Brain Organoids as a model to study Zika virus and SARS-CoV-2 infections

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Abstract

In recent years, we are living through different viral pandemics that result in neurological impairments. Given the human-specific nature of brain development, physiology, and pathology, it is imperative to use human models to investigate the neurological impact of viral infections, such as Zika virus and SARS-CoV-2. Brain organoids are powerful *in vitro* platforms for the analysis of the effects of viral infections on brain development and function, with prospective application to new emerging viral threats. Using brain organoids, it was possible to show that Zika virus infects neural stem cells, disrupting the cell cycle and neurogenesis, leading to microcephaly, a severe reduction of the brain. On the other hand, while it is still under investigation how SARS-CoV-2 might enter and alter the brain, organoid studies are helping to characterize its neurotropism and potential mechanisms of neurovirulence. Here we describe a method for the infection of human brain organoid cultures with Zika and Sars-CoV-2 viruses that can be used to study neurodevelopmental phenotypes, alteration in neuronal functionality, host-pathogen interactions as well as for drug testing.

Key Words:

Brain, COVID-19, development, human, infection, in vitro, iPSC, microcephaly, Organoids, SARS-CoV-2, ZIKV, Zika virus.

1. Introduction

Viral infections have a major impact in the central nervous system development and function[1]. During embryonic development, viruses such as Rubella, Cytomegalovirus and Zika virus, together with other STORCH pathogens, are now well known to be transmitted from mother to foetus, crossing the placental barrier, and resulting in brain malformations such as microcephaly. In practice, microcephaly is defined as a

rare clinical sign, a severe reduction of the brain caused by genetic abnormalities or teratogens[2]. Microcephalic patients have the head circumference measurements significantly below the mean (2-3 standard deviation) of individuals with same sex and age[3]. In research, as access to human embryonic tissue is extremely difficult, human brain organoids are considered an excellent model to study this malformation as it recapitulates the early human corticogenesis stages[4]. It has been shown that brain organoids can be used to model microcephaly caused by distinct viruses[5,6].

In addition, the recent coronavirus pandemics highlighted the use of brain organoids to investigate the possible impacts of SARS-CoV2 in the central nervous system[7–10]. It is well-accepted that the COVID-19 virus, once thought to trigger a severe acute respiratory syndrome (SARS), induces a multi-organ dysfunction, including the nervous system[11]. In this context, approximately one third of the COVID-19 patients developed neurological symptoms[12]. Here we will summarize the tested methods and step-by-step protocols employed to study viral infection using brain organoids[5,6]. Specifically, we will describe protocols of Zika virus and SARS-CoV-2 as examples of viral infections that can be studied using brain organoids models.

Materials

1.1 Brain organoid preparation:

Equipment

- Aspirator
- Class II biological safety cabinet
- CO2 incubator equipped with an orbital shaker in a BSL2 or BSL3 facility (depending on biosafety regulations)
- Light Microscope
- Matrigel Matrix
- Neubauer chamber
- Pipet controller and serological pipettes
- Pipettes, Multichannel pipette, and tips (p1000, p200, p10)
- Small scissors
- Tweezers

- Water bath (37ºC)

Plasticware

- 1,5 ml tubes
- 10 cm petri dishes
- 15 ml and 50ml conical tubes
- 6-well and 12-well culture plates
- Parafilm
- Reagent reservoir

Reagents/Cells

- 70% ethanol spray bottle
- Accutase
- Dulbecco's Phosphate Buffered Saline (D-PBS) without Ca2+ and Mg2+
- Human pluripotent stem cell (hPSC) culture
- ROCK inhibitor, Y-27632
- STEMdiff™ Cerebral Organoid Kit STEMCELL Technologies
- Trypan blue

1.2 Viral preparation

Equipment

- Aspirator
- Autoclave
- Centrifuges and rotors: a low-speed and a highspeed centrifuge rotor
- Class II or Class III biological safety cabinet (depending on viral regulations)
- CO2 incubator
- Microscope
- Neubauer chamber
- Pipet controller and serological pipettes
- Pipettes, Multichannel pipette, and tips (p1000, p200, p10).
- Thermoblock (58ºC)

- Water bath (37°C)

Plasticware

- 1,5 ml tubes
- 15 ml and 50ml conical tubes
- 6-well, 24-well and 96-well plates
- Parafilm
- Reagent reservoir
- T75 flasks

Reagents/Cells

- 0.5% Crystal violet
- 10% neutral-buffered formalin (Sigma-Aldrich, catalogue number: HT501128)
- 3% Carboxymethylcellulose
- Alpha-MEM
- Cell maintenance and Infection medium
- Clone C6/36 cells (ATCC CRL 1660)
- Infection medium
- Leibovitz's L15 medium (Thermo Fisher, catalogue number: 11415056)
- MEM Non-essential amino acids solution 100 x – (Thermo Fisher, catalog number: 11140076)
- sodium bicarbonate 7.5% solution (Thermo Fisher, catalog number: 25080102)
- Trypsin 0.25%-EDTA
- Tryptose broth solution (Sigma-Aldrich, catalogue number: T8159)
- Vero E6 cells (ATCC® CRL -1586™)
- Virus stock (See Note 1)
- Mock medium (See Note 2)

2. Methods

Infection experiments in organoids are typically done by making a few adjustments to organoids grown in regular conditions. We briefly describe the generation of brain organoids according to the Lancaster protocol; more details of this method can be found in several papers[13–16]. Here, we add the adjustments necessary for performing infection experiments, including the modification of: plastic ware (e.g. culturing organoids in individual plates, using multiwell plates for infections), media volumes to ensure the same volume/organoid ratio across different conditions, matrigel removal - depending on the organoid stage, working on a BSL2/3 containment (e.g. biosafety rules, ethical approval, organoid shaker needed in the BSl2/3, imaging station for organoid documentation). Next, we will explain how to prepare the viral stocks and exposure to inoculum, how to calculate the ratio of viral particles per cell, and the assays used for viral detection.

2.1 Brain organoid culture

The protocol used for the brain organoid culture was originally developed and published by Lancaster et al., 2013[4] and adapted by Garcez et al., 2016[6] and Krenn et al., 2021[5] to infect brain organoid cultures. All liquids used should be sterile and equilibrated at room temperature before use.

Day 0 - Embryoid bodies preparation (suspension):

a) Prepare the EB medium and seeding medium. Mix 40 ml basal medium 1 to 10 ml supplement A. Mix 70 µl of 5 mM ROCK inhibitor to 7 ml of EB Formation Medium (final concentration 50 µM).

b) Cultivate human pluripotent stem cells until it reaches a semi-confluent state. Aspirate the regions where the cells are differentiating and aspirate the medium, wash with PBS.

c) Add 0.5 ml of accutase and incubate the plate at 37°C for 5 minutes to detach the cells.

d) Resuspend cells 5-10 times with a 1ml pipette and add the cell suspension to a 15 ml sterile tube.

e) Centrifuge the cells at 300 x g for 5 minutes and discard the supernatant

f) Add 1 ml of EB seeding medium and resuspend cells 5-10 times with a 1 ml pipette and count the cells. You can use trypan blue to exclude the dead cells and count the live cells on a Neubauer camera.

g) Calculate 9,000 cells/well for one embryoid body preparation. Adjust the volume of cell suspension to seed the cells in a 96-well plate. Final volume is 150µl per well and plates should be incubated at 37°C, 5% CO2. See Note 3.

h) After 3 days, change the media using the EB medium (without the ROCK inhibitor). Aspirate the medium and add 150µl of ED medium per well.

Day 6 - Neural induction (suspension):

a) Prepare the neural induction medium and warm to room temperature (15 - 25°C). Mix 49.5 ml basal medium 1 to 0.5 ml of supplement.

b) Prepare a 96-well plate with 0.2 ml of neural induction medium per well.

c) Add 1 EB per well using a wide bore p200 tip.

d) Incubate the plate at 37°C, 5% CO2

e) Change media daily.

Day 11 – Expansion (Matrigel):

a) Sterilize with 70% ethanol a piece of parafilm, place it on a petri dish.

b) Prepare the Matrigel as following the manufacturer's instructions (calculate 15µl per EB).

c) Prepare expansion medium by adding 0.25 ml supplement C and 0,5 ml supplement D to 24.25 ml of basal medium 2.

d) Transfer the EB with a wide bore 200 µl pipette to the Matrigel surface at the parafilm. Gently remove the medium. Coat each EB with 5µl of Matrigel.

e) Incubate the plate at 37°C, 5% CO2 for 30 minutes.

f) After the Matrigel is polymerized, use expansion medium to flush the EB into a well of 6-well plate, final volume of 3 ml per well or 10 cm dishes, final volume of 12 ml per well.

g) Incubate the plate at 37°C, 5% CO2 for 72 hours.

Day 15 – Differentiation (suspension):

a) Prepare maturation medium by adding 1 ml of Supplement E in 49 ml of basal medium 2.

b) Gently remove the medium from the wells.

c) Add 3 ml of maturation medium per well in a 6-well plate. Alternatively, use 10 cm dishes, with final volume of 12 ml per well.

d) Use an orbital shaker in a 37°C, 5% CO2 incubator.

e) Change media every four days.

2.2 Brain organoid infection

To select a brain organoid age to perform the viral infection experiments, it is recommended to take in consideration that each organoid stage will display specific cell types and processes that are summarized in Table 1 (see Note 4). In this protocol, we will perform infections at day 45, a stage that allows for targeting radial glia cells (Figure 1).

For a 45 days old brain organoid, consider 10^4 PFU as approximately an MOI (multiplicity of infection) of 1, which would be one viral particle per cell. However, it is important to notice that with a MOI=1 not all cells will be infected after 24 hours. See Note 6 for information about the MOI range applied for infectious disease modelling using brain organoids. For a more accurate MOI calculation, you can dissociate a regular size organoid and count the number of cells. To calculate the amount of virus needed to perform an infection, calculate the number of cells multiplied by the desired MOI and you will know the number of PFU (Plaque Forming Units) needed. Calculate the volume of virus for each well: divide the PFU needed by the titer of your viral stock. Dilute this volume in 1 ml of infection media per well.

Formula: Volume of virus (ml) = (number of cells x desired MOI)/titer of virus stock.

For example: To infect 10e5 cells at an MOI of 0.1 with a stock of virus with a titer of 1 x 10e6 PFU/ml: vol (ml)= (10e5 x 0.1)/ (1x 10e6) = 0.01 ml.

a) On day 45, remove Matrigel if needed (see Note 7)

b) Randomly allocate the organoids to the “infected” and “mock” control wells (see Note 8).

c) Transfer the work to a BSL2/3 facility. (see Note 5)
d) Prepare the infection medium thawing the viral stock on ice. Use 1mll per well of a 6-well plate.
e) Remove the medium from the wells.
f) Add the infection medium. It should be an infection film, only 1 ml per well of a 6-well plate.
g) Incubate the plate at 37°C, 5% CO2 for 1 hour. (See Note 14)
h) Discard the infection medium in an inactivation disposal that contains hypochlorite solution 20%.
i) Replace with maturation media (3ml per well).
j) Incubate the plate at 37°C, 5% CO2 for 3-20 days, depending on the analysis (see Note 9).
k) Monitor and document organoid growth, morphology, and appearance of debris in the culture medium using a light microscope (Figure 2).
l) Collect the samples for viral detection.

2.3 Viral detection

Multiple methods could be employed to detect viruses.

Plaque assay is the most elegant biological assay to evaluate infectivity. This assay aims to quantify infectious virus particles in a sample. Plaque assay is based on the cytopathic effect, specifically the cell death induced by viral infection. Confluent monolayers of susceptible and permissive cells are exposed to serial dilutions of the sample and overlaid with semisolid medium. Semisolid media restrict virus dispersion in the wells and consequently the viral progeny released from an infected cell target neighbouring/surrounding cell. This ultimately leads to the formation of an area of dead cells (plaques) because of the infection of a single cell with a single virus particle. Plaques are visualized as clear holes or areas in the stained monolayer. Serial dilutions yield discernible plaques.

Another method used to detect viruses is the RT-qPCR, applied to quantify viral RNA. Supernatants of infected cultures can be collected and stored at -80 to detect viral RNA (Figure 3). Immunocytochemistry can also be utilized to label viral proteins or components and co-localized with the host cells. Multiple antibodies can be used to

detect ZIKV. A commonly used is a pan flavivirus antibody that targets the envelope protein, also known as 4G2. It is not specific for ZIKV, as it also detects other flavivirus. Another non-specific antibody is the J2 from Scicons. It targets double-stranded RNA, which detects RNA viruses (double-stranded RNA viruses or replication intermediate forms of single strand RNA viruses. Figure 4 shows examples of Zika detection in infected organoids using two different antibodies raised against Zika Envelope protein (mouse monoclonal and rabbit polyclonal).

2.4 Plaque assay

Prepare the following solutions:

3% Carboxymethylcellulose

- 6 g carboxymethylcellulose
- 200 ml distilled water
- Sterilize by autoclaving at 121°C for 15 min
- Store at room temperature

0.5% Crystal violet

- 0.5 g crystal violet
- 20 ml absolute ethanol
- 80 ml distilled water
- Filter sterilize
- Store at room temperature protected from light

Cell maintenance medium

- 500 ml DMEM High Glucose with L-glutamine
- 50 ml FBS
- Store at 4°C

Day 1: Cell preparation

a) Seed Vero cells at 1 x 10^5 cells/well in 12-well tissue culture plates.
b) Incubate cells for 48 hours.

Day 3: Sample titration

a) Check the cell monolayer on the microscope. On the day of sample titration, the monolayer should be confluent.
b) Thaw the virus on ice.
c) Prepare tenfold serial dilutions (10^{-1} to 10^{-6}) of the sample in serum-free medium in 1.5 ml tubes. For a 10^{-1} dilution, dilute 50 ul of the original sample in 450 ul serum-free medium. For posterior dilutions, dilute 50 ul of the previous dilutions in 450 ul serum-free medium. Discard tips in between dilutions to avoid virus carryover. To escalate the number of samples, a deep-well plate can be used.
d) Add 200 ul inoculum of each dilution into duplicate wells
e) Incubate the plates for 1 hour. Rock the plate every 15 min to assure an evenly distribution of the virus inoculum in the cell monolayer and prevent cells from drying.
f) Discard the inoculum and add 1 ml of the semisolid medium.
g) Incubate at 37 C with 5% CO_2 for 3 days (for SARS-CoV-2) and 5 days (for ZIKV). During the incubation period, the plate should not be disturbed, and incubation periods vary for different viruses and virus strains.

Day 8: Fixation and plaque visualization

a) Add 1 ml/well of 10% formalin.
b) Incubate at room temperature for 30 min.
c) Discard the medium.
d) Rinse the plate with tap water
e) Add 0.5 ml crystal violet dye solution.
f) Incubate for 5 minutes at room temperature.
g) Wash the plate with tap water to remove excess stain.
h) Count the plaques in each well where individual plaques are discernible (20-100 PFU). See Note 15.
i) Convert the result into plaque-forming units (PFU/ml).

Formula: virus titer of the sample = (average number of plaques per well x dilution factor)/volume of the inoculum (in ml)

Figure 5 summarizes the plaque assay protocol and Figure 6 are examples of real plaque plates. See Mendoza et al., 2020[24] for alternative SARS-CoV-2 plaque assays.

2.5 Viral propagation

Viruses are propagated in susceptible and permissive cells. ZIKV is an arthropod-borne flavivirus transmitted between *Aedes* mosquitoes and primates. For viral propagation, ZIKV can be either inoculated in C6/36, an *Aedes albopictus* line, or in Vero cells, a mammalian cell line. SARS-CoV-2 is propagated in Vero cells. For propagation, low MOI (0.0001 to 0.1) is used to avoid the production of defective viral particles. Multiple rounds of infection yield high virus titers. Cytopathic effects of viral infection might negatively impact virus titers and therefore, virus harvest must proceed soon after its appearance. In this chapter, we describe the protocol for ZIKV propagation in C6/36 cells at an MOI of 0.1. C6/36 cells are grown in their appropriate medium in a B.O.D. incubator at 28 C in the absence of CO_2.

Prepare the following solutions:

C6/36 medium:

- 500 ml Leibovitz's L15 medium (Thermo Fisher, catalogue number: 11415056)
- 50 Tryptose broth solution (Sigma-Aldrich, catalogue number: T8159)
- 5 ml L-glutamine 200 mM (Thermo Fisher, catalogue number: 250030081)
- 5 ml MEM Non-essential amino acids solution 100 x – (Thermo Fisher, catalogue number: 11140076)
- 5 ml sodium bicarbonate 7.5% solution (Thermo Fisher, catalogue number: 25080102)

Day 1:

a) Seed cells at 6 x 10^6 cells/75 cm^2. The number of T75 flasks depends on the volume needed. Use at least one flask for mock (to mock-infect control cells), one flask for ZIKV infected, and one extra flask to establish the amount of virus required on the day of infection.

Day 2 - Viral infection

a) Use the extra flask to count cells and to determine the amount of virus required to infect the cells at the desired MOI. 50-80% cell confluence is desired on the day of infection

To count the cells:

a) Discard the culture medium from the flask.

b) Rinse the cells with PBS

c) Add 2 ml 0.25% trypsin solution to detach cells

d) Resuspend cells in 4 ml C6/36 medium supplemented with 5% FBS.

e) Count cells in a Neubauer chamber

To calculate the amount of virus to a MOI = 0.1

a) Calculate the amount of virus inoculum using the formula: Volume of virus inoculum (ml) = [(number of cells/flask) x MOI]/titer of virus stock

E.g.: To infect a flask with 7.5 x 10^6 cells with ZIKV at a MOI=0.1 using a virus stock with a titer of 5 x 10^7 PFU/ml

Volume of virus inoculum (ml) = (7.5 x 10^6 x 0.1)/(5 x 10^5) = 0.015 ml

b) To prepare the inoculum, thaw a vial of ZIKV stock and collect the volume calculated previously in a sterile tube.

c) Complete the virus inoculum with serum-free C6/36 medium to 3.5 ml for each T75 flask.

ZIKV infection:

a) Add virus inoculum to each flask. Use at least one flask for uninfected cells. In this flask, add 3.5 ml serum-free C6/36 medium.
b) Incubate flasks at 28°C for 1 hour. Gently rock each flask every 15 min to prevent cells from drying and to spread the virus evenly.
c) Discard virus inoculum
d) Add 10 ml of C6/36 medium L15 supplemented with 2% FBS. The low concentration of foetal bovine serum increases the efficiency of infection (See Note 12).

e) Incubate at 28°C for 5 days. Check cells every day under the microscope to verify the cytopathic effect (CPE).

Day 7: Harvest conditioned medium

a) Collect the conditioned medium.
b) Centrifuge the conditioned medium at 300 x g for 10 min.
c) Filter through a 0.22 μm filter to remove cellular debris.
d) Aliquot and store at -80. (See Note 13)

3. Notes

1. Virus stock should have a concentration above 10e6 PFU.
2. Mock stock medium is the supernatant from mock-infected cells, using same parameters as described in section 2.5, viral propagation protocol.
3. The number of seeded cells can range from 6000 to 20000 per well and we suggest to empirically test the ideal number for each PSC line you are using. For example, if EBs generated with 9000 cells appear small and with a lot of debris, increase the number of cells.

4. Cell type compositions and processes described in Table 1 refer to studies mostly using the cerebral organoid protocol based on Lancaster et al. 2013[4]. However, protocols modifications may impact organoid development, so it is important to empirically validate the presence of cell type and process of interest before setting up the infections.
5. It is important to check with biosafety officers the requirements for manipulating each virus. SARS-CoV-2, for instance, is a biological safety level 3 (BSL-3) pathogen, so all the practices should be carried on BSL-3 facilities, using the advised personal protective equipment to mitigate contamination risks.
6. MOI range most used is between 0.1-10, depending on the desired number of days post infection. High MOI is recommended for short infection experiments to ensure that the target cells will be infected.
7. Removal of matrigel (or part of it) can be done manually using sterile tips. This step will facilitate the organoid manipulation and dissociation for RT-qPCR analysis.
8. For infections of multiple organoids, we recommend using 4-6 organoids per well. Alternatively, it is possible to infect individual organoids placed in smaller wells (12-wells).
9. The timepoints can vary depending on the aim of the experiment. For study of neurotropism, early timepoint (1-4 days) should be used, while for studies of the neurodevelopmental impact, infections can be protracted for 20-24 days.
10. Organoid countering can be done using ImageJ either using the freehand selection tool or by creating a mask via thresholding. area measurement can be done with "Measure" function in Image J.
11. Other methods to assess growth/size is by calculating the organoid volume using light sheet microscopy as described in Li et al. 2017[25] or counting the number of cells by FACS. Both methods require fixation of the infected material to inactivate the virus.
12. In general, for adsorption purposes serum-free medium is used to infect cells but for viruses that use TAM receptors (TYRO, AXL and MER), an adapter protein that is present in serum is required for virus-receptor binding. This is the case for ZIKV - so, the infection medium supplemented with 2% FBS.
13. Virus stocks should be maintained at low passage number. After thawing a vial of virus, discard the vial. Do not freeze and store the vial. Virus transport must be

performed in dry ice in an appropriate container and according to biosafety regulations. For each virus stock, use 3-5 vials to perform plaque assay.

14. The infection period recommended allows virus adsorption to the cells, i.e., virus binding to the cells. It is recommended that medium volume should be minimal to enhance the chance of virus-cell interaction, but enough to make sure that all the cells in the well (or another recipient) will be covered in liquid and will not dry out.

15. In plaque assay, plaque overlap can lead to an underestimation of viral titers. The wells that are chosen for counting plaque number should not overlap. Figure 6 depict examples of real plaque plates.

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Brain organoid age	Target cell	Target process	References
1-3 weeks	Neuroepithelial cells	Polarity establishment Neuroepithelial proliferation	Kadoshima et 2013[17]; Krenn et al. 2021[5]
1-2 months	Radial glia Intermediate Progenitors Early-born neurons Interneurons Microglia	Radial glia proliferation Neurogenesis Interneuron specification Interneuron migration	Lancaster et al. 2013[4]; Kadoshima et al. 2013[17]; Qian et al. 2016[14]; Renner et al. 2017[18]; Bagley et 2017[19]; Ornel et al., 2018[20]
3-4 months	Outer radial glia Late-born neurons Choroid Plexus	oRG specification Interneuron migration	Renner et al.2017[18]; Bershteyn et al. 2017[21]; Bajaj et al. 2021[22]; Watanabe et al., 2017[23]
5-6 months	Astrocytes	Gliogenesis Spontaneous Network activities	Qian et al. 2016[14]; Watanabe et al., 2017[23]

Table 1: A summary of the main cellular events that unfold during brain organoid development.

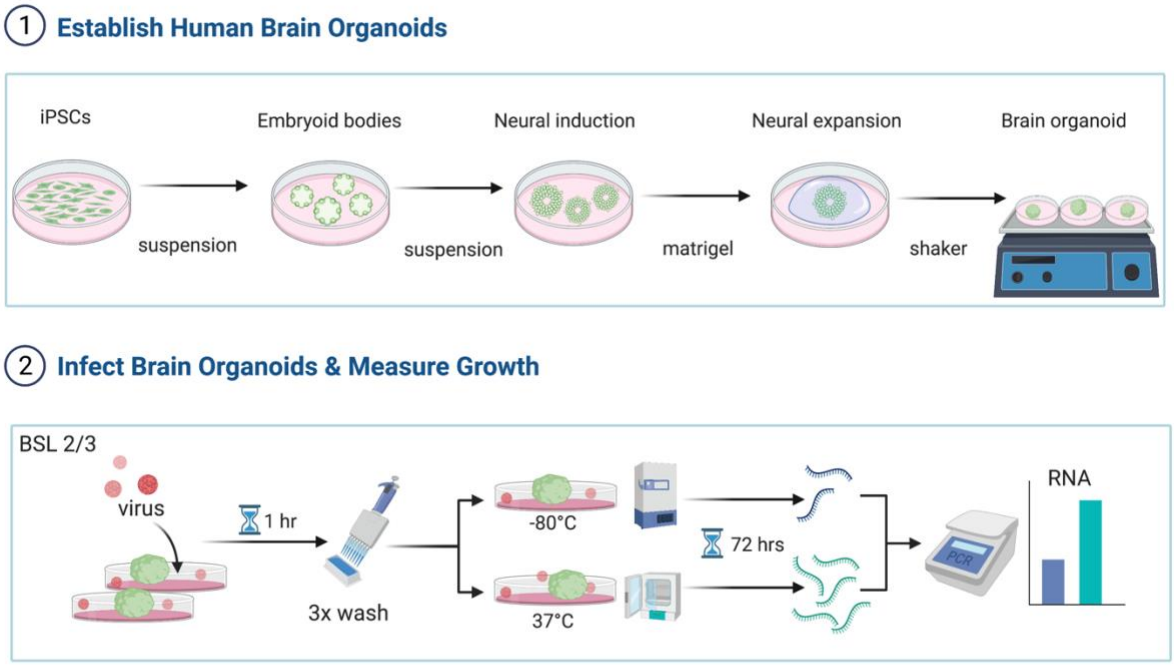

Figure 1: Schematic representation of brain organoids establishment and infection.

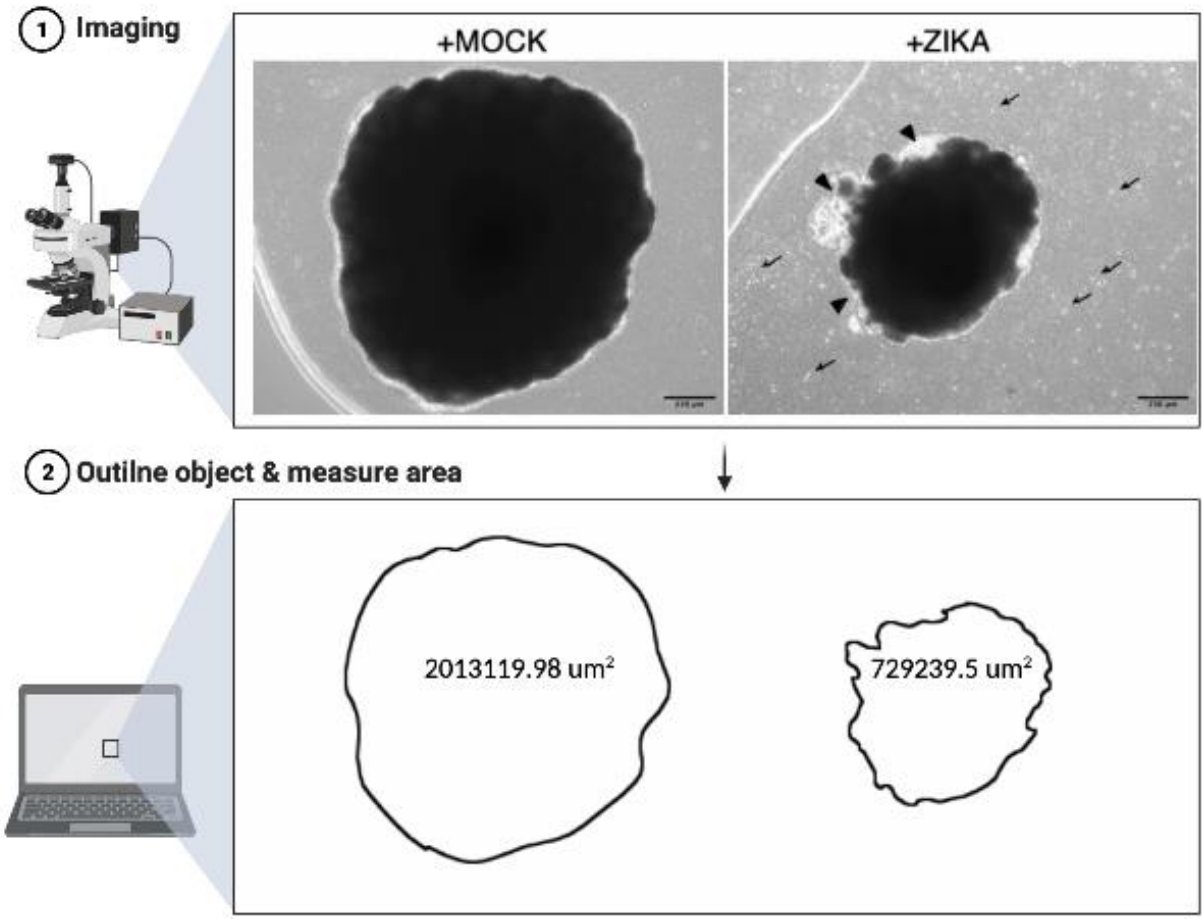

Figure 2: Monitoring organoid size. 1. Example of organoids imaged using a cell culture microscope in the BSL2 room 12 days after exposure to MOCK or Zika virus. Note the appearance of irregularities on the organoid surface (arrowheads) and debris in the medium (arrows). 2. Example of organoid area reduction revealed by the area measurement of the object derived from organoid contouring (See Note 10 and 11).

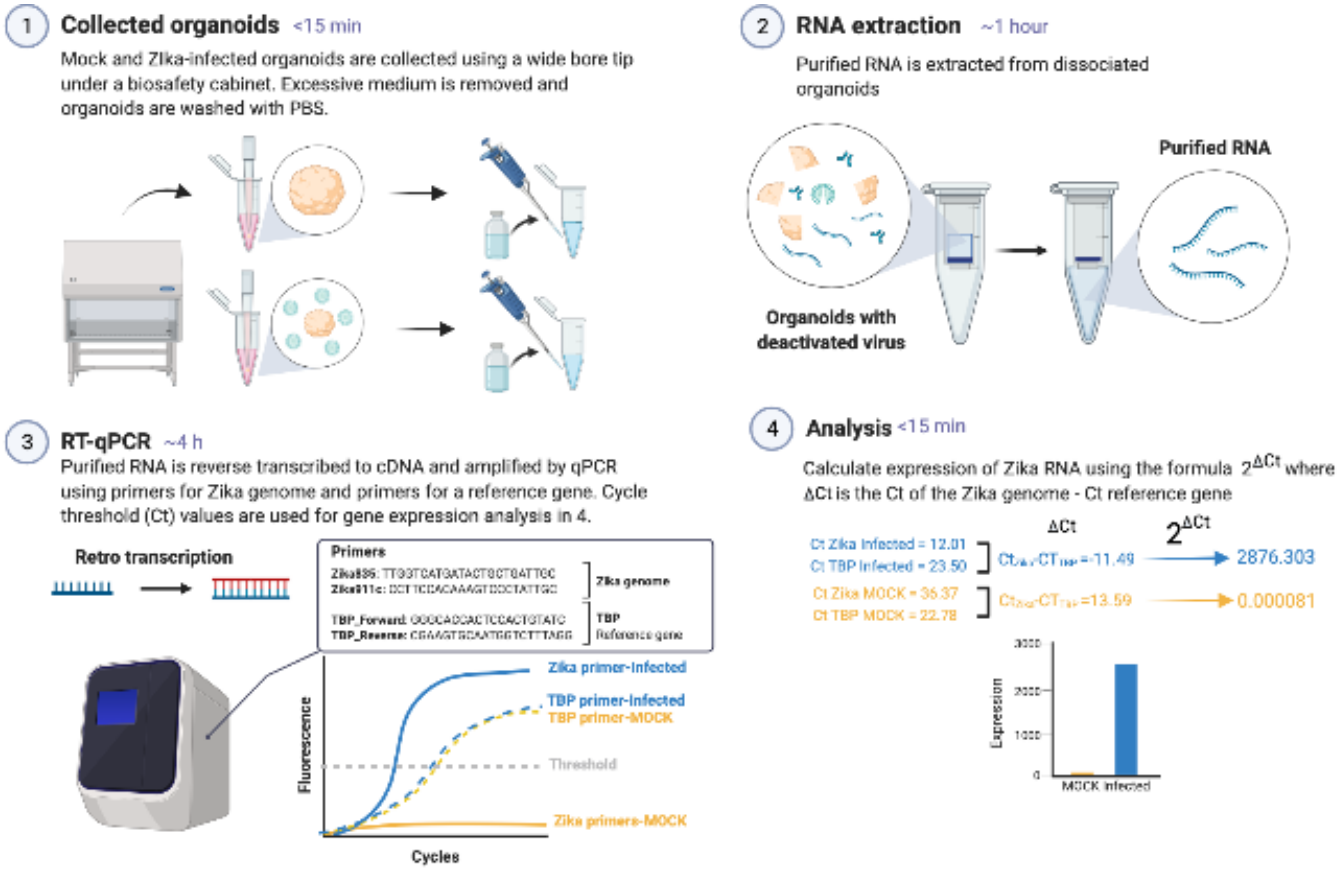

Figure 3: Viral detection through RT-PCR.

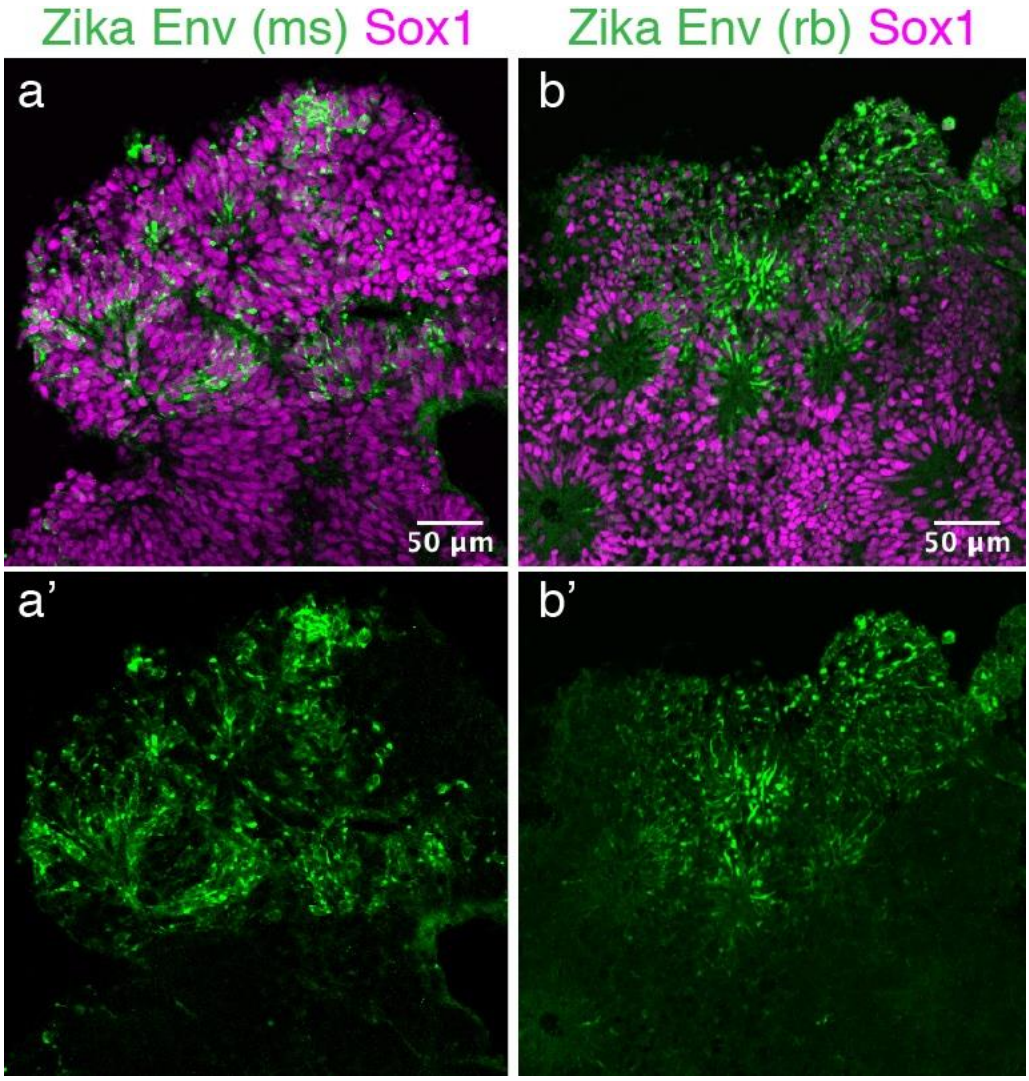

Figure 4: Zika detection using Immunocytochemistry in infected organoids. In green, antibodies raised against Zika Envelope protein, the mouse monoclonal Millipore clone D1 MAB10216-I(A) and the Rabbit polyclonal GeneTex GTX133314 (B). Sox1 is used to label neural stem cells.

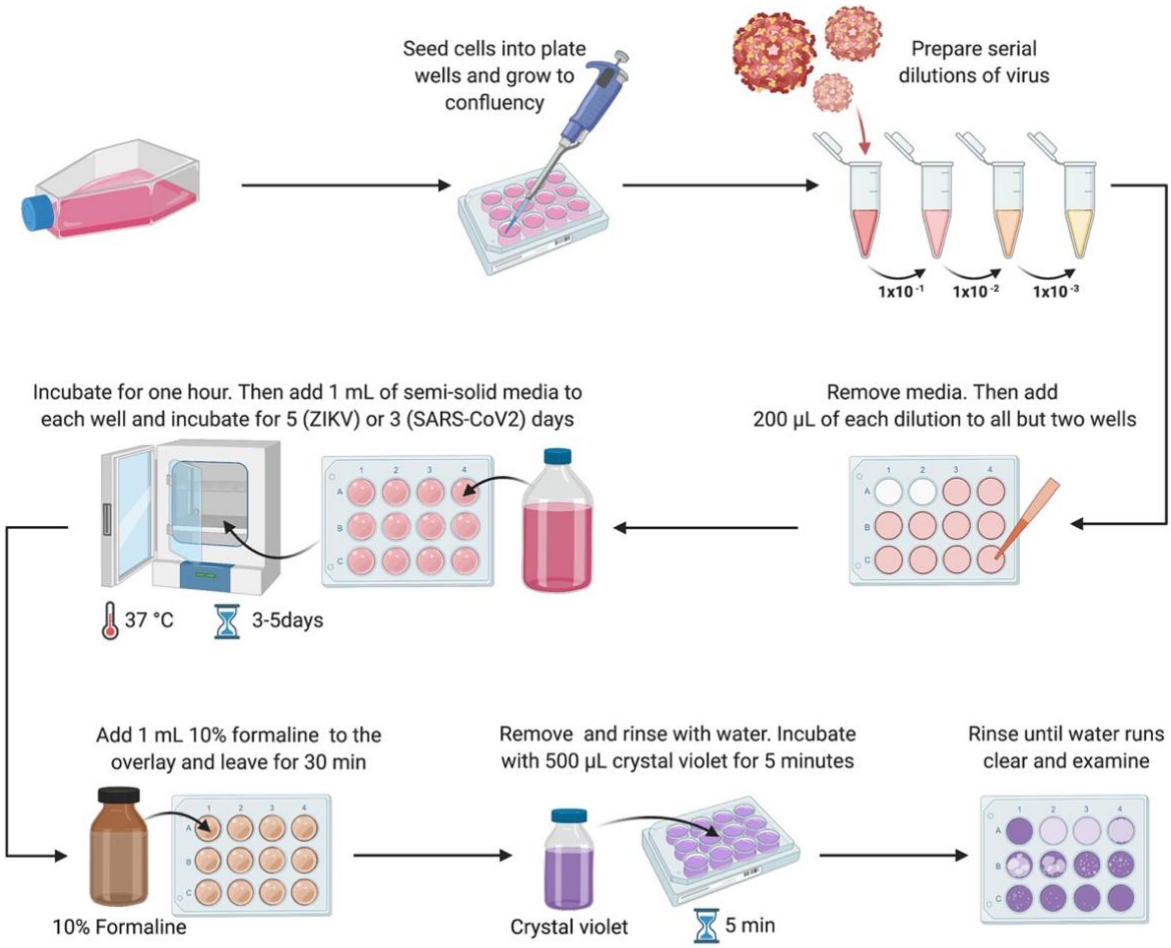

Figure 5: Summary of plaque assay protocol.

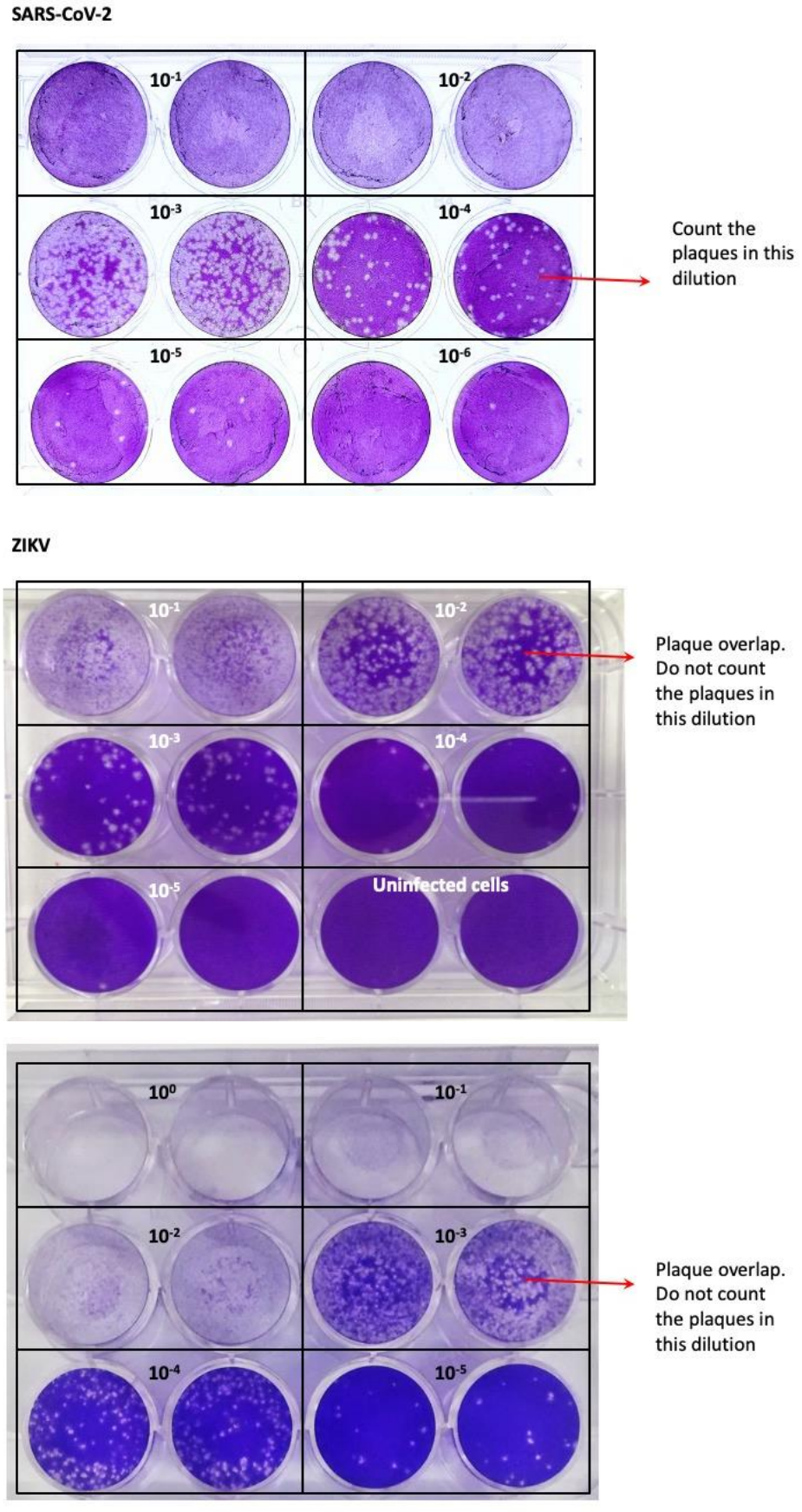

Figure 6: Examples of plaque assay plates infected with ZIKV and SARS-CoV2.