Journal of Visualized Experiments

Adapting Gastrointestinal Organoids for Pathogen Infection and Single Cell Sequencing under Biosafety Level 3 (BSL-3) Conditions

--Manuscript Draft--

Article Type:	Invited Methods Collection - JoVE Produced Video
Manuscript Number:	JoVE62857R3
Full Title:	Adapting Gastrointestinal Organoids for Pathogen Infection and Single Cell Sequencing under Biosafety Level 3 (BSL-3) Conditions
Corresponding Author:	Megan Stanifer Heidelberg University Hospital Heidelberg, GERMANY
Corresponding Author's Institution:	Heidelberg University Hospital
Corresponding Author E-Mail:	m.stanifer@dkfz.de
Order of Authors:	Megan Stanifer
	Steeve Boulant
Additional Information:	
Question	**Response**
Please specify the section of the submitted manuscript.	Immunology and Infection
Please indicate whether this article will be Standard Access or Open Access.	Open Access ($3900)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Heidelberg, Germany
Please confirm that you have read and agree to the terms and conditions of the author license agreement that applies below:	I agree to the Author License Agreement
Please provide any comments to the journal here.	
Please confirm that you have read and agree to the terms and conditions of the video release that applies below:	I agree to the Video Release

TITLE:

Adapting Gastrointestinal Organoids for Pathogen Infection and Single Cell Sequencing under Biosafety Level 3 (BSL-3) Conditions

AUTHORS AND AFFILIATIONS:

Megan L. Stanifer[1,2*], Steeve Boulant[2,3,4*]

[1]Department of Infectious Diseases, Molecular Virology, Heidelberg University Hospital, 69120 Heidelberg, Germany.
[2]Current address, Department of Molecular Genetics and Microbiology, College of Medicine, University of Florida, Gainesville, Florida.
[3]Department of Infectious Diseases, Virology, Heidelberg University Hospital, 69120 Heidelberg, Germany.
[4] Research Group "Cellular Polarity and Viral Infection", German Cancer Research Center (DKFZ), 69120 Heidelberg, Germany.

Email addresses of co-authors:
Megan L. Stanifer (megan.stanifer@med.uni-heidelberg.de, m.stanifer@ufl.edu)
Steeve Boulant (steeve.boulant@med.uni-heidelberg.de, s. boulant@ufl.edu)

*Corresponding authors:
Megan L. Stanifer (megan.stanifer@med.uni-heidelberg.de, m.stanifer@ufl.edu)
Steeve Boulant (steeve.boulant@med.uni-heidelberg.de, s.boulant@ufl.edu)

KEYWORDS:

human intestinal organoids, single-cell RNA sequencing, biosafety level 3, organoid infection, enteric pathogens, apical and basolateral infection

SUMMARY:

This protocol describes how to infect human intestinal organoids from either their apical or basolateral side to characterize host/pathogen interactions at the single-cell level using single-cell RNA sequencing (scRNAseq) technology.

ABSTRACT:

Human intestinal organoids constitute the best cellular model to study pathogen infections of the gastrointestinal tract. These organoids can be derived from all sections of the GI tract (gastric, jejunum, duodenum, ileum, colon, rectum) and, upon differentiation, contain most of the cell types that are naturally found in each individual section. For example, intestinal organoids contain nutrient-absorbing enterocytes, secretory cells (Goblet, Paneth, and enteroendocrine), stem cells, as well as all lineage-specific differentiation intermediates (e.g., early or immature cell types). The greatest advantage in using gastrointestinal tract-derived organoids to study infectious diseases is the possibility of precisely identifying which cell type is targeted by the enteric pathogen and to address whether the different sections of the gastrointestinal tract and their specific cell types similarly respond to pathogen challenges. Over

the past years, gastrointestinal models, as well as organoids from other tissues, have been employed to study viral tropism and the mechanisms of pathogenesis. However, utilizing all the advantages of using organoids when employing highly pathogenic viruses represents a technical challenge and requires strict biosafety considerations. Additionally, as organoids are often grown in three dimensions, the basolateral side of the cells is facing the outside of the organoid while their apical side is facing the inside (lumen) of the organoids. This organization poses a challenge for enteric pathogens as many enteric infections initiate from the apical/luminal side of the cells following ingestion. The following manuscript will provide a comprehensive protocol to prepare human intestinal organoids for infection with enteric pathogens by considering the infection side (apical vs. basolateral) to perform single-cell RNA sequencing to characterize cell-type-specific host/pathogen interactions. This method details the preparation of the organoids as well as the considerations needed to perform this work under biosafety level 3 (BSL-3) containment conditions.

INTRODUCTION:

Studying the cell type-specific tropism and cell type-specific immune response to human enteric viruses has been historically challenging due to the lack of primary human cellular models. This limitation has now been partially eradicated with the development of organoids[1]. In the case of the gastrointestinal tract, gastric and intestinal organoid models have been developed for humans and several other species (e.g., murine, bovine, feline, bat)[2–6]. Intestinal organoids reproduce the structural architecture of the human intestinal epithelium and contain crypt and villi-like structures, functional intestinal lineages and have even been used to identify previously unknown cell lineages. Two different approaches can be used to grow intestinal organoids. First, intestinal stems cells containing crypts are isolated from tissue resections or biopsies and grown under specific culture conditions (e.g., Wnt3A, R-spondin, Noggin, and EGF) to expand, and then differentiate the stem cells to most intestinal cell lineages (e.g., enterocytes, Paneth cells, Goblet cells, enteroendocrine cells)[7]. This method allows for the isolation of organoids from all sections of the gastrointestinal tract (e.g., stomach, duodenum, jejunum, ileum, and colon). The second method relies on human-induced pluripotent or embryonic stem cells, which are then differentiated in a stepwise process into intestinal epithelial cells[8]. These induced stem-cell-based organoids are often described as being more embryonic in nature as compared to patient-derived organoids. While all these organoid models have been critical to unraveling developmental cues needed to form the intestinal tract, their use in infectious disease research is still in its infancy.

Enteric virus is a broad term covering all viruses, which infect through the gastrointestinal tract, such as picornaviruses (e.g., EV-71), reoviruses (e.g., rotavirus), and caliciviruses (e.g., norovirus)[9]. Enteric viruses initiate their infectious life cycle through the ingestion of contaminated food and water, which leaves people in developing countries at high risk due to the discharge of untreated waste into the environment and lack of medical care after the onset of infection[10]. Depending on the type of pathogen, the infection can lead to gastroenteritis, vomiting, and/or watery diarrhea due to leakage of the intestinal lining. Human noroviruses are a highly prevalent and highly infectious enteric pathogen, that lead to over 600 million infections and 15 million hospitalizations worldwide[11]. Organoids have been key to norovirus

research as they support the infection and replication of human norovirus, which was previously unable to be cultured in standard cell culture models[12].

Over the past two decades, coronaviruses have emerged as key human pathogens[13]. This family includes the highly pathogenic MERS, SARS-CoV-1, and SARS-CoV-2, which require strict safety level containments when performing research on these viruses. Interestingly, while all three of these pathogens are mostly recognized for the induced respiratory symptoms and distress, it is now evident that these viruses do not solely infect the respiratory tract but also other organs. An important pathology induced in SARS-CoV-2 infected patients besides the respiratory distress is the presence of gastrointestinal symptoms[14]. A fraction of SARS-CoV-2 infected patients displays such symptoms, ranging from very mild to severe diarrhea. Additionally, SARS-CoV-2 genomes can be detected in stool and gastrointestinal tract biopsies of infected patients[15]. Importantly the presence of gastrointestinal symptoms is not limited to SARS-CoV-2 as they were also observed in MERS and SARS-CoV-1 infected patients. To understand how SARS-CoV-2 induces gastrointestinal distress and precisely identify the tropism of SARS-CoV-2 in the gastrointestinal tract, human intestinal organoids have been a key tool and are now exploited to unravel cell type-specific responses to this pathogen[16,17].

Transcriptional profiling of a cell population (bulk RNA sequencing) has been standard practice when evaluating pathogen infections of both immortalized cell lines as well as with organoids. While this allows us to determine global changes in response to pathogens (e.g., upregulation of cytokines), bulk RNAseq does not allow us to determine why specific cells in a population are more prone to infection than others. Single-cell RNA sequencing (scRNAseq) has become a powerful tool to unravel cell lineage-specific transcriptional programs and can be used to determine how these programs support or repress virus infection[18,19]. The first description of scRNAseq was in 2009 and was used to evaluate the transcription profiles of the different cells found in a mouse blastomere[20]. These technologies have now been explored and can be implemented through several different platforms. Early versions of this technology applied fluorescence-activated cell sorter (FACS) to separate individual cells for sequencing, which was often limited to 96- or 384-well plates, thereby giving 300 individual cells to analyze per sample[21]. These methods have now been advanced by the single-cell sequencing platforms, which use a microfluidic device to encapsulate single cells into individual droplets with barcode containing beads. This technology allows for up to 10,000 cells to be captured per sample condition.

Combining organoids technology with scRNAseq allows us to study how enteric pathogens impact the gastrointestinal tract in a cell type-specific manner. However, several technical and biosafety considerations need to be taken. First and foremost, classical organoids culture methods (3-dimensional (3D) organoids, embedded in an extracellular matrix (ECM)) expose the basolateral side of the epithelial cells to the outside of the organoid. As enteric pathogens initiate their infection through ingestion of contaminated food/water, the infection most often initiates from the apical side of the cells, which is not accessible in these 3D intestinal organoids. Therefore, organoids need to be prepared to make the apical side accessible for pathogen infection either through 2D seeding, thereby directly exposing the apical side of the

cells, or through microinjection[22,23]. Second, to perform scRNAseq of infected biological samples, it is important to consider their infectious nature. While methods to fix cells and inactivate pathogens prior to single-cell isolation for subsequent RNAseq have been proposed, these methods often lead to a decrease in sequencing quality[18]. The protocol below will describe several approaches to infect intestinal organoids with enteric viruses considering the infection side (apical vs. basolateral infection) (**Figure 1**). Additionally, the protocol will include a workflow to dissociate and isolate single cells from organoids infected with highly pathogenic viruses for scRNAseq. The protocol will highlight the key steps that need to be implemented when working under biosafety level-3 (BSL-3) containment conditions to avoid the generation of aerosols and potential contamination.

PROTOCOL:

Human tissue was received from colon resection or ileum biopsies from the University Hospital Heidelberg for the following protocol. This study was carried out under the recommendations of the University Hospital Heidelberg with informed written consent from all subjects in accordance with the Declaration of Helsinki. All samples were received and maintained in an anonymized manner. The protocol was approved by the Ethics commission of the University Hospital Heidelberg under protocol S-443/2017.

1. Maintenance and passaging of intestinal and colon organoids

CAUTION: Human intestinal organoids are derived from human tissue or from induced pluripotent/embryonic stem cells, and as such, ethical approval is required. Country-specific regulations need to be followed. Human material is generally not tested and is therefore often considered BSL-2 material. Proper containment conditions need to be confirmed in the country in which the experiment takes place.

1.1 Prepare intestinal and colon organoids from isolated tissues and/or biopsies using previously described methods[2]. Additionally, more technical details on how to prepare organoids from patient-derived material or from iPSCs can be found in Lees et al. and Mahe et al.[24,25].

1.2 Once organoid cultures are established, follow the below described splitting routine to prepare organoids to perform infections with enteric pathogens.

1.3 Seed 20–100 organoids in 50 μL of 100% ECM solution in 24-well non-tissue culture treated plates. Add 500 μL of growth media (**Table 1**) per well.

1.4 Change the media every 2 days by removing 250 μL of the old media and adding 250 μL of the fresh growth media to each well. Warm the media to room temperature before changing the media.

CAUTION: Cold media will liquefy the ECM solution containing organoids.

1.5 Passage the organoids once per week when the interior begins to become dark due to the accumulation of dead cells. An example of this can be found in **Figure 2**.

1.6 On the day of splitting, remove the ECM solution from -20 °C and thaw on ice.

1.7 Place the new empty cell culture plate that will be used to seed the organoids after splitting at 37 °C (this requires a minimum of 1 h to be warm and could be warmed overnight). Warm the culture media to room temperature.

1.8 Remove the growth media with a P1000 pipette and add 500 μL of cold 1x phosphate-buffered saline (PBS) to each well for 3 min to partially liquefy the ECM solution and dissociate from the plate.

1.9 To ensure full disruption of the ECM solution, use a P1000 pipette (set-up at 450 μL). Pipette up and down 10 times to resuspend the PBS, ECM solution, and organoids, transfer the resuspended organoids into a 15 mL conical tube and place them on ice.

1.10 Collect multiple wells of the same organoids into the same conical tube.

1.11 If multiple different organoids (different donors, different sections, different pre-treatment, etc.) are split at the same time, collect them in different conical tubes. While collecting, maintain the conical tubes on ice.

1.12 Spin the samples at 500 x *g* for 5 min at 4 °C. Carefully remove the PBS with a pipette to maintain the organoid pellet at the bottom.

1.13 Avoid using a vacuum waste system with a fitted pipette as the pellet of organoids is very loose and can easily be aspirated when removing the PBS too quickly.

1.14 Add 1 mL of 0.05% trypsin to the conical tube and resuspend organoids by pipetting up and down 10 times with a P1000 pipette. Incubate the conical tube containing organoids at 37 °C for 3 min.

1.15 Add 2 mL of DMEM/F12 media containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin to stop the digestion and resuspend to disrupt the organoids by pipetting up and down 10 times with a P1000 pipette.

1.16 Spin the samples at 500 x *g* for 5 min at 4 °C. Remove conical tubes from the centrifuge and store them on ice.

1.17 Carefully remove media/trypsin with a 5 mL disposable pipette to maintain the organoid pellet at the bottom. Leave around 500 μL of media and remove this with a P1000 pipette.

1.18 Once media/trypsin has been removed from all conical tubes, remove the 24-well non-cell culture treated plate from the 37 °C incubator and place it under the cell culture hood.

1.19 Add 100% ECM solution (kept on ice) to the conical tube with the split organoids at 1:3 to 1:5 ratio depending on the growth habits of the individual donor organoids. (For example, if one well containing 20–100 organoids in a 50 μL drop of ECM solution was passaged, resuspend the organoid pellet into 150–250 μL of ice-cold ECM solution.)

1.20 Seed 50 μL of ECM solution/organoid mix per well of the 24-well non-cell culture treated plate pre-warmed to 37 °C.

NOTE: ECM solution will polymerize very quickly once it has warmed. Keep the ECM solution cold at all times (stored on ice) during use. When organoids have been resuspended, seed immediately on a new plate. For beginners, it is recommended to store a box of pipette tips at -20 °C to allow extra time for the seeding.

1.21 Incubate the 24-well plate at 37 °C for 10–15 min to allow the ECM solution to polymerize.

1.22 Following polymerization, add 500 μL of growth media (**Table 1**)[26] to each well and incubate the organoids at 37 °C. Check the organoids daily by microscopy. Change the media every 2 days as in step 1.4.

2. Preparation of organoids in two-dimensions (2D) for apical infection

NOTE: The following protocol will describe how to seed intestinal organoids as a monolayer of cells in a cell culture plate to infect intestinal epithelium cells from their apical side. Use the 48-well plate for the sequencing experiments and the 8-well glass-bottom chamber slide to control infection using immunofluorescence approaches.

2.1. Grow and maintain organoids as described in section 1.

2.2. Prior to seeding organoids in 2D, coat the 48-well plates and 8-well glass-bottom chamber slide with 200 μL of 2.5% human collagen in water per well for 1 h at 37 °C.

NOTE: Intestinal organoids are best seeded in 48-well plates and in 8-well glass-bottom chamber slide. Experience has shown that they do not infect well in 96-well plates. Transwell inserts can also be used to allow for both apical and basolateral infection in 2D. If transwells are used, monitor the trans-epithelial electrical resistance (TEER) prior to infection to confirm a confluent monolayer. Normally, intestinal organoids have a tight barrier with a TEER of 450–600 Ohm/cm^2.

2.3. Seed 100–150 organoids in each well of a 48-well plate or for one well of an 8-well glass-bottom chamber slide.

2.4. To estimate the number of organoids, count the number of organoids present in the 50 μL of ECM solution drop of the 24-well plate prepared in section 1. On average, 1–2 wells are needed, which will give approximately 15,000–30,000 cells.

2.5. To disrupt the ECM solution and trypsinize the organoids, follow steps 1.8–1.17.

2.6. Remove human collagen from the 48-well plates and the 8-well glass-bottom chamber slide.

2.7. Resuspend the organoid pellet in the conical tube in 250 μL of growth media/well and add the mixture to the collagen-coated wells. Place the plate in a 37 °C incubator.

NOTE: When performing the experiments, multiple conditions are compared (mock *vs.* infected +/- treatment of interest). To minimize variability between each well of 2D seeded organoids, collect the total number of necessary organoids in the same conical tube. Collecting organoids from up to 12 wells of a 24-well plate can use 1 mL of trypsin and 2 mL of neutralizing media. When using 12–24 wells, increase this to 2 mL of trypsin and 4 mL of neutralizing media.

2.8. After 48 h, remove the plate from the incubator and place it in the cell culture hood. Remove the growth media and replace it with 250 μL per well of differentiation media (**Table 1**).

2.9. Next, replace the differentiation media with 250 μL per well of fresh differentiation media after 48 h and place the plate back in the 37 °C incubator.

2.10. After 48 h, confirm differentiation (four days post-switch to differentiation media) by extracting RNA, making cDNA with 250 ng of RNA, and performing SYBR green based qPCR using primers for stem cells (OLFM4 and/ or SMOC2), goblet cells (MUC2), enteroendocrine cells (CHGA), and enterocytes (SI and/or CYP3A4) (**Figure 3**).

NOTE: Upon switching from growth media to differentiation media, observe a decrease in the expression of stem cell markers (OLFM4 and/or SMOC2) and an increase in goblet cells (MUC2), enteroendocrine cells (CHGA), and enterocytes (SI and/or CYP3A4) markers by qPCR. Prepare an extra well and maintain with growth media to compare the expression level of each cell-type-specific marker in growth *vs.* differentiation media.

2.11. Upon confirmation of differentiation, organoids are ready for apical infection. Move the organoids to the biosafety level containment required for the pathogen of choice.

CAUTION: Refer to the institute's local regulations and standard operating procedures when handling pathogens under BSL-2 or BSL-3 containment.

2.12. To infect the intestinal organoids grown in 2D from their apical side, remove media with

a P1000 pipette and add pathogen diluted at the multiplicity of infection required for the experiment in differentiation media (minimum volume of pathogen/media mix is 50 μL per well and maximum volume is 250 μL per well).

2.13. Allow the infection to proceed for 2 h at 37 °C with either a rocker table located in the cell culture incubator or by manually rocking the plate every 15–20 min. Optimize this time based on the pathogen of choice.

2.14. After 2 h, remove the media with a P1000 pipette and replace it with 250 μL per well of fresh differentiation media. Incubate cells at 37 °C until the time point of single-cell sequencing.

2.15. To prepare cells for single-cell sequencing, proceed to section 4.

3. Preparation of organoids in three-dimensions (3D) for apical and basolateral infection

3.1. Grow organoids as described in section 1 in a 24 well, non-cell culture treated plate.

3.2. Two days post-passaging, remove the plate from the incubator and place in a cell culture hood.

3.3. Remove the growth media with a P1000 pipette, replace it with 500 μL/well of differentiation media pre-warmed to room temperature and place the plate in the 37 °C incubator.

3.4. After 48 h, replace with fresh differentiation media (500 μL/well).

NOTE: Organoids are maintained in differentiation media for a total of 4 days prior to infection.

3.5. Confirm differentiation as described in step 2.10.

3.6. Upon confirmation that differentiation has occurred, organoids are ready for infection.

3.7. Thaw ECM on ice. Warm the differentiation media to room temperature and warm a 24-well plate at 37 °C

3.8. To perform infections, remove the organoids from the ECM solution.

NOTE: Remove as much ECM solution as possible as viruses prefer to stick to the ECM solution rather than cells. If there is too much ECM solution remaining around the organoids, then infectivity will be severely impacted.

3.9. Disrupt the ECM and trypsinize the organoids as mentioned in steps 1.8–1.17.

NOTE: To minimize variability between each infection condition, combine the organoids coming

from several wells of a 24-well plate into the same conical tube. For example, if eight conditions for infection are required, eight wells of a 24-well plate (containing ~100 organoids each) will be combined and subsequently divided into eight wells of a new 24-well plate following needle disruption (see step 3.12).

3.10. Resuspend the organoids in 1 mL of differentiation media. For apical and basolateral infection follow step 3.10.1 and for basolateral infection only follow step 3.10.2.

3.10.1. Attach a 27 G needle to a 1 mL syringe and resuspend the pellet six times to allow disruption of organoids and for infection to occur at both the apical and basolateral sides. Proceed to step 3.11.

NOTE: The organoids should be the classical cyst-looking and octopus-budded organoids with a dark center when observed prior to needle disruption. Following the disruption, these organoids will appear smaller with less to no dark interior. When performing infection, there will always be a minimum of two conditions (mock and infection), a minimum of two wells of a 24-well plate will be initially combined into a 15 mL conical tube for needle-based disruption (explaining why a minimum of 1 mL is used for resuspension). When performing more than two conditions, combine up to 10 wells of a 24-well plate in a single 15 mL conical tube and resuspend in 1 mL of differentiation media for needle disruption. Following the disruption, add 500 μL of differentiation media per n + 1 (for example, if using 10 wells, then top up with 4.5 mL to have 5.5 mL total). If more than 10 conditions are needed, use several conical tubes. A 1 mL syringe is recommended as organoids are disrupted better with this volume of the syringe. An alternative to a syringe is to employ a well-flattened P1000 tip.

3.10.2. Resuspend the organoids in 500 μL of differentiation media per well. Be careful not to disrupt the organoids and make sure that the apical side is not accessible. Proceed to step 3.11.

3.11. Transfer 500 μL of organoid suspensions per well to a 24-well cell culture plate using a P1000 pipette and move the plate to the biosafety level containment required for the pathogen of choice.

CAUTION: Refer to the local regulations and the institute's standard operating procedures when handling pathogens under BSL-2 or BSL-3 containment. Always perform the needle disruption of the organoids outside the BSL-3 to avoid potential accidents with the needle. Local regulations may also prevent the use of needles in the BSL-3.

3.12. Add the pathogen diluted in differentiation media to reach the multiplicity of infection required for the experiment. Do not exceed a total volume of 500 μL (to have 1 mL total in the 24-well plate).

3.13. Allow the infection to proceed for 2 h (this time will need to be adapted to the pathogen of choice) at 37 °C with either a rocker table located in the cell culture incubator or by manually rocking the plate every 15–20 min.

3.14. Following the 2 h incubation, collect the organoids into one 1.5 mL tube per condition and spin at 500 x *g* for 5 min at 4 °C.

3.15. Remove the media containing pathogen with a P1000 pipette and store it on ice. Wash the organoids pellet once with PBS.

3.16. Resuspend the organoids in 50 µL of 100% ECM solution (which was previously thawed on ice), plate into a 24-well non-cell culture treated plate pre-warmed to 37 °C and incubate the 24-well plate at 37 °C for 10–15 min to allow the ECM solution to polymerize.

3.17. Following polymerization, add 500 µL of differentiation media at room temperature to each well and incubate at 37 °C until harvesting for single-cell sequencing.

NOTE: If harvesting needs to occur more than 48 h post-seeding, change the media every 2 days by removing the old media and replacing it with 500 µL of fresh differentiation media. However, experience has shown that since organoids are grown under differentiation media, they will not survive much longer than 48 h.

4. Preparation of single cell solution and preparation of Gel beads-in-emulsion (GEMs) in biosafety level 3 (BSL-3) conditions

NOTE: Completion of the following steps requires that the single cell sequencing equipment (**Table of Materials**) and a PCR machine capable of handling 100 µL reactions are present inside a BSL-3 facility. Organoids are grown as described in section 1 and infected as described in sections 2–3 depending on the pathogen and entry route. At a pre-determined time post-infection, organoids are harvested. Below the method used to harvest intestinal organoids is described.

4.1. Prior to harvesting infected organoids, remove the Single Cell Gel beads (**Table of Materials**) from -80 °C and warm to room temperature (at least 30 min prior).

4.2. Additionally, equilibrate the RT reagent, Reducing Agent B and RT enzyme C (all stored at -20 °C) to room temperature. Resuspend the template switch oligo as described in the manufacturer's instructions.

NOTE: All the following steps need to be performed in respect to the local biosafety regulations following the established standard operating procedure for the considered pathogen. As a rule of thumb for BSL-3 work, it must be ensured that the exterior of all vessels (plates, tubes, etc.) exiting a cell culture hood are properly disinfected. The same applies for the hands/gloves of the person performing the experiments.

4.3. To perform infections on organoids seeded in 2D (section 2) proceed to step 4.4. To perform infections on 3D organoids (section 3) proceed to sections 4.5.

4.4. For infections of 2D organoids, bring the cell culture plate into the hood and remove the differentiation media with a P1000 pipette.

4.4.1. Add 250 µL of 1x PBS at room temperature to each well.

4.4.2. Remove the PBS with a P1000 pipette and add 250 µL of room temperature dissociation enzyme (e.g., TrypLE Express) to each well of a 48-well plate. Change gloves, clean the plate, and place the plate with the dissociation enzyme in the 37 °C incubator.

4.4.3. Observe the cell dissociation by microscopy every 5 min.

NOTE: Approximately, it takes 15 min to dissociate intestinal organoids cultured in 2D into single cells. This time will need to be adjusted as it will depend on how many organoids were initially seeded and infected as well as on the pathogen as some pathogens are more cytopathic and cause organoids to dissociate much faster than others.

4.4.4. Following confirmation of an apparent single cell suspension, bring the plate back into the cell culture hood and stop the digestion by adding of 250 µL of DMEM/F12 media containing 10% FBS per well of a 48-well plate.

4.4.5. Collect the cells into a 15 mL conical tube using a P1000 pipette.

4.4.6. Change gloves, clean the tube, and spin the samples at 500 x *g* for 5 min at 4 °C.

4.4.7. Carefully remove the media/dissociation enzyme with a P1000 to maintain the cell pellet at the bottom. Resuspend single cells in a minimal volume of PBS containing 0.1% BSA. For intestinal organoids, resuspend in 250 µL for each well of the 48-well plate.

4.4.8. Pass the cell suspensions into a FACS tube with a filter to remove any large clumps and place the FACS tube containing cells on ice.

4.4.9. Proceed to step 4.6 to continue with cell counting and preparation of the master mix.

4.5. For infections of 3D organoids, remove the plate from the incubator and place it in the cell culture hood.

4.5.1. Remove the differentiation media from each well of the 24-well plate with a P1000 pipette. Add 500 µL of ice-cold 1x PBS to each well and incubate for 3 min on ice.

4.5.2. To ensure full disruption of the ECM solution, use a P1000 pipette (set to 450 µL). Pipette up and down 10 times to resuspend the PBS, ECM solution and organoids; transfer the resuspended organoids into a 15 mL conical tube and place on ice. Collect each infection

condition in its own 15 mL conical tube.

NOTE: Using a 15 mL conical tube gives a better, more distinct cell pellet than a 50 mL conical tube or a 1.5 mL tube.

4.5.3. Change gloves and remove the tube from the cell culture hood. Clean the outside of the tube.

4.5.4. Spin the samples at 500 x *g* for 5 min at 4 °C.

4.5.5. Move the tube back into the cell culture hood and remove PBS with a P1000 pipette to avoid resuspending the organoids pellet from the bottom of the tube.

4.5.6. Resuspend the pellet in 1 mL of dissociation enzyme (e.g., TrypLE Express). Change gloves, clean the tube, and incubate the samples at 37 °C.

4.5.7. Every 10 min for 30 min, move the tube back in the cell culture hood and resuspend the organoids by pipetting up and down with a P1000 pipette 10 times.

NOTE: Normally, intestinal organoids require around 30 min to form a single cell suspension when infected in 3D.

4.5.8. To determine when organoids are dissociated to single cells, take 10 µL of the organoid suspension using a p10 pipette.

4.5.9. Place the suspension in a disposable plastic cell counter slide. Seal the sample input port with clear tape.

4.5.10. Change gloves and clean the outside of the cell counter. Use a brightfield microscope to determine whether a single cell suspension is made.

4.5.11. Following confirmation of a single cell suspension, stop the digestion by adding 1 mL of DMEM/F12 media containing 10% FBS and pipette up and down 10 times with a P1000 pipette.

4.5.12. Change gloves, clean the tube, and spin the samples at 500 x *g* for 5 min at 4 °C.

4.5.13. Carefully remove the media/dissociation enzyme with a P1000 pipette to avoid resuspending the cell pellet from the bottom of the tube.

4.5.14. Resuspend the single cells in a minimal volume of PBS containing 0.1% BSA. For intestinal organoids resuspend in 250 µL.

4.5.15. Pass the cell suspensions into an FACS tube with a filter to remove any large clumps and place the FACS tube containing cells on ice.

4.5.16. Proceed to step 4.6 to continue with cell counting and preparation of the master mix.

4.6. Determine the number of cells per μL by adding 10 μL of the cell suspension to a disposable plastic cell counting chamber.

4.7. Seal the sample input port with clear tape before removing the sample from the cell culture hood, as at this stage, the cell suspension is still infectious.

4.8. Remove the gloves, clean the cell counting chamber, and count the cell number using a brightfield microscope.

4.9. Inside the cell culture hood, prepare a master mix of RT Reagent, Template Switch Oligo, Reducing Agent B, and RT enzyme C in a 1.5 mL tube as per the manufacturer's instructions depending on the number of samples in the experiment. For each sample, aliquot 33.4 μL of master mix into a PCR tube and store on ice.

4.10. Add the cells and water to the master mix according to the target cell number as described in the manufacturer's instructions.

NOTE: 50%–60% of the cells are normally recovered (i.e., when loading 10,000 cells on the chip, 5,000–6,000 cells are used for analysis). Therefore, always load 10,000 cells. If the cell density does not allow this, centrifuge the cells and resuspend in a lower volume. Take care not to overload the chip as this will result in clogging of the chip. Additionally, if cells are not properly dissociated, there will be an increased risk of getting multiple cells per bead, which will need to be removed in downstream bioinformatics processing.

4.11. Change the gloves, move the single-cell controller into the cell culture hood, and prepare the chip as the chip is only covered with a gasket that is not sealed.

NOTE: The machine needs to be inside the hood to prevent exposure to infected cell suspensions and potential aerosols.

4.12. Add the single-cell chip to the chip holder and fill the unused lanes with 50% glycerol.

4.13. Add the master mix, beads, and partitioning oil to the lanes used for samples as per the manufacturer's instructions.

4.14. Cover the chip with the gasket, load the chip into the controller, and start the program.

NOTE: It is recommended to only load six of the eight lanes. Issues with improper emulsions often occur in lanes one and eight. The company says that all lanes are independent, and this should not occur; however, if possible, avoid these two lanes and run two chips if eight samples are needed.

4.15. Upon completion of the program, remove the chip and the gasket.

4.16. Use a multichannel pipette and transfer 100 µL of the emulsions to a clean PCR tube. Ensure that each well has a uniform white color indicating a complete emulsion has occurred.

4.17. Change gloves, clean the tubes, and transfer the PCR tube to a PCR machine that can support 100 µL reactions. Run the program: 53 °C for 45 min; 85 °C for 5 min.

4.18. At completion, store the samples at 4 °C. At this point, process the reaction according to the manufacturer's instructions or store at 4 °C for 3 days or -20 °C for 1 week.

4.19. After 5 min at 85 °C, most enveloped viruses will be inactivated, remove the cDNA from the BSL-3 according to the normal operating procedure and perform the library preparations in a BSL-1 laboratory.

NOTE: This experiment needs to be performed as three biological replicates (e.g., on three different days) because the extent of the differentiation of each cell type can slightly vary between each experiment. The resulting sequencing libraries could be differently indexed and sequenced together in one sequencing run.

REPRESENTATIVE RESULTS:

Preparation of organoids for single-cell sequencing

Single-cell sequencing results are highly dependent on using good quality cells. To ensure that organoids are of good quality, they should be properly maintained and observed on a daily basis to determine when they are ready to be split (**Figure 2**). The timing of splitting organoids is donor-dependent; some donors grow more quickly and need to be split every 5 days, while others are slower and need to be split every 10 days. On average, organoids are split one time per week when the centers become dark (**Figure 2B**). If organoids are allowed to become too large and accumulate too many dead cells in the center, the organoid will die.

Organoids are maintained in a media that contains high amounts of Wnt3A. This supports the stem cell niche and promotes the organoids to continue to grow and proliferate. Under these growth conditions, the organoids contain high amounts of stem cells and transit-amplifying cells and a lower amount of differentiated cell populations such as mature enterocytes, Goblet cells, and enteroendocrine cells. However, to mimic the cellular complexity found within the human intestine, it is important to push cell differentiation and produce more of these cells. This is accomplished by changing the media conditions and removing Wnt3A, and reducing R-Spondin and Noggin (**Table 1**). Normally, cellular differentiation toward enterocytes, Goblet cells, and enteroendocrine cells requires 4 days of differentiation media (**Figure 3**). It is key to obtain a good differentiation; otherwise, evaluating pathogen tropism and cell type-specific responses will become difficult.

Single-cell sequencing results

To evaluate how SARS-CoV-2 infects human colon and ileum organoids, single-cell sequencing was performed. Organoids were prepared as described above and infected in a 2D format to allow for apical infection by SARS-CoV-2. Infected cells were harvested at 12 h and 24 h post-infection and were processed for single-cell sequencing as described above. Analysis of the single-cell sequencing data allowed us to determine that only a subpopulation (immature enterocyte 2) of human intestinal epithelial cells supported the infection of SARS-CoV-2 (**Figure 4**). Additionally, as not all cells in a population were infected, both infected cells and non-infected bystander cells were analyzed (**Figure 5**). These results showed that SARS-CoV-2 induced a pro-inflammatory signal cascade in infected cells while non-infected bystander cells showed an interferon-mediated immune response. Additionally, scRNA-Seq showed that infected cells were unable to sense interferons due to virus-mediated blockage of the pathway (**Figure 5**). It was not possible to obtain this information when using bulk RNA sequencing.

Confirmation of BSL-3 pathogen inactivation

Full inactivation must be confirmed with the pathogen of choice and validated that it is safe to remove the cDNA from the BSL-3. For SARS-CoV-2, full inactivation of the virus was validated by taking 100 μL of SARS-CoV-2 and incubating it in a PCR machine for 5 min at 85 °C. The virus was then added back to naïve Vero cells, and virus infection was compared to non-heat treated virus by immunofluorescence and plaque assays at 24 h, 48 h, and 72 h post-infection to ensure that all particles were no longer infectious. These results were sent to the local regulatory agency, and upon their approval the single-cell experiment and cDNA processing was performed.

FIGURE AND TABLE LEGENDS:

Figure 1: Schematic depicting the three different methods to prepare human intestinal organoids for infection with enteric pathogens. Apical infection can be achieved through seeding intestinal organoids in 2D. An apical and basolateral infection can be performed by disrupting the 3D organoid. Finally, a basolateral only infection can be performed by infecting intact 3D intestinal organoids. Each of these methods can be used to generate samples for single cell sequencing.

Figure 2: Organoid maintenance schematic and representative brightfield images. (**A**) Schematic for maintenance and passaging of human intestinal organoids. (**B**). Representative brightfield images of days 1, 3, 5, and 7 post-splitting. By day 7 the organoids become large and dark due to the accumulation of dead cells and are ready to be split. Scale bar indicates 25 μm.

Figure 3: Representative qPCR of human intestinal organoids 4 days post-switching to differentiation media. Intestinal organoids were maintained in growth media or switched to differentiation media for 4 days. RNA was harvested and qPCR was performed for markers of stem cells (OLFM4), Paneth cells (LYZ), Goblet cells (MUC2), and enterocytes (SI). N = 5.

Figure 4: Identification of the cell population infected by SARS-CoV-2. Human colon and ileum derived organoids were infected with SARS-CoV-2. After 12 and 24 h post-infection cells were harvested and subjected to single-cell RNA sequencing to identify which cell populations

supported SARS-CoV-2 infection. Virus infection was found to increase over time and mainly infect immature enterocyte 2. This figure has been modified from Triana et al.[19].

Figure 5: Determination of intrinsic innate immune response. Human colon derived organoids were infected with SARS-CoV-2. After 12 and 24 h post-infection cells were harvested and subjected to single cell RNA sequencing to determine the intrinsic innate immune response in both virally infected and non-infected bystander cells. SARS-CoV-2 infected cells displayed a strong pro-inflammatory response while non-infected bystander cells displayed an interferon-mediated response. Figure modified from Triana et al.[19].

Table 1: Media composition for growth and differentiation media.

DISCUSSION:

Enteric pathogens most often initiate their lifecycle by infecting intestinal epithelial cells from their apical side facing the lumen of the gut. While organoids are well recognized to be a good model to reproduce the cellular complexity and organization of the intestinal epithelium, their organization as three-dimensional, closed structures make their apical membrane inaccessible to the pathogen. This protocol described methods for infecting intestinal organoids from their apical side, their basolateral side, or both with BLS-3 pathogens. These protocols can easily be adapted to study any enteric pathogen under BSL-2 or BLS-3 containment or any other organoid model by following a few critical steps that are highlighted below. The method described above is for the isolation and preparation of single-cell droplets in accordance with the regulations in Germany. As a disclaimer, this protocol does not describe the biosafety handling measures (standard operating procedures) that need to be taken while working under BSL-3 conditions. It is also important to insist that the regulations may vary in other countries and that the local authorities must be contacted to make sure that all local regulations are respected.

One of the critical steps in seeding organoids in two dimensions for apical infection is controlling that cells will similarly differentiate compared to when grown as classical three-dimensional organoids. Depending on the enteric pathogen, tropism could be restricted to very rare cells or to cells that need to be highly differentiated. In this case, using a two-dimensional organoid that did not fully differentiate could result in the misconclusion that this enteric pathogen cannot infect intestinal organoids. It is suggested, if possible, to perform infections using the three configurations of this protocol: 2D organoid for apical infection only (section 2), cracked open 3D organoids for apical and basolateral infection (section 3), and full 3D organoids for basolateral infection only (section 3). This approach will help to discern the entry route of the pathogen (apical *vs.* basolateral) and will also control that a similar level of differentiation has been achieved. An alternative for 2D apical infection is microinjection, which will use a 3D organoid but deliver the pathogen directly into the apical side (see Bartfeld et al.[27] for details). This method requires a skilled injector to ensure that the pathogen is properly placed, and the organoids remain intact. Microinjection is commonly used in BSL-2 containment and may not be suitable for BSL-3 containment.

An additional important consideration when performing infection experiments in 2D seeded

organoids is the final cell density. As mentioned in step 2.3, 100–150 organoids will be seeded in one well of a 48-well plate or one well of an 8-well glass-bottom chamber slide. Depending on the organoid line and on the person handling the organoids, the size of these organoids can be significantly different. This could result in very different cell densities in the 48-well plate or 8-well glass-bottom chamber slide. Depending on the enteric virus, some viruses prefer more sparse cells, while others will also be able to infect confluent cells. The molecular origin of such differences in infectivity for different cell confluences is not clear; therefore, pilot experiments aiming at finding the best cell density for the enteric pathogen of choice should be performed prior to performing further downstream characterization.

Often FACS sorting is performed prior to performing the single-cell droplet emulsion. This step is often used to separate dead from live cells and single cells from doublets. When working with BSL-3 pathogens, it requires that the facility is equipped with an appropriate FACS sorter, which is not often the case. Further, not all cells in an organoid have the same size, and it is often hard to discriminate between a doublet or a larger cell, thereby causing a risk of negatively selecting against a specific cell type. Also, there is still discussion in the field whether the time needed for sorting between 5,000–10,000 for each sample could result in a significant modification of the transcript profile of the individual cells. While cell fixation methods compatible with single-cell sequencing (e.g., methanol and RNAassist) have been described, it was observed that this leads to a decrease in the quality of sequencing[18]. Finally, it is suspected that sorting cells using cell death markers can also lead to a bias. Given the directional proliferation and differentiation of the cells through the crypt-villi axis, the most differentiated cells, which are going to be shed and released, are located at the tip of the villi. These cells are often positive for different markers of cell death pathways (e.g., apoptosis, necrosis, and necroptosis); however, when looking at rotavirus infection of mouse intestine, the tip of the villi is the most infected area[28]. Thus, filtering out cells that may look positive for death markers would result in a negative selection of the infected cells that may represent the physiological infection. Currently, there is no good solution for sorting and fixing organoids prior to single-cell sequencing. Using live, unsorted cells is recommended as further studies are needed to find suitable alternative protocols.

Single-cell sequencing has revolutionized how cellular responses can be evaluated. This technique allows for the identification of cell lineage-specific responses both in basal conditions and under pathogen infections. This method has opened doors in many fields that were previously limited by bulk readouts. While this method is very powerful, it has its limitations. A key limitation is the extensive bioinformatic analysis that is required downstream of the sequencing. This is especially key when analyzing tissues and assigning cell types where there is currently no annotation. Having a skilled bioinformatician is required to support all single-cell studies.

This protocol describes how to seed and handle human intestinal organoids, infect them with enteric pathogens, and perform scRNAseq. Adapting this approach to other organs is now possible, as organoid model systems have been developed for most organs. Lung and liver organoids are similarly organized compared to intestinal organoids, and as such, using an

analogous approach could be transposed to these organoids. The critical control will be to validate that when grown in two dimensions or cracked open, these organoids achieve similar differentiation as their 3D organoid counterparts. The specific features and genes that define a differentiated status are specific for each organ model. Other organoid models such as kidney and vascular organoids, large dense structures, will need additional methods to serially dissociate these structures into single cells.

ACKNOWLEDGMENTS:

Megan Stanifer and Steeve Boulant were supported by research grants from the Deutsche Forschungsgemeinschaft (DFG): (Project number 240245660, 278001972, 415089553, and 272983813 to Steeve Boulant and 416072091 to Megan Stanifer), the state of Baden-Wuerttemberg and the Bundesministerium für Bildung und Forschung (BMBF) within the ORGANOSTRAT funding program. Schematics were created in BioRender.

DISCLOSURES:

The authors declare no competing financial interests.

REFERENCES:

1. Kretzschmar, K., Clevers, H. Organoids: Modeling development and the stem cell niche in a dish. *Developmental Cell*. **38**, 590–600 (2016).
2. Sato, T. et al. Long-term expansion of epithelial organoids from human colon, adenoma, adenocarcinoma, and Barrett's epithelium. *Gastroenterology*. **141**, 1762–1772 (2011).
3. Sato, T. et al. Single Lgr5 stem cells build crypt-villus structures in vitro without a mesenchymal niche. *Nature*. **459**, 262–265 (2009).
4. Tekes, G., Ehmann, R., Boulant, S., Stanifer, M. L. Development of feline ileum- and colon-derived organoids and their potential use to support feline coronavirus infection. *Cells*. **9** (2020).
5. Derricott, H. et al. Developing a 3D intestinal epithelium model for livestock species. *Cell and Tissue Research*. **375**, 409–424 (2019).
6. Zhou, J. et al. Infection of bat and human intestinal organoids by SARS-CoV-2. *Nature Medicine*. **26**, 1077–1083 (2020).
7. Sato, T., Clevers, H. Growing self-organizing mini-guts from a single intestinal stem cell: mechanism and applications. *Science*. **340**, 1190–1194 (2013).
8. Spence, J. R. et al. Directed differentiation of human pluripotent stem cells into intestinal tissue in vitro. *Nature*. **470**, 105–109 (2011).
9. de Graaf, M., van Beek, J., Koopmans, M. P. Human norovirus transmission and evolution in a changing world. *Nature Reviews Microbiology*. **14**, 421–433 (2016).
10. Leshem, E. et al. Real-world effectiveness of pentavalent rotavirus vaccine among bedouin and jewish children in southern Israel. *Clinical Infectious Diseases : An Official Publication of the Infectious Diseases Society Of America*. **62 Suppl 2**, S155–S160 (2016).
11. Karst, S. M. The influence of commensal bacteria on infection with enteric viruses. *Nature Reviews Microbiology*. **14**, 197–204 (2016).
12. Ettayebi, K. et al. Replication of human noroviruses in stem cell-derived human enteroids. *Science*. **353**, 1387–1393 (2016).

13. de Wit, E., van Doremalen, N., Falzarano, D., Munster, V. J. SARS and MERS: recent insights into emerging coronaviruses. *Nature Reviews Microbiology.* **14**, 523–534 (2016).
14. Scaldaferri, F. et al. The thrilling journey of SARS-CoV-2 into the intestine: From pathogenesis to future clinical implications. *Inflammatory Bowel Diseases*. **26**, 1306–1314 (2020).
15. Kipkorir, V., Cheruiyot, I., Ngure, B., Misiani, M., Munguti, J. Prolonged SARS-CoV-2 RNA detection in anal/rectal swabs and stool specimens in COVID-19 patients after negative conversion in nasopharyngeal RT-PCR test. *Journal of Medical Virology*. **92**, 2328–2331 (2020).
16. Lamers, M. M. et al. SARS-CoV-2 productively infects human gut enterocytes. *Science*. **369**, 50–54 (2020).
17. Stanifer, M. L. et al. Critical role of type III interferon in controlling SARS-CoV-2 Infection in human intestinal epithelial cells. *Cell Reports*. **32**, 107863 (2020).
18. Triana, S. et al. Single-cell transcriptomics reveals immune response of intestinal cell types to viral infection. *bioRxiv* (2020).
19. Triana, S. et al. Single-cell analyses reveal SARS-CoV-2 interference with intrinsic immune response in the human gut. *Molecular Systems Biology*. **17**, e10232 (2021).
20. Tang, F. et al. mRNA-Seq whole-transcriptome analysis of a single cell. *Nature Methods*. **6**, 377–382 (2009).
21. Hwang, B., Lee, J. H., Bang, D. Single-cell RNA sequencing technologies and bioinformatics pipelines. *Experimental & Molecular Medicine*. **50**, 1–14 (2018).
22. García-Rodríguez, I., Sridhar, A., Pajkrt, D., Wolthers, K. C. Put some guts into it: Intestinal organoid models to study viral infection. *Viruses*. **12**, 1288 (2020).
23. Stanifer, M. L. et al. Asymmetric distribution of TLR3 leads to a polarized immune response in human intestinal epithelial cells. *Nature Microbiology*. **5**, 181–191 (2020).
24. Lees, E. A. et al. Using human induced pluripotent stem cell-derived intestinal organoids to study and modify epithelial cell protection against salmonella and other pathogens. *Journal of Visualized Experiments: JoVE*. **147**, 59478 (2019).
25. Mahe, M. M., Sundaram, N., Watson, C. L., Shroyer, N. F., Helmrath, M. A. Establishment of human epithelial enteroids and colonoids from whole tissue and biopsy. *Journal of Visualized Experiments: JoVE*. **97**, 52483 (2015).
26. Fujii, M. et al. Human Intestinal organoids maintain self-renewal capacity and cellular diversity in niche-inspired culture condition. *Cell Stem Cell*. **23** (6), 787–793.e6 (2018).
27. Bartfeld, S., Clevers, H. Organoids as model for infectious diseases: Culture of human and murine stomach organoids and microinjection of Helicobacter Pylori. *Journal of Visualized Experiments: JoVE*. **105**, 53359 (2015).
28. Hernandez, P. P. et al. Interferon-lambda and interleukin 22 act synergistically for the induction of interferon-stimulated genes and control of rotavirus infection. *Nature Immunology*. **16**, 698–707 (2015).

Figure 1

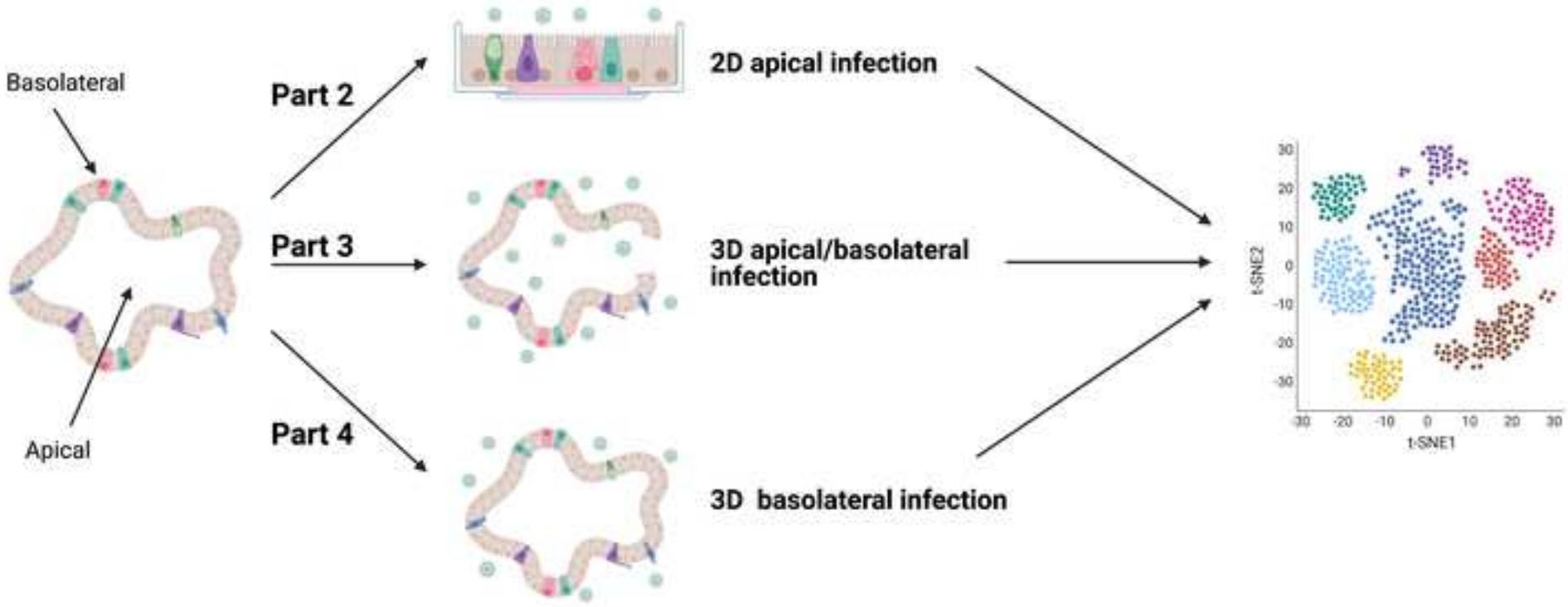

Figure 2

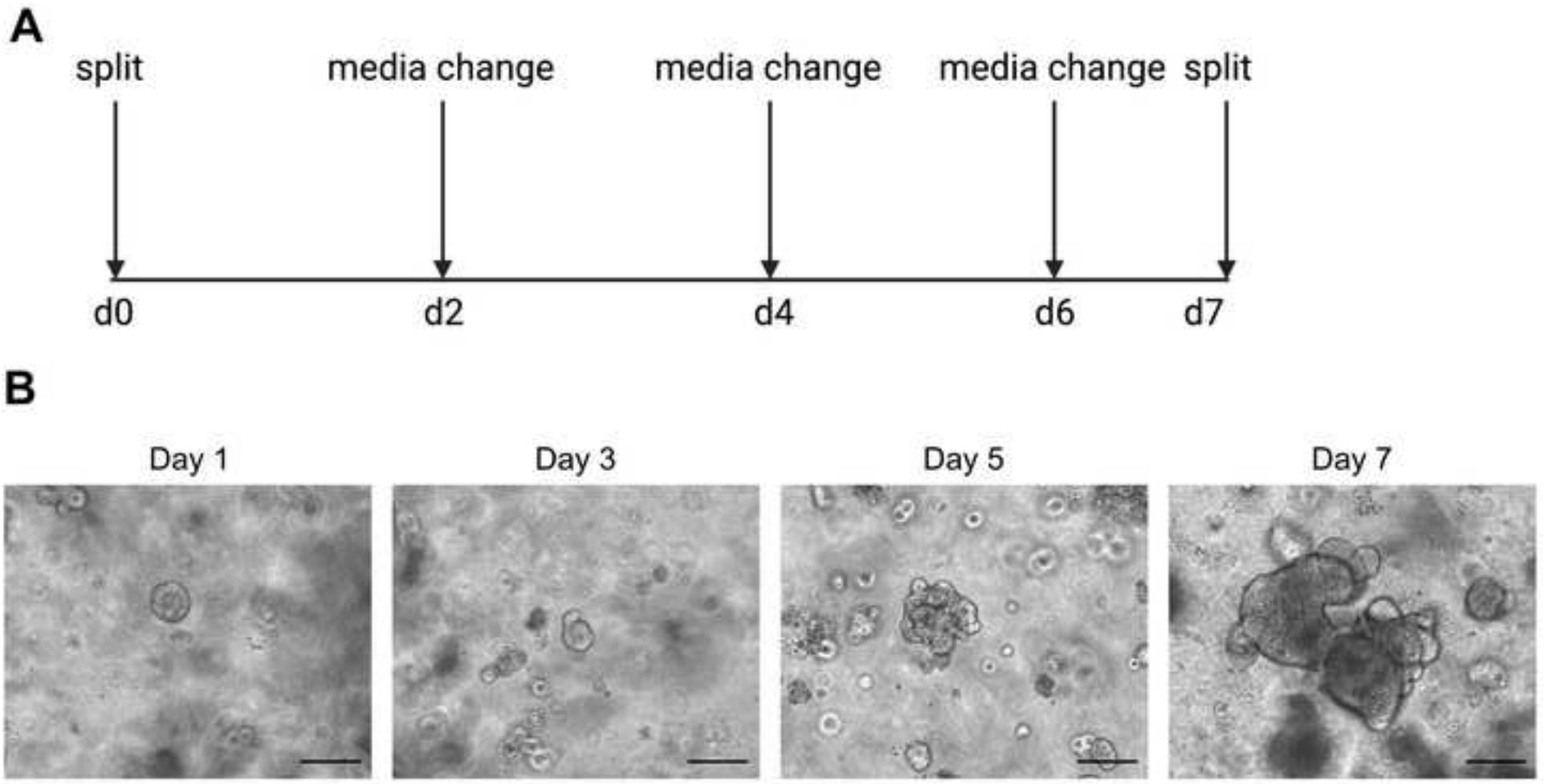

Figure 3

Click here to access/download;Figure;Figure 3.jpg

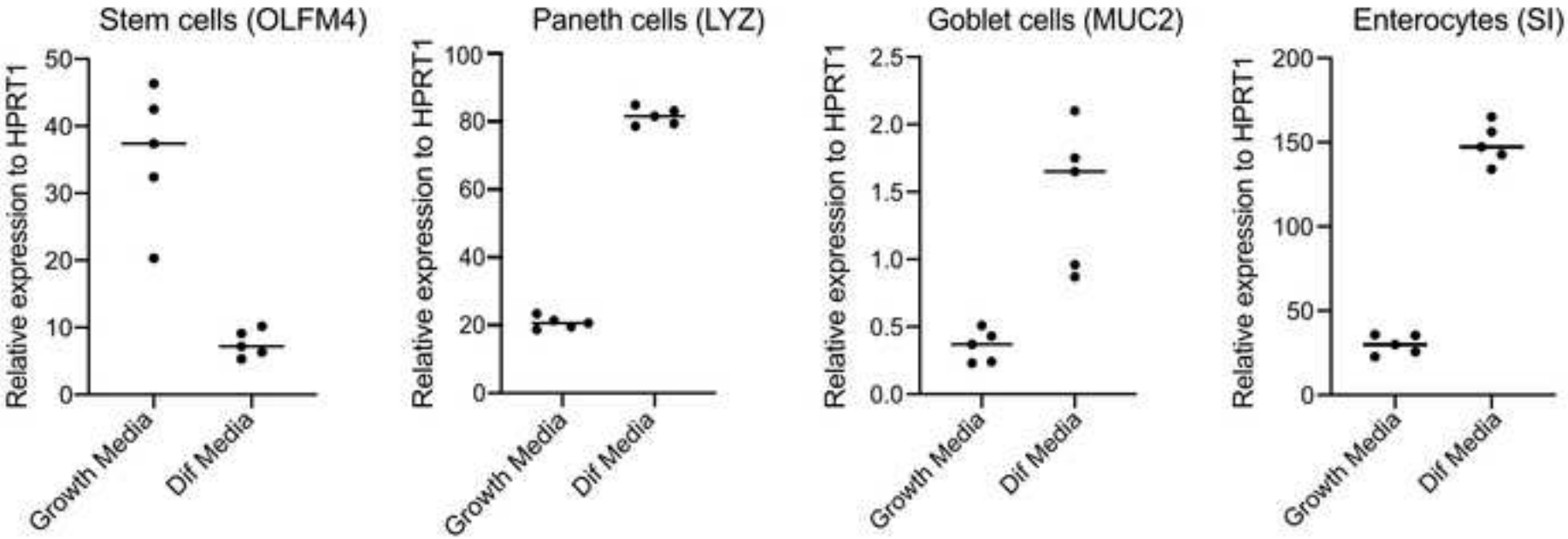

Figure 4

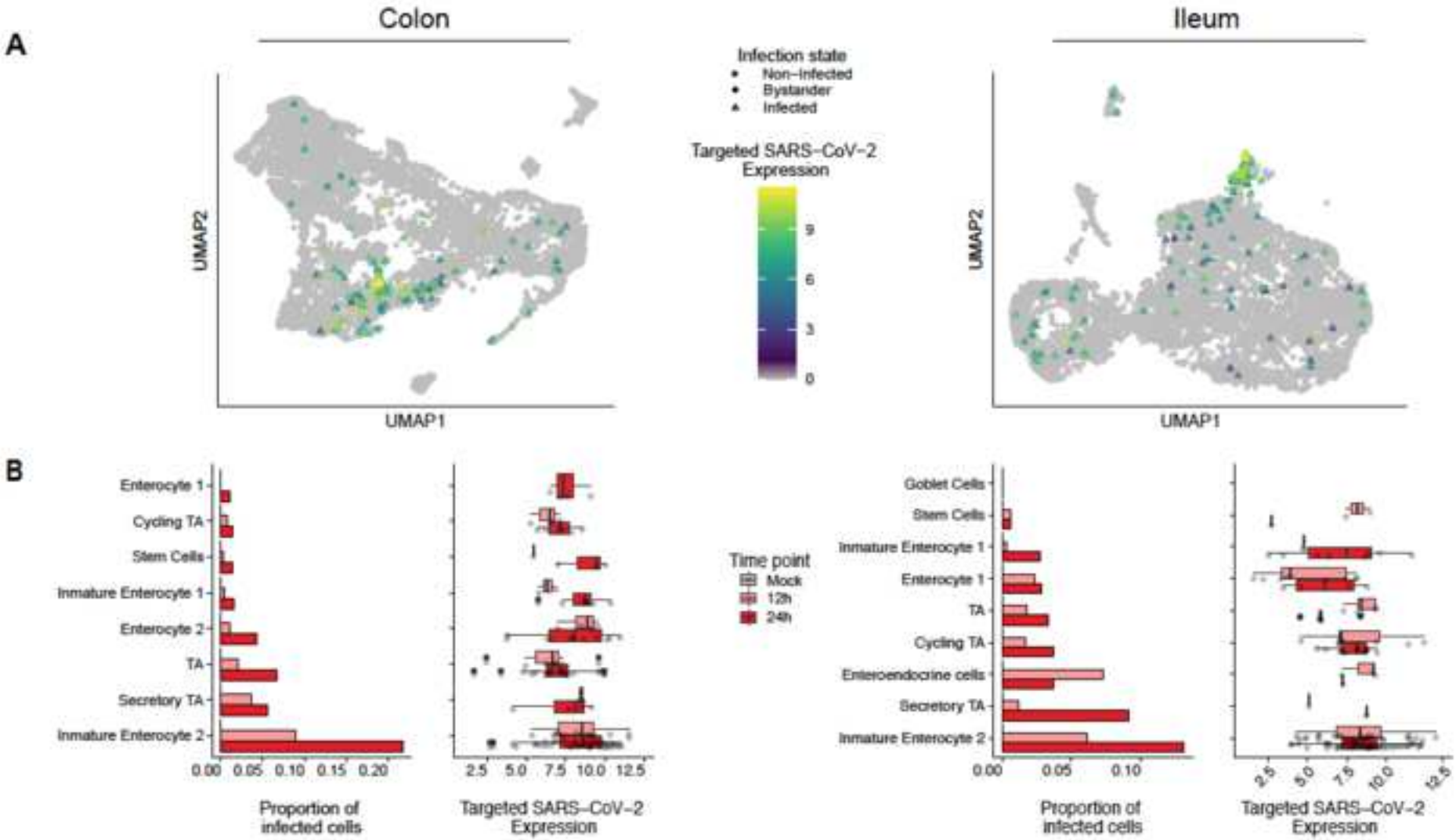

Figure 5

Click here to access/download;Figure;Figure 5.png

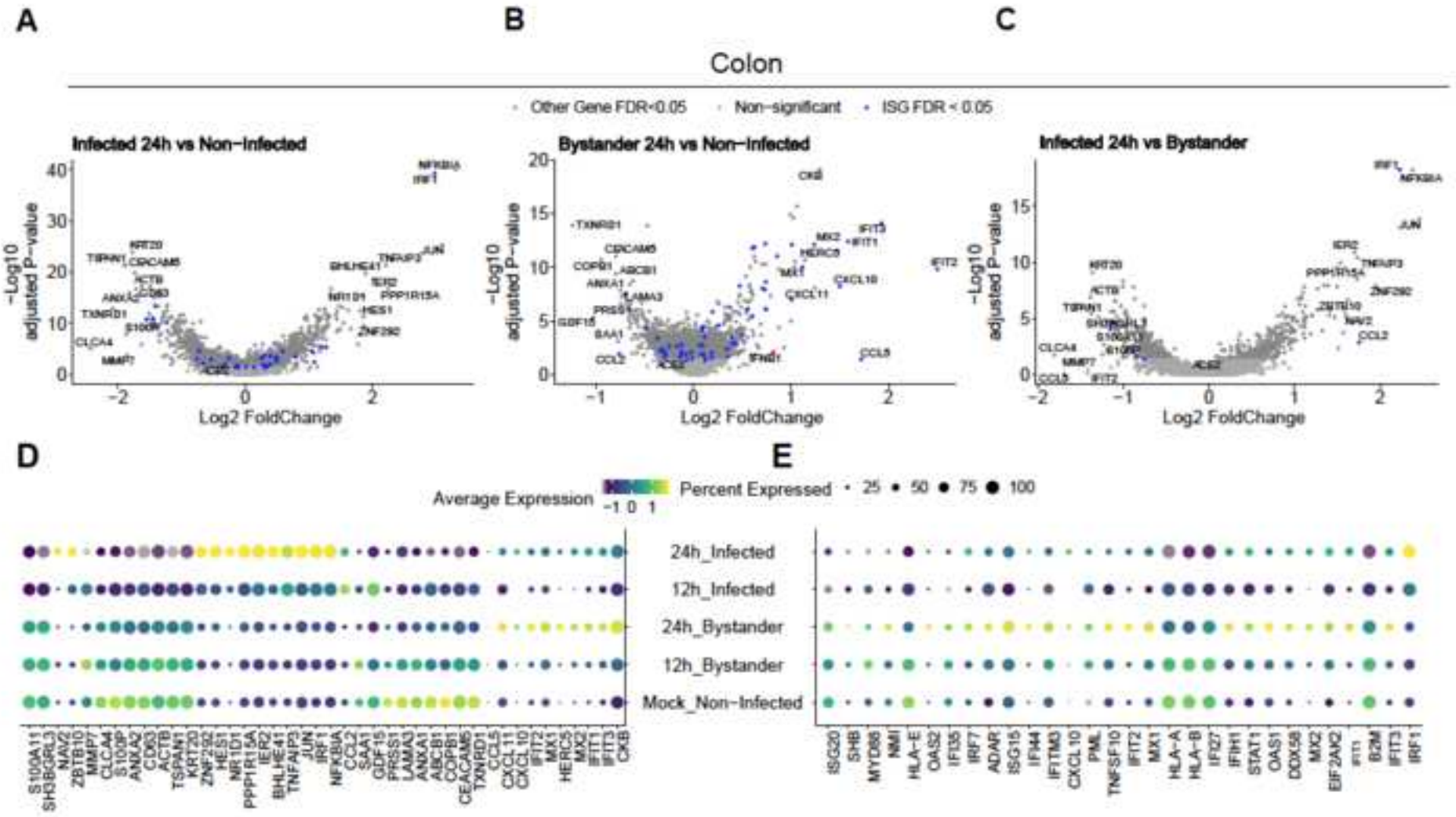

Table 1 Click here to access/download;Table;Table 1 -62857_R3.xlsx

Growth Media	
Compound	**Final concentration**
Ad DMEM/F12	GlutaMAX (1X)
+GlutaMAX	HEPES 1 mM
+HEPES	Pen 10 U/mL
+P/S	Strep 10 µg/mL
L-WRN	50% by volume
B27	1:50
N-acetyl-cysteine	1 mM
EGF	50 ng/mL
A83-01	500 nM
IGF-1	100 ng/mL
FGF basic	50 ng/mL
Gastrin	10 mM
Differentiation Media	
Compound	**Final concentration**
Ad DMEM/F12 +GlutaMAX +HEPES +P/S B27	GlutaMAX (1x) HEPES 1 mM Pen 10 U/mL Strep 10 µg/mL 1:50
N-acetyl-cysteine	1 mM
R-spondin	5% by volume
Noggin	50 ng/mL
EGF	50 ng/mL
Gastrin	10 mM
A83-01	500 nM

Dear JOVE editorial board,

Thank you for taking the time to go through our manuscript and updated the formatting. We have answered all remaining questions and believe that the article should be ready for publishing.

Best regards,
Megan Stanifer and Steeve Boulant

Editorial comments:

1. Please note that the article has been formatted to fit the journal standard. Comments to be addressed are included in the manuscript.

Comments have been addressed in the manuscript.

2. Please note that the protocol section, with the correct formatting, should not exceed a length of 10 pages. For this purpose, the steps repeated in different sections of the protocol are referenced as the step numbers to avoid repeating the steps. Section 3 and section 4 of the protocol had only one step different, so the sections were merged. The specific step in both sections is shown in step 3.9 (3.9.1 for apical and basolateral, 3.9.2 for basolateral only). Please check and verify.

Thanks for combining these, they are correct.

3. Figure 4 and Figure 5 are damaged and cannot be downloaded. Please upload figures that can be downloaded and viewed in full resolution.

New Figure 4 and 5 have been uploaded.

This is the website showing that EMBO press allows authors to retain copyrights and reuse their images with citation.
https://www.embopress.org/page/journal/17444292/authorguide#chargesguide