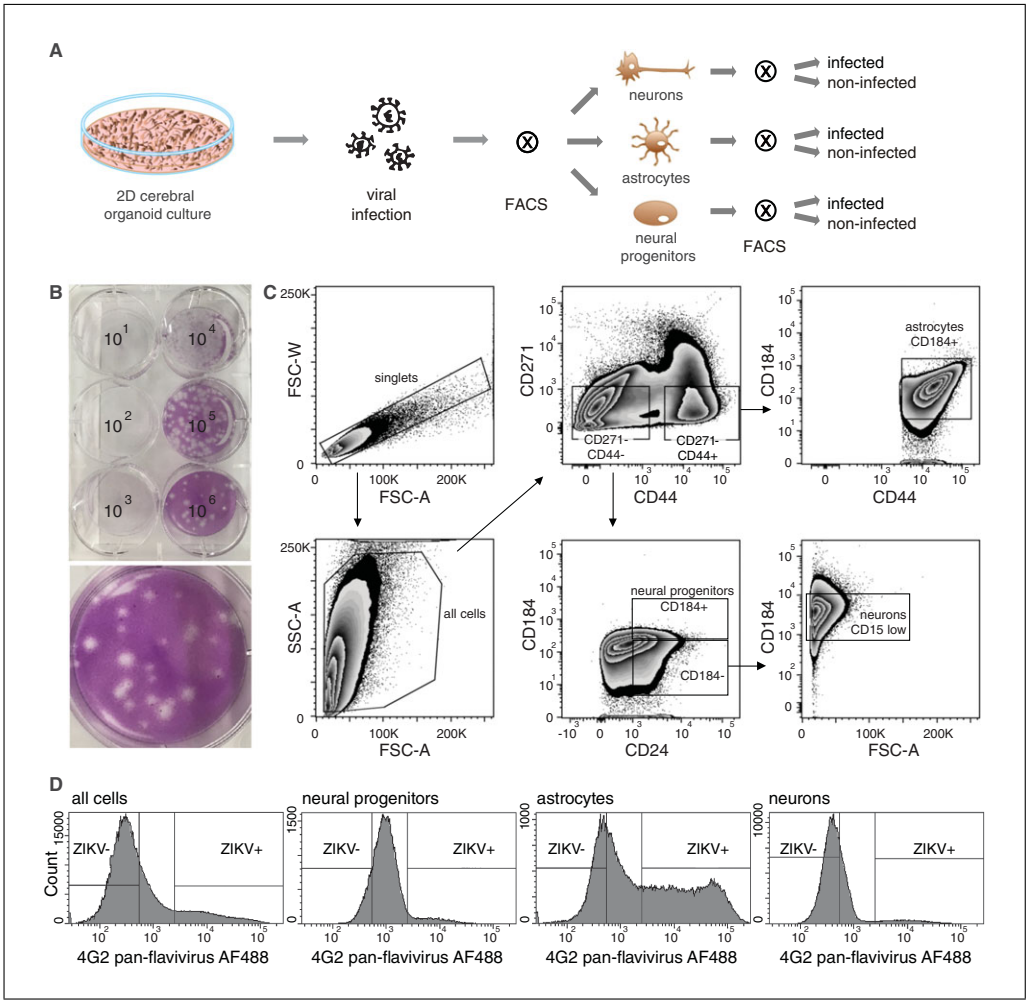

Figure 2 Virus infection and fluorescence-assisted cell sorting. (**A**) Schematic overview of virus infection and sorting of cerebral organoid cultures. (**B**) Example of a plaque assay readout. Vero cells were infected with 10-fold serial dilutions as indicated. (**C**) Examples of cell sorting plots for isolating astrocytes, neurons, and neural progenitor cells from hESC-derived cerebral organoid cultures. (**D**) Illustration of ZIKV$^-$ and ZIKV$^+$ FACS gating for all cells and sorted neural progenitors, astrocytes, and neurons.

(env-defective HIV) and pseudotyped with the VSV-G envelope is described below (see Alternate Protocol). The env-defective HIV virus was generated by cloning the CMV EGFP expression cassette into the ENV position of the virus, while preserving the remaining full length with intact gag/pol and accessory proteins. This insertion restricts the virus to a single round of infection, since HIV virus particles defective for the envelope gene are generated.

Materials

VERO cells (ATCC CCL-81)
Vero cell culture medium (see recipe)
Zika virus strain, e.g., Uganda 1947 (MR 766, ATCC 1838) or Puerto Rico 2015 (PRVABC59, ATCC VR-1843)
Vero cell infection medium (see recipe)
2× overlay medium (see recipe)
1% (w/v) DEAE dextran (MP Biomedicals, cat. no. 195133) in distilled water (sterilize by filtration)
5% (w/v) sodium bicarbonate (Fisher Scientific, cat. no. L-14639) in distilled water
2% Oxoid agar stock solution (see recipe)

4% paraformaldehyde (PFA) in phosphate-buffered saline (Boston BioProducts, cat. no. BM-155)
Dulbecco's phosphate-buffered saline (DPBS, no calcium or magnesium, Invitrogen, cat. no. 14190144)
1% (w/v) crystal violet (Fisher Scientific, cat. no. C581-100) in distilled water
Methanol
2D cerebral organoid culture of 7 days (see Basic Protocol 1)
Cerebral organoid medium (see recipe)
Penicillin/streptomycin (Sigma, cat. no. P4333)
Fetal bovine serum (FBS, HyClone, cat. no. SH30071.03)
EDTA solution (Invitrogen, cat. no. 15575-038)
PermWash buffer (BD Biosciences, cat. no. 554723)
Accutase (Innovative Cell Technologies, cat. no. AT104)
DMEM/F12 (Gibco, cat. no. 11330-032)
Fluorochrome-conjugated antibodies (see Table 1)

T175 culture flasks
2-ml cryogenic vials
6-well flat-bottom cell culture plates
Phase-contrast microscope
50-ml polypropylene centrifuge tubes
Cell scrapers (*optional*)
1.5-ml microcentrifuge tubes

Additional reagents and solutions for trypsinizing cells

Prepare Zika virus supernatant

1. Grow Vero cells in Vero cell culture medium to ~80% confluence. Dissociate with EDTA/trypsin and seed 7.5×10^6 cells in a T175 culture flask.

   Cell supernatant from uninfected cells can be prepared in parallel as a mock infection control.

2. After 16–24 hr, remove medium from the flask and replace with 1 multiplicity of infection (MOI) of virus prepared in fresh Vero cell infection medium. Return to the 37°C, 5% CO_2 incubator.

   Multiplicity of infection (MOI) = plaque forming units (pfu) of virus used for infection/number of cells. The Zika virus titer is determined by plaque assay on Vero cells as described below.

   Vero cell infection medium contains lower FBS levels to prevent reduced infection efficiency from serum-induced antiviral activity.

3. After 72 hr of infection, collect the medium and centrifuge 10 min at $400 \times g$ to remove cellular debris.

4. Keep the supernatant and store as ~1-ml aliquots in 2-ml cryogenic vials at −80°C (can be stored for years).

Measure Zika virus titer by plaque assay

5. Seed Vero cells at 1×10^5 cells per well in a 2-ml volume using a 6-well flat-bottom plate. Seed six wells per sample to allow for serial dilution of viral supernatant.

6. After 24 hr, verify if Vero cells have formed confluent monolayers without any gaps.

   If necessary, cells can be cultured up to another 24 hr to form confluent monolayers.

7. Prepare 10-fold serial dilutions of viral culture supernatant (step 4) in Vero cell infection medium ranging from $10\times$ to $10^6\times$.

8. Remove culture medium from the Vero cells and add 500 µl of each dilution from step 7, moving from highest ($10^6\times$) to lowest ($10\times$). Return plates to the incubator for 1 hr, keeping the cells covered with virus by tilting the plates in different directions every 15 min.

9. During incubation, prepare fresh agar overlay. Microwave 2% Oxoid agar stock solution until completely liquid, then combine the following:

   8.5 ml distilled water
   25 ml of $2\times$ overlay medium
   0.5 ml 1% DEAE dextran
   1 ml 5% sodium bicarbonate
   15 ml of 2% Oxoid agar

   Let the mixture cool until it can be handheld.

10. After 1 hr of infection, remove the virus-containing medium from cells and apply 2 ml agar overlay per well. Allow to solidify for 5 min at room temperature before moving the plates to the incubator.

11. At 48 hr post-inoculation, count viral foci using a phase-contrast microscope. Calculate the viral titer as pfu per ml.

12. Fix cells by applying 4 ml of 4% PFA on the agar overlay and incubating for 15 min at room temperature.

13. During fixation, prepare fresh crystal violet staining solution as follows:

    40 ml 1% crystal violet solution
    80 ml 100% methanol
    380 ml distilled water

    Staining will require 2 ml/well.

14. Wash cells/overlay once with 5 ml DPBS, then remove the agar overlay by flicking the plate upside down.

15. Stain cells for 5 min with 2 ml per well fresh crystal violet staining solution for clear visualization of plaques.

    Figure 2B shows an example of a plaque assay after crystal violet staining.

Infect cerebral organoid cultures

16. Approximately 1 hr before infection, remove half of the medium from cerebral organoid cultures using a 1-ml pipet and replace with cerebral organoid medium supplemented with penicillin/streptomycin (1:50 dilution). Return to the 37°C incubator.

17. Thaw the appropriate amount of virus and control supernatant on ice. Using a 20-µl pipet, drip 5–20 µl virus or control supernatant (equaling the desired MOI) into the appropriate wells in a cross pattern to enhance spread throughout the whole well.

    In order not to harm the axonal and dendritic cell connections, it is recommended not to shake the tissue culture plates to spread the virus.

18. Return the culture to the 37°C incubator for 72 hr.

Table 1 Antibody Conjugates for Flow Cytometry and Fluorescence-Activated Cell Sorting

Antibody	Conjugate	Supplier (cat. no.)	Dilution
Anti-flavivirus group E-antigen 4G2	AF488	Millipore (MAB10216); conjugated with Invitrogen antibody labeling kit (A20181)	1:200
Anti-human CD15	V450	BD Biosciences (561584)	1:100
Anti-human CD24	BUV395	BD Biosciences (563818)	1:100
Anti-human CD44	PERCP-Cy5.5	BD Biosciences (560531)	1:100
Anti-human CD184	APC	BD Biosciences (555976)	1:50
Anti-human CD271	PE	BD Biosciences (557196)	1:50

Collect cells and immunostain for FACS

19. Prepare fresh FACS buffer: 1% FBS and 2 mM EDTA in DPBS.
20. Dilute 10× BD Perm/Wash buffer in distilled H_2O to make a 1× solution.
21. Gently aspirate all medium from the culture with a pipet and add 0.5 ml Accutase per well. Incubate for 5 min at 37°C.
22. Add 1 ml DMEM/F12 per well and pipet up and down using a 1-ml pipet to dislodge the cells.

    When cells do not easily dislodge, using a cell scraper can facilitate collection of the cells.

23. Transfer cells to 50-ml tubes containing 3 ml DMEM/F12 medium for every well that is collected. Wash each well one time with 1 ml DMEM/F12 to collect any remaining cells.
24. Centrifuge at 450 × *g* for 10 min at room temperature. Remove supernatant and gently resuspend cells in 5 ml FACS buffer.

    Washing volumes are anticipated on cell pellets derived from approximately two to four plates.

25. Centrifuge at 450 × *g* for 5 min at room temperature. Remove supernatant and gently resuspend cells in 1 ml FACS buffer. Transfer to 1.5-ml microcentrifuge tubes.
26. Prepare 200 µl per sample of cell surface marker antibody mixture by adding the appropriate concentrations of fluorophore-conjugated antibodies for CD15, CD24, CD44, CD184, and CD271 to FACS buffer.

    See Table 1 for antibody conjugates and dilutions used for flow cytometry and FACS.

27. Centrifuge cells at 450 × *g* for 5 min at room temperature. Remove supernatant, gently resuspend cells in 200 µl antibody mixture, and incubate 45 min in the dark at room temperature.

    Since fluorophores are light sensitive, exposure of samples to light should be avoided from this step on.

28. Wash cells by adding 500 µl FACS buffer, centrifuging at 450 × *g* for 5 min at room temperature, removing the supernatant, and gently resuspending the cells in 500 µl FACS buffer.
29. Centrifuge at 450 × *g* for 5 min at room temperature, remove supernatant, and gently resuspend cells in 2% PFA (1:1 dilution of 4% PFA in DPBS). Incubate 5 min in the dark at room temperature.

30. Centrifuge at 450 × *g* for 5 min at room temperature, remove supernatant, and gently resuspend cells in 500 µl PermWash buffer. Repeat.

31. Centrifuge at 450 × *g* for 5 min at room temperature, remove supernatant, and gently resuspend cells in 200 µl PermWash buffer supplemented with antibody against ZIKV (fluorophore-conjugated anti-E-antigen, 4G2, Table 1). Incubate 1 hr in the dark at room temperature.

32. Centrifuge at 450 × *g* for 5 min at room temperature, remove supernatant, and gently wash cells with 500 µl PermWash buffer. Repeat at least once.

33. Centrifuge at 450 × *g* for 5 min at room temperature, remove supernatant, and gently resuspend cells in 1 ml FACS buffer. Place samples on ice and shield from light for transportation to the flow cytometer.

FLUORESCENCE-ASSISTED CELL SORTING

BASIC PROTOCOL 3

This protocol describes how to set the compensation parameters for multicolor FACS and set the appropriate gates to separate the cerebral organoid–derived cells by cell type (neurons, neural progenitors, and astrocytes) and infection based on their fluorescent labeling.

Materials

Fluorophore-conjugated antibodies (see Table 1)
Ultracomp eBeads for compensation (Thermo Fisher Scientific cat. no. 01-2222-42)
Dulbecco's phosphate-buffered saline (DPBS, no calcium or magnesium, Invitrogen, cat. no. 14190144)
FACS buffer: 1% FBS and 2 mM EDTA in DPBS, freshly prepared
Stained and infected cells and appropriate controls for sorting (see Basic Protocol 2)
Lysis buffer for RNA or DNA extraction (*optional*)

Fluorescence-assisted cell sorter and software (FACSARIA III with FACSDiva software, BD Biosciences)
Falcon test tubes with cell strainer snap caps (Corning, cat. no. 08-771-23)
15-ml Falcon tubes

Prepare compensation beads and determine sorter settings

1. Make single-stain controls for every antibody used. Vortex compensation beads for 5 min before use. To 1 drop of compensation beads from the dispenser bottle, add the same amount of conjugated antibody (or less) as used for staining one cell sample. For dilutions of conjugated antibodies, see Table 1.

   Binding of antibodies to compensation beads does not rely on the antibody's specificity or affinity, and therefore less antibody than required for staining one cell sample can be used. This is especially recommended when background staining of the negative bead population is too high. According to the manufacturer, 0.125 mg or less of staining antibody can be used with the compensation beads if 0.03-1 mg of antibody is used to stain one cell sample.

2. Incubate 15 min in the dark at 4°C.

3. Wash beads once by centrifuging at 600 × *g* for 5 min, discarding the supernatant, and resuspending in 400 µl fresh FACS buffer.

4. Use unstained cells and compensation beads to determine optimal settings for forward and side scatter, as well as for fluorescence detectors by adjusting the voltages for the photomultiplier tubes (PMTs) until you see beads and cells.

5. Analyze all single-stain controls to ensure that the positive peak for every antibody-fluorophore is on scale. Change PMT voltage, if necessary. Try to have a maximum spread between the positive and negative peaks for every control.

6. Once optimal PMT voltages for all channels are determined, run all single-stain controls again and record all data for compensation setup. Do not change PMT voltages anymore at this point.

   If you need to change PMT voltages later, you will have to rerun all single-stain controls again with the adjusted PMT voltages.

7. Calculate the compensation matrix using the software provided with the flow cytometer (FACSDiva).

Prepare samples, set gates, and sort samples

8. Filter samples by pipetting the cell solution on top of the cell strainer caps on the test tubes. Add 0.5–1 ml DPBS on top of the strainer to ensure all single cells go through the strainer.

9. Remove the cap and place the test tube into the tube holder of the sorter. Sort infected from non-infected cells based on detection of flavivirus E protein. Mock and infected cells are used to set the gates for positive and negative cells. In order to avoid false-negative cells as much as possible, move the negative gate far to the left. Sorting efficiency should be 90% or higher. Collect virus-positive and virus-negative cells in 15-ml Falcon tubes.

   Illustration of infection and non-infection gates is shown in Figure 2D.

   Cells can be sorted first for cell type or infection depending on personal preference. If it is necessary to concentrate the cells after the first sorting round, centrifuge the samples at 450 × g and resuspend in FACS buffer.

10. Sort the different cell types (astrocytes, neural progenitor cells, neurons) from virus-positive and virus-negative cells using the gating scheme below and in Figure 2C.

    a. Apply gates for CD271 and CD44 first.
    b. Gate $CD271^-$ $CD44^+$ cells for CD184+ to obtain cells with the astrocyte marker signature.
    c. Gate $CD271^-$ $CD44^-$ cells for CD184, CD24, and CD15 to separate neural progenitor cells ($CD271^-$ $CD44^-$ $CD184^+$ $CD24^+$) and neurons ($CD271^-$ $CD44^-$ $CD184^-$ $CD24^+$ $CD15^{low}$).

11. Collect cells in three 5-ml FACS tubes with sorting efficiency of 90% or higher. Transfer cells to 15-ml Falcon tubes for further downstream applications.

Process samples for downstream applications

12. Centrifuge sorted cells at 450 × *g* for 10 min at 4°C. Keep on ice and remove supernatant without disturbing the pellet.

13. Directly add lysis buffer to the pellet for downstream DNA or RNA extraction or store cells at –80°C until further use.

    Examples of Zika virus infection rates per cell type are shown in Figure 3A.

ALTERNATE PROTOCOL

SORTING OF PSEUDOTYPED HIV-INFECTED CEREBRAL ORGANOID CULTURES

To illustrate adaptation of the protocol to any virus that infects human neuronal cells, this protocol describes flow cytometry after infection with a single-cycle HIV lentiviral

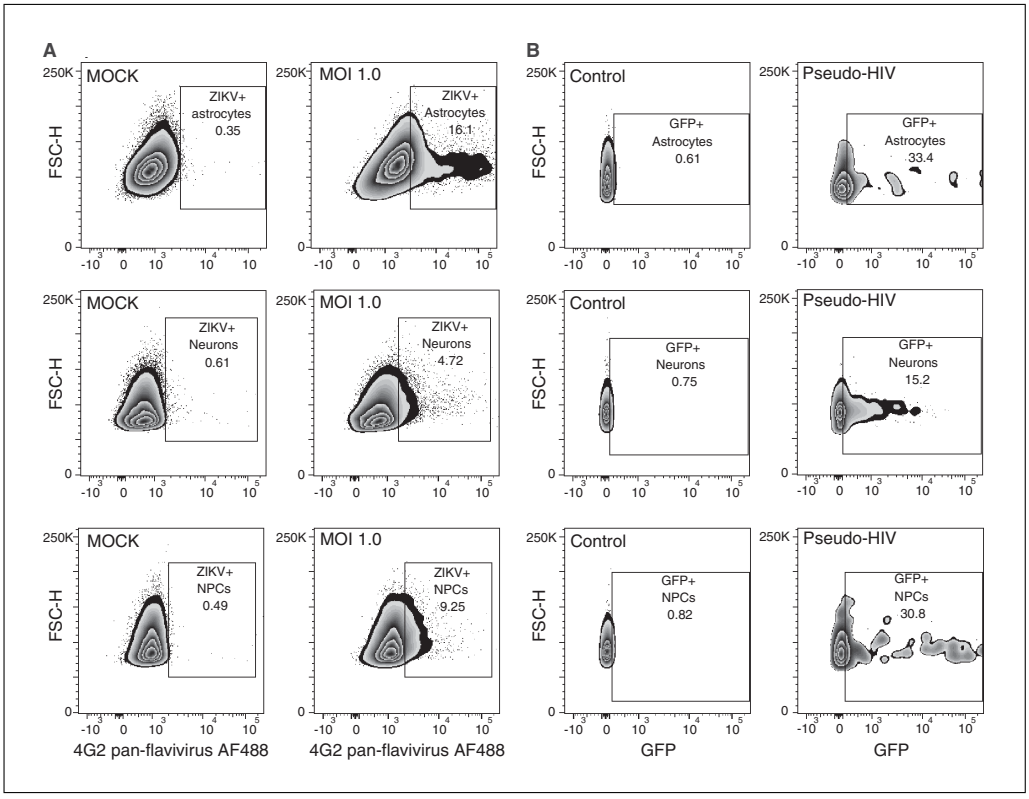

Figure 3 Examples of FACS gating and percentages of virus-infected cells among isolated astrocytes, neurons, and neural progenitor cells (NPCs). (**A**) Cerebral organoid cultures infected with ZIKV. (**B**) Cerebral organoid cultures infected with pseudotyped HIV.

reporter virus. HIV1 lacking a functional Env gene and expressing a GFP reporter gene under the control of a CMV promoter was pseudotyped with the vesicular stomatitis virus (VSV-G) envelope glycoprotein to mediate cell entry but limit replication to a single round. The flow cytometry plots in Figure 3B show expression of the CMV promoter–driven GFP reporter gene in neural progenitors, neurons, and astrocytes, indicating that lentiviral integration is supported in these cell types.

CAUTION: Appropriate biosafety precautions including standard operating procedures for work with lentiviruses need to be implemented prior to work with lentiviruses. The described experiments were conducted in a BSL2+ facility by trained personnel.

Materials

HEK293T cells (ATCC CRL-3216)
HEK293T cell culture medium (see recipe)
Plasmids: molecular clone HIV R7/3 Δenv CMV-EGFP, pHCMV-G coding for the G glycoprotein of VSV-G (Lloyd, Ng, Muesing, Simon, & Mulder, 2007)
Polyethylenimine (PEI 25K, Polysciences, cat.no. 23966)

T75 culture flask
Sterile disposable 0.45-μm filter units
2-ml cryogenic vials

Produce single-cycle lentiviral reporter virus

1. Seed 5×10^6 HEK293T in 15 ml HEK293T cell culture medium in a T75 culture flask and culture overnight.

2. The next day, transfect cells overnight with 20 μg HIV R7/3 Δenv CMV-EGFP (replication defective) and 3 μg pHCMV-G using 3 μg/ml polyethylenimine.

3. In the morning, replace cell medium with HEK293T cell culture medium.
4. On days 2 and 3 after transfection, collect culture supernatant and centrifuge at 450 × *g* for 5 min to remove cellular debris.
5. Combine supernatants and filter through a 0.45-μm filter. Store as ∼1-ml aliquots in 2-ml cryogenic vials at −80°C (can be stored for years).

Infectivity titers of the viral stocks can be determined by infecting TZM-bl reporter cells (RRID:CVCL_B478) with serial dilutions in triplicate.

Infect cells, prepare samples, and perform sorting

6. Infect cells with HIV R7/3 Δenv CMV-EGFP culture supernatant and proceed through fixation with PFA as described above (see Basic Protocol 2, see steps 16–29).

Since the HIV reporter virus expresses GFP, which is detectable with the same settings as for AF488 or FITC fluorophores, there is no need to permeabilize the cells to allow for intracellular viral protein staining.

7. Centrifuge at 450 × *g* for 5 min at room temperature, remove supernatant, and gently resuspend cells in 1 ml FACS buffer. Repeat twice for a total of three washes.
8. Place samples on ice and shield from light for transportation to the flow cytometer.
9. Sort cells using the same flow cytometer settings and gating strategy described in Basic Protocol 3. Use uninfected cells to determine the background and setting the gate for virus-positive cells.

REAGENTS AND SOLUTIONS

Cerebral organoid medium

125 ml DMEM/F12 (Gibco, cat. no. 11330-032)
125 ml Neurobasal medium (Gibco, cat. no. 21103049)
1.25 ml N-2 supplement (Gibco, cat. no. 17502001)
62.5 μl Insulin solution (Sigma, cat. no. I9278)
2.5 ml GlutaMAX supplement (Gibco, cat. no. 35050061)
1.25 ml MEM-NEAA (Gibco, cat. no. 11140050)
2.5 ml B27 supplement (Gibco, cat. no. 17504044)

After combining above ingredients, prepare a 1:100 dilution of 2-mercaptoethanol (Sigma, cat. no. M3148) in DMEM/F12 and add 87.5 μl of this to the medium. Sterilize by filtration and store up to 2 weeks at 4°C. Equilibrate to room temperature before use.

Recipe according to Lancaster & Knoblich (2014).

HEK293T cell culture medium

Dulbecco′s modified Eagle′s medium (DMEM, Corning, cat. no. 10-013-CV)
10% fetal bovine serum (FBS, HyClone, cat. no. SH30071.03)
1:100 penicillin/streptomycin (Sigma, cat. no. P4333)
Sterilize by filtration
Store up to 4 weeks at 4°C
Equilibrate to room temperature before use

Overlay medium, 2×

200 ml 10× MEM (Gibco, cat. no.21430020)
20 ml 200 mM L-glutamine (Corning, cat. no. 25-005-CR)
48 ml 5% (w/v) sodium bicarbonate (Fisher Scientific, cat. no. L-14639)

20 ml 1 M HEPES (Fisher Scientific, cat. no. BP310-500)
20 ml penicillin/streptomycin (Sigma, cat. no. P4333)
20 ml fetal bovine serum (HyClone, cat. no. SH30071.03)
672 ml distilled H_2O
Sterilize by filtration
Store up to 4 weeks at 4°C

Oxoid agar stock solution, 2% (w/v)

Prepare a 2% (w/v) solution of Oxoid agar (Oxoid, cat. no. LP0028) in distilled water. Sterilize by autoclaving and store up to 4 weeks at room temperature.

The agar overlay will become solid when cooled to room temperature and needs to be reheated before use.

Vero cell culture medium

Dulbecco′s modified Eagle′s medium (DMEM, Corning, cat. no. 10-013-CV)
10% fetal bovine serum (FBS, HyClone, cat. no. SH30071.03)
1:100 penicillin/streptomycin (Sigma, cat. no. P4333)
Sterilize by filtration
Store up to 4 weeks at 4°C
Equilibrate to room temperature before use

Vero cell infection medium

Dulbecco′s modified Eagle′s medium (DMEM, Corning, cat. no. 10-013-CV)
2% fetal bovine serum (FBS, HyClone, cat. no. SH30071.03)
1:100 penicillin/streptomycin (Sigma, cat. no. P4333)
Sterilize by filtration
Store up to 4 weeks at 4°C
Equilibrate to room temperature before use

COMMENTARY

Background Information

The human brain is unique in its structure and functionality compared to other mammals. To circumvent the limitations of animal models, ample effort has been made to establish human cell–based models to study brain development, function, and disorders. Preceded by many protocols describing the generation of neural rosettes, neural progenitors, and different types of mature neuronal cells, the most comprehensive model resembling the human brain to date is the human stem cell–derived cerebral organoid model, which generates three-dimensional brain-like tissues *in vitro* that mimic not only the diversity of neuronal cell types but also the structure and cellular interactions typical of human brain tissue. The fact that cell development is similar to that in the *in vivo* environment, allowing establishment of heterotypical neuronal interactions, distinguishes this model from the direct differentiation of human stem cells into one specific neural cell type.

Challenges in studying these more complex multicellular tissues include potentially higher variability and increased complexity of data analyses. To facilitate the analysis of defined cell types in complex brain organoids upon virus infection, we developed a FACS-based protocol. FACS has been proven useful for sorting neuronal cells from adult rat brains and for distinguishing cell populations during neural induction of human embryonic stem cells (Yuan et al., 2011). Choosing FACS over microscopy-based cell identification not only reduces output quantification errors, but also expands choices for downstream analyses of the identified cells. In this protocol, we use six-color cell sorting, which leaves the option to include more colors if the FACS/flow cytometer laser and filter setup allow for it. For example, inclusion of a viability dye to separate live from dead cells could reduce noise and improve separation of cell populations. However, one should be cautious about interference of the dead cell reagents with further downstream processing. The protocol we describe includes cell fixation either to allow intracellular staining of viral proteins and/or as a safety measure. Although not tested by

Table 2 Troubleshooting Guide for Common Problems with FACS of Virus-Infected Cerebral Organoid Cultures

Problem	Possible cause	Solutions
Basic Protocol 1		
Low reattachment and/or survival after dissociation	Too long incubation with dissociation agent	Use shorter incubation time
	Dissociation of organoids into single cells; cells are replated too sparsely	Reserve bigger cell clumps; increase seeding density
	Cells damaged during centrifugation	Use lower centrifugation speed
Basic Protocol 2		
Excessive cell death upon infection	Too high concentration of virus; contamination of virus with toxic agents	Check virus stock concentration; harvest virus before massive cell death occurs or grow virus in insect cells to reduce toxins during virus production
Low infection efficiency	Too low concentration of virus; unhealthy cerebral organoid culture	Check virus stock concentration; check cell culture for mycoplasma or other "hidden" contaminants
Basic Protocol 3		
No events displayed in FSC-SSC scatter plot	Voltages of PMTs need to be adjusted depending on cell size and granularity	Adjust voltages for FSC and SSC PMT
No or very few cells after sorting	Too short centrifugation time of cells diluted in high volumes	Increase centrifugation time appropriately when working with high volumes
	Clogging of FACS system	Carefully monitor system during sorting for any anomalies
Low sorting efficiency	Cells are too concentrated and/or flow settings are too high for efficient sorting	Reduce flow speed and/or dilute cell sample

us, a modified version of this protocol without fixation, if staining of intracellular markers is not required, is anticipated to allow live cell sorting.

Critical Parameters and Troubleshooting

The protocol includes three critical stages: preparing organoid cultures for optimal infection, obtaining well-prepared virus stocks, and sorting of immunostained cell samples. Due to the advanced-level sorting with at least six markers, guidance or assistance from a FACS specialist might be required for users who do not have advanced FACS skills. A guide to common problems with possible causes and solutions is summarized in Table 2.

Anticipated Results

In a typical experiment, starting from ∼15 organoids seeded in four 6-well plates, with an overall infection efficiency of 15%, one can expect to obtain 20,000 to 200,000 cells per group. For downstream applications, we have successfully extracted DNA suitable for bisulfite sequencing, or RNA suitable for whole transcriptome amplification and subsequent qRT-PCR from samples as low as ∼4000 cells (Janssens et al., 2018).

Time Considerations

The protocol starts from mature organoids, which will take up to 12 weeks to develop. Growing a virus stock and performing plaque assays usually takes 5 days. Preparing the infected organoid culture will take 10 days. The last day of the protocol, which includes cell

collection, immunostaining, and FACS, should be anticipated to be labor intensive and may take up to 10 hr. It is therefore recommended to split some of the tasks between two people to lower the work load and reduce the time spent between collection and final freezing of the cells.

Acknowledgments

We acknowledge Christopher Bare and the flow cytometry and microscopy cores at the Icahn School of Medicine at Mount Sinai for assistance. We thank Dr. Ana Fernandez-Sesma, Dr. Rafael Fenutria, Dr. Vinod Balasubramaniam, and Ms. Megan M. Schwarz for sharing conjugated anti-flavivirus group E-antigen 4G2 antibody and virus. This work was supported by the Huffington Foundation (T.P.Z.), NIH grants (NIH/NIAID U19AI118610 and R21AI129486 to A. G.-S. and R21 AI125236 to V. S.), and the Belgian American Educational Foundation (fellowships to S. J. and M. S.). The authors declare no competing financial interests.

Literature Cited

Camp, J. G., Badsha, F., Florio, M., Kanton, S., Gerber, T., Wilsch-Bräuninger, M., ... Treutlein, B. (2015). Human cerebral organoids recapitulate gene expression programs of fetal neocortex development. *Proceedings of the National Academy of Sciences of the United States of America*, *112*(51), 15672–15677. doi: 10.1073/pnas.1520760112.

Choi, S. H., Kim, Y. H., Hebisch, M., Sliwinski, C., Lee, S., D'Avanzo, C., ... Kim, D. Y. (2014). A three-dimensional human neural cell culture model of Alzheimer's disease. *Nature*, *515*(7526), 274–278. doi: 10.1038/nature13800.

Garcez, P. P., Loiola, E. C., Madeiro da Costa, R., Higa, L. M., Trindade, P., Delvecchio, R., ... Rehen, S. K. (2016). Zika virus impairs growth in human neurospheres and brain organoids. *Science*, *352*(6287), 816–818. doi: 10.1126/science.aaf6116.

Janssens, S., Schotsaert, M., Karnik, R., Balasubramaniam, V., Dejosez, M., Meissner, A., ... Zwaka, T. P. (2018). Zika virus alters DNA methylation of neural genes in an organoid model of the developing human brain. *mSystems*, *3*(1), e00219-17. doi: 10.1128/mSystems.00219-17.

Lancaster, M. A., & Knoblich, J. A. (2014). Generation of cerebral organoids from human pluripotent stem cells. *Nature Protocols*, *9*(10), 2329–2340. doi: 10.1038/nprot.2014.158.

Lancaster, M. A., Renner, M., Martin, C.-A., Wenzel, D., Bicknell, L. S., Hurles, M. E., ... Knoblich, J. A. (2013). Cerebral organoids model human brain development and microcephaly. *Nature*, *501*(7467), 373–379. doi: 10.1038/nature12517.

Lloyd, A. G., Ng, Y. S., Muesing, M. A., Simon, V., & Mulder, L. C. F. (2007). Characterization of HIV-1 integrase N-terminal mutant viruses. *Virology*, *360*(1), 129–135. doi: 10.1016/j.virol.2006.10.007.

Mariani, J., Coppola, G., Zhang, P., Abyzov, A., Provini, L., Tomasini, L., ... Vaccarino, F. M. (2015). FOXG1-dependent dysregulation of GABA/glutamate neuron differentiation in autism spectrum disorders. *Cell*, *162*(2), 375–390. doi: 10.1016/j.cell.2015.06.034.

Qian, X., Nguyen, H. N., Song, M. M., Hadiono, C., Ogden, S. C., Hammack, C., ... Ming, G.-L. (2016). Brain-region-specific organoids using mini-bioreactors for modeling ZIKV exposure. *Cell*, *165*(5), 1238–1254. doi: 10.1016/j.cell.2016.04.032.

Yuan, S. H., Martin, J., Elia, J., Flippin, J., Paramban, R. I., Hefferan, M. P., ... Carson, C. T. (2011). Cell-surface marker signatures for the isolation of neural stem cells, glia and neurons derived from human pluripotent stem cells. *PLoS One*, *6*(3), e17540. doi: 10.1371/journal.pone.0017540.