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TITLE:

Generation, Maintenance, and Characterization of Human Pluripotent Stem Cell-derived Intestinal and Colonic Organoids

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SUMMARY:

Here, detailed methods for generating, maintaining, and characterizing human pluripotent stem cell-derived small intestinal and colonic organoids are described. These methods are designed to improve reproducibility, expand scalability, and decrease the working time required for plating and passaging of organoids.

ABSTRACT:

Intestinal regional specification describes a process through which unique morphology and function are imparted to defined areas of the developing gastrointestinal (GI) tract. Regional specification in the intestine is driven by multiple developmental pathways, including the bone morphogenetic protein (BMP) pathway. Based on normal regional specification, a method to generate human colonic organoids (HCOs) from human pluripotent stem cells (hPSCs), which include human embryonic stem cells (hES) and induced pluripotent stem cells (iPSCs), was developed. A three-day induction of BMP signaling sufficiently patterns mid/hindgut tube cultures into special AT-rich sequence-binding protein 2 (SATB2)-expressing HCOs containing all of the main epithelial cell types present in human colon as well as co-developing mesenchymal cells. Omission of BMP (or addition of the BMP inhibitor NOGGIN) during this critical patterning period resulted in the formation of human intestinal organoids (HIOs). HIOs and HCOs morphologically and molecularly resemble human developing small intestine and colon, respectively. Despite the utility of HIOs and HCOs for studying human intestinal development, the generation of HIOs and HCOs is challenging. This paper presents methods for generating, maintaining, and characterizing HIOs and HCOs. In addition, the critical steps in the protocol and troubleshooting recommendations are provided.

INTRODUCTION:

Studying human colon development is difficult due to restrictions on the use of human fetal

tissue. Animal models have been invaluable and historically used for genetic approaches in mice to study intestinal development. However, differences between mouse and human intestinal development limit the applicability of mice as a model system. For instance, although crypt formation in the small intestine and colon of mice occurs postnatally, humans are born with fully formed crypts[1]. Furthermore, the human small intestine and colon contain cell types that are not found in mice, including motilin (MLN)-expressing enteroendocrine cells in the small intestine[2] and mucin 5B (MUC5B)-expressing goblet cells in the colon[3,4]. For this reason, it is important to have a cell culture system that accurately models the dynamic molecular events that define the early stages of colon development. Therefore, directing hPSCs to generate cells with colon characteristics provides a powerful model for the study of human colon development.

Protocols have been developed to facilitate the reproducible[5], synchronous, and efficient formation of intestine-like[6] and colon-like organoids[7] from hPSCs. These protocols use a stepwise differentiation procedure that mimics the development of the fetal intestine and colon (**Figure 1**). The first definitive endoderm is generated from human pluripotent stem cells by treatment with Activin A, a Nodal mimetic. Exposure of the definitive endoderm to high levels of WNT and fibroblast growth factor (FGF) induces morphogenesis into $CDX2^+$ mid/hindgut tube spheroids. Midgut/hindgut spheroids are then embedded in extracellular matrix (ECM) and patterned into either HIOs or HCOs through a transient manipulation of BMP signaling. Inhibiting BMP signaling using NOGGIN or adding growth medium alone results in the formation of HIOs, which resemble the human proximal small intestine.

By activating BMP signaling using BMP2, mid/hindgut spheroids are patterned into HCOs, which retain patterning in the epithelium and mesenchyme[7]. HCOs contain colon-enriched, MUC5B-expressing goblet cells and are competent to generate colon-specific insulin-like 5 (INSL5)-expressing enteroendocrine cells. Isolated mesenchyme from HCOs expresses homeobox A13 (HOXA13) and HOXD13, which are also expressed in human primary colon mesenchyme[8]. It is important to remember that the patterning step occurs during days 7–10 of the differentiation protocol. This three-day period is sufficient to induce colonic patterning that is maintained following extended *in vitro* culture.

The protocols described below are for researchers who are familiar with feeder-free hPSC culture. For researchers who are not familiar with this type of hPSC culture, a training course on hPSCs such as those offered by Stem Cell Technologies or the Pluripotent Stem Cell Facility (PSCF) at Cincinnati Children's Hospital is recommended. The quality of the starting hPSCs is critical and can affect all downstream steps. The protocol that follows would begin with hPSCs that have been grown for 4 days and are ready to split.

PROTOCOL:

1. Generation of human intestinal and colonic organoids

1.1. Preparing ECM-coated plates

1.1.1. Add 50 mL of cold DMEM medium into a 50 mL conical tube.

1.1.2. Remove an aliquot of 4x hESC-qualified ECM (see the **Table of Materials**) from the -80 °C freezer and thaw on ice.

NOTE: Refer to the product's certificate of analysis to determine the volume of ESC-qualified ECM required to prepare a 4x stock.

1.1.3. If hESC-qualified ECM is not fully thawed, take 750 µL of DMEM from the 50 mL conical tube and mix it with the ECM.

1.1.4. Transfer the DMEM/hESC-qualified ECM mixture to the 50 mL conical tube with DMEM and mix well. Add 0.5 mL per well into each of 4 x 24-well cell culture plates.

1.1.5. Shake the plates to spread the ECM evenly throughout the well to ensure that the entire surface is covered. Using parafilm, seal the ECM-coated plates and leave them at room temperature in a biosafety cabinet for at least 1 h. Store ECM-coated plates at 4 °C for up to 2 weeks or until needed.

1.2. hPSCs single-cell plating

1.2.1. Place an ECM-coated 24-well plate inside a biosafety cabinet for 30 min to allow it to reach room temperature.

1.2.2. Place mTeSR1 complete medium, cell detachment solution, and advanced DMEM inside a 37 °C water bath and allow them to warm up for 30 min.

1.2.3. Verify that hPSCs are at least 85% confluent with minimal differentiation. Remove any differentiated cells if necessary.

1.2.4. Prepare the plating medium in a 50 mL conical tube as follows: 13 mL of mTeSR1 and 13 µL of 10 mM Y-27632 Rho-associated protein kinase (ROCK) inhibitor.

NOTE: Y-27632 inhibits anoikis and increases the survival of single cells.

1.2.5. To collect cells from a 6-well plate, aspirate the medium from 3 to 4 wells and wash once with 2 mL of advanced DMEM per well.

1.2.6. Aspirate the advanced DMEM and dispense 1 mL of the cell dissociation solution into each well. Incubate the plate for 5–7 min inside a 5% CO_2, 37 °C incubator. Check under the microscope that cells are in suspension.

1.2.7. Dissociate any remaining clumps of cells by pipetting up and down 4–5 times using a 5-mL pipette.

1.2.8. Add 2 mL of advanced DMEM into each well, gently pipette up and down 4–5 times, and transfer to a 15 mL conical tube. Spin down the cells at 300 × *g* for 3 min at room temperature.

1.2.9. Aspirate the medium from the tube without aspirating the cell pellet and add 6 mL of the prepared medium of mTeSR1 plus ROCK inhibitor. Gently resuspend the cells by pipetting up and down 3–4 times and then transfer the suspension to the rest of the mTeSR1/ROCK inhibitor medium inside the 50 mL tube. Resuspend vigorously 4–5 times and count the cells using a hemacytometer.

1.2.10. Aspirate the ECM from the 24-well plate just before plating the cells. Resuspend the cells again by pipetting up and down 2–3 times and dispense 0.5 mL of the cell suspension in each well.

NOTE: The optimal plating density needs to be determined by the experimenter. Here, the optimal cell number is 80,000–200,000 cells per well.

1.2.11. Gently rock the plate 3 times clockwise, 3 times counterclockwise, 3 times forward and back, and 3 times side to side to evenly disperse the cells.

1.2.12. Transfer the plate to a 37 °C, 5% CO_2 incubator and incubate for 24 h.

NOTE: Do not disturb the plate for the first few hours to ensure proper dispersal of cells within the wells.

1.2.13. After 24 h (**Figure 2A**), aspirate the spent medium, add 0.5 mL per well of mTeSR1, incubate again at 37 °C, 5% CO_2 for 24 h (**Figure 2B**), and then proceed to the next step.

1.3. Differentiation of definitive endoderm (DE) from hPSCs

1.3.1. In a 15 mL conical tube, add 13 mL of Activin Day 1 medium (see the **Table of Materials**), 13 µL of 100 µg/mL Activin A, and 1.95 µL of 100 µg/mL BMP4. Warm the medium in a 37 °C water bath.

1.3.2. Aspirate the mTeSR1 medium from the 24-well plate and add 0.5 mL of Activin Day 1 medium per well. Place the plate in a 37 °C, 5% CO_2 incubator and incubate for 24 h. Check the cells after 24 h.

NOTE: Extensive cell death should be apparent, as depicted in **Figure 2C**. Although the monolayer will appear sparse, colonies of cells will have expanded.

1.3.3. Prepare Activin Day 2 complete medium by adding 12.5 µL of 100 µg/mL Activin A into 12.5 mL of Activin Day 2 medium in a 15 mL conical tube. Place the tube in a 37 °C water bath.

1.3.4. Take the 24-well differentiation plate out of the CO_2 incubator and remove the spent medium. Dispense 0.5 mL of prewarmed Activin Day 2 medium per well and place the plate back inside the CO_2 incubator for 24 h.

NOTE: Care should be taken when dispensing the medium. Do not dispense medium directly in the center of the well, as this will detach cells in the monolayer. Carefully dispense medium down the side of the well. The next day, a monolayer of cells is formed with negligible cell death. Cells should now be ~90 to 95% confluent (**Figure 2D**).

1.3.5. Prepare Activin Day 3 complete medium by adding 12.5 µL of 100 µg/mL Activin A into 12.5 mL of Activin Day 3 medium in a 15 mL conical tube. Place the tube in a 37 °C water bath.

1.3.6. Remove the spent medium and dispense 0.5 mL of Activin Day 3 medium per well.

NOTE: Care should be taken when dispensing medium. Do not dispense medium directly in the center of the well as this will detach cells in the monolayer. Carefully dispense medium down the side of the well. The next day, the monolayer should reach full confluency with little to no cell death at this stage. Do not attempt to generate mid-hindgut spheroids if the monolayer is not confluent 24 h after adding Activin Day 3 medium. Refer to **Figure 2E** for the ideal morphology of the DE monolayer before proceeding to the generation of mid-hindgut spheroids.

1.3.7. Perform immunofluorescence (IF) staining of the monolayer for the expression of forkhead box A2 protein (FOXA2) and sex-determining region Y (SRY)-box transcription factor 17 (SOX17) when optimizing DE differentiation.

1.4. Differentiation of DE into mid-hindgut spheroids

1.4.1. In a 50 mL conical tube, add 25 mL of mid-hindgut induction medium with FGF4 (no CHIR99021) and place it in a 37 °C water bath for 30 min.

1.4.2. To prepare the complete mid-hindgut induction medium, add 7.5 µL of CHIR99021 after the medium is warm.

1.4.3. Remove the spent medium, dispense 0.5 mL of mid-hindgut induction medium per well and incubate at 37 °C, 5% CO_2 for 24 h.

1.4.4. After condensation of cells within the monolayer has occurred the next day (**Figure 3A**), replace the spent medium with fresh mid-hindgut induction medium and place the plate back in a 37 °C, 5% CO_2 incubator for 24 h.

NOTE: Tubular structures will be noticeable after 48 h of mid-hindgut induction, as shown in **Figure 3B**. Mid-hindgut spheroids will start budding off the monolayer on day 3, as depicted in **Figure 3C.**

1.4.5. To avoid discarding the floating spheroids while changing the medium, transfer the old medium into a 15 mL tube and centrifuge at 300 × *g* for 1 min. Resuspend the spheroids in 12.5 mL of fresh mid-hindgut induction medium, add 0.5 mL per well into the same 24-well plate, and incubate at 37 °C, 5% CO_2 for 24 h.

1.4.6. On day 4 of hindgut induction (**Figure 3D**), harvest floating spheroids from the plate wells by collecting the medium into a 15 mL tube followed by centrifugation at 300 × *g* for 1 min. Proceed to the next step for embedding mid-hindgut spheroids in ECM.

1.5. Plating and patterning of mid/hindgut spheroids in ECM

1.5.1. Thaw ECM basement membrane matrix at 4 °C overnight before the embedding.

NOTE: This ECM is different from the hESC-qualified ECM. The ECM should be put on ice, and the appropriate volume of ECM can be aliquoted into prechilled 1.5 mL microcentrifuge tubes on ice. **Table 1** contains information about ECM volume. Ensure that the volume of ECM is at least 75% in the droplet in which the spheroids will be embedded.

1.5.2. Warm up a 24-well plate in a 37 °C incubator.

1.5.3. Collect the floating spheroids from all the 24-wells using a 1000 µL pipet and transfer them to a 15 mL conical tube. Spin down the spheroids at 300 × *g* for 1 min at room temperature. Aspirate most of the medium but leave the volume required for plating (**Table 1**).

1.5.4. Prepare 1000 µL and 200 µL pipette tips by cutting their ends. Take out the prewarmed 24-well plate.

1.5.5. Mix the floating spheroids, transfer the appropriate volume to the ECM tube placed on ice, and then resuspend the spheroids and ECM by pipetting to mix them well.

1.5.6. Take 65 µL of the mixture of spheroids and ECM with the cut 200 µL pipette tips and load to the center of each well in the 24-well plate. To make sure a good ECM droplet is formed, lift the pipet gently and slowly while the mixture is dispensed.

NOTE: Plate 30–100 spheroids per well.

1.5.7. Gently transfer the plate to a 37 °C, 5% CO_2 incubator and incubate for 5 min.

1.5.8. Flip the plate upside down and incubate for 15–25 min, which will help the ECM droplets to maintain a dome-like structure.

1.5.9. During the incubation, prepare the required medium and warm it up. Once the ECM is solidified, add 0.5 mL of HIO- or HCO-patterning medium in each well and incubate at 37 °C, 5% CO_2. See **Figure 3E** for an image of spheroids in ECM.

1.5.10. Culture the spheroids in the patterning medium for 3 days.

1.5.11. After 3 days, change the HIO- or HCO-patterning medium to normal growth medium and incubate at 37 °C, 5% CO_2.

NOTE: The organoids at this time point (Day 10) are early-stage organoids. Based on the project goal, some early-stage organoids can be used for early patterning analysis, as detailed in section 2.

1.6. Outgrowth and passaging of human intestinal and colonic organoids

1.6.1. After day 10, change the growth medium every 3 days.

NOTE: Depending on the plating density and the growth of the organoids, more frequent media changes may be required. Medium changes should be done before the phenol red (pH indicator) in the medium becomes yellow.

1.6.2. Incubate the organoids until Day 21 (**Figure 3F**).

NOTE: BMP2 treatment will result in a reduced number of organoids (~3-fold less than HIOs) that grow from spheroids. Therefore, a larger number of spheroids needs to be plated for the generation of HCOs.

1.7. Splitting of organoids on Day 21

1.7.1. Inspect the organoids.

NOTE: The organoids need to be split on Day 21 as the ECM is almost degraded by Day 21 due to organoid growth and expansion.

1.7.2. Prepare the 1000 µL and 200 µL pipette tips by cutting their ends.

1.7.3. Warm up a 24-well Nunc plate in a 37 °C incubator.

1.7.4. Aliquot the appropriate volume of ECM into the prechilled 1.5 mL tubes (**Table 1**).

1.7.5. Gently scrape the ECM droplet with the organoids with a 1000 µL pipette tip and pipette the mixture up and down several times to break up the ECM.

1.7.6. Transfer the mixture to a 60 mm Petri dish and check the organoids under a microscope. If necessary, separate the organoids from each other using sterile forceps. Ensure that the separation does not damage the epithelium of the organoids.

1.7.7. Transfer the organoids to a 15 mL conical tube and centrifuge at 300 × *g* for 30 s. Aspirate the supernatant and leave ~1 mL of medium in the tube.

NOTE: The volume of the medium can be changed based on the purpose of the experiment. If more organoids are required per ECM droplet, the medium volume can be decreased. However, if fewer organoids are required, the medium volume can be increased.

1.7.8. Mix the organoids well, transfer the appropriate volume to the ECM tube placed on ice, and then resuspend the organoids and ECM by pipetting to mix them well.

1.7.9. Add 65 µL of the mixture of spheroids and ECM to the center of each well of the 24-well plate using the cut 200 µL pipette tips.

NOTE: It is critical to take up the organoids first and then the ECM during the pipetting. Thus, while dispensing the mixture, the organoids are at the top of the ECM droplet.

1.7.10. Put the plate in a 37 °C, 5% CO_2 incubator for 5 min.

1.7.11. Flip the plate upside down for another 15–25 min to help the ECM droplets maintain a dome-like structure.

1.7.12. Add 0.5 mL of HIO/HCO outgrowth medium in each well and incubate at 37 °C, 5% CO_2.

NOTE: Outgrowth medium is the same for both HIOs and HCOs after patterning.

1.7.13. Change the medium every 2–3 days until Day 35 (**Figure 3G**).

2. Verifying the patterning of organoids by reverse-transcription quantitative polymerase chain reaction (RT-qPCR)

2.1. Before collecting RNA, prepare 1x RNA lysis buffer following the manufacturer's instructions.

2.2. Discard the spent medium and add 350 µL of lysis buffer to each well of the 24-well plate. Lyse the organoids by pipetting up and down using a 1 mL pipette. For better lysis, vortex briefly at maximum speed for 5 s.

2.3. Keep all samples at -80 °C until ready for RNA extraction. Once ready, remove the RNA samples from the -80 °C freezer and thaw them on ice for 10 min. Vortex the tubes vigorously at room temperature for 10–15 min to ensure complete lysis of the samples.

2.4. Place the samples on ice again and proceed to RNA extraction as instructed by the manufacturer. Transfer the RNA samples to a -80 °C freezer if not ready to proceed to the next step.

2.5. Perform RNA extraction, DNAse treatment, cDNA synthesis, and RT-qPCR using standard methodology. Refer to **Table 2** for a list of primer names and sequences.

3. Verifying the patterning of organoids by immunofluorescence

3.1. Collection and fixation of organoids

3.1.1. Aspirate HIO or HCO medium from the appropriate wells and add 1 mL of ice-cold phosphate-buffered saline (PBS) to each well. Dissociate the organoids from the ECM using a cut 200 µL pipette tip. Pipette up and down to dissociate large chunks of ECM from the organoids.

3.1.2. Transfer the organoids to a 15 mL conical tube and fill the rest of the tube with ice-cold PBS and mix gently by inverting the tube. Allow the organoids to settle by gravity and aspirate the PBS.

NOTE: If the organoids do not settle by gravity, centrifuge at 300 × *g* for 1 min.

3.1.3. Aspirate the PBS and add 1 mL of ice-cold ECM-degrading solution. Keep the tube on ice for 10–15 min on a rotating platform with gentle shaking.

NOTE: Tilt the tube at a 45° angle to allow proper mixing of the organoids and the Cell Recovery Solution. After 10–15 min, the organoids should settle to the bottom of the tube, indicating the complete digestion of the ECM.

3.1.4. Add cold PBS in the tube up to 15 mL; mix well by inverting the tube several times. When the organoids settle to the bottom of the tube, aspirate the PBS and add 1 mL of prechilled 4% paraformaldehyde to fix the organoids. Incubate the organoids with 4% paraformaldehyde on ice for 1 h.

3.1.5. Fill the rest of the tube with ice-cold PBS and place it horizontally on a rocking platform at 4 °C overnight. The next day, dispose of the paraformaldehyde/PBS in the specified waste container and wash once with 15 mL of ice-cold PBS, as described in step 3.1.2.

3.1.6. Aspirate the PBS and fill the tube with 30% sucrose in PBS. Place the tube horizontally on a rocking platform at 4 °C overnight.

3.1.7. The next day, embed the organoids in a 7 mm x 7 mm x 5 mm base model with OCT medium and flash-freeze using a dry ice/ethanol bath.

3.1.8. Dry the blocks with laboratory wipes, wrap them in a paper towel, and keep them in a -80 °C freezer overnight. The next day, cut 5 µm sections onto microscope slides using a cryostat. Store the slides at -80 °C.

3.2. Immunofluorescence staining of the organoids

3.2.1. Process the slides from the -80 °C freezer and perform IF staining using standard protocols.

3.2.2. Apply coverslips to the slides and dry them at room temperature, protected from light for at least 2 h or overnight before imaging.

3.2.3. Image the slides using a 25x objective of a confocal microscope.

REPRESENTATIVE RESULTS:

The successful generation of spheroids during the mid/hindgut induction stage is indicative of successful patterning. Perform IF staining for CDX2 on floating spheroids and on the monolayer to confirm that patterning is correct. Although staining at the definitive endoderm (DE) stage can indicate the effectiveness of DE induction, spheroid generation is not possible without efficient DE induction. To test the efficiency of DE induction, perform IF staining and/or RT-qPCR for FOXA2 and SOX17.

Following the patterning stage, the expression of HOX factors is the best indicator of successful patterning. HOX factors are primarily expressed in the intestinal and colonic mesenchyme[8,9]. Therefore, HOX factor expression will reflect the patterning of the mesenchyme. The expression of mRNA of the anterior HOX factor *HOXD3* should be highest in NOGGIN-treated HIOs, less in epithelial growth factor (EGF)-treated HIOs, and lowest in BMP-treated HCOs. Conversely, *HOXA13* and *HOXD13* mRNA expression should be low in HIOs and high in HCOs (**Figure 4**). In addition, perform RT-qPCR for *MSX2*, a direct target of BMP signaling at day 10. *SATB2* expression can be seen in the epithelium of HCOs at day 10; however, examination of *SATB2* by RT-qPCR is not a reliable indicator of patterning as HIOs contain populations of neurons[10,11] that can also express SATB2[12,13]. Therefore, use RT-qPCR to examine HOX factor expression to determine if patterning was successful. Perform immunofluorescence staining for SATB2, CDX2, and CDH1 to determine if HCO epithelium was properly patterned (**Figure 5**).

FIGURE AND TABLE LEGENDS:

Figure 1: Schematic of the differentiation protocol for HCOs. The general timeline of HCO differentiation is shown. Although HIO differentiation is not shown, it would be the same except for the patterning stage in which NOG+EGF or EGF alone would be used from days 7–10. Abbreviations: hPSCs = human pluripotent stem cells; HCOs = human colonic organoids; HIOs = human intestinal organoids; EGF = epithelial growth factor; BMP2 = bone morphogenetic protein 2; NOG = Noggin.

Figure 2: Generation of the definitive endoderm monolayer from hPSCs. Morphology of hPSCs is shown before (**A, B**) and after Activin A Day 1 (**C**) after Activin A Day 2 (**D**) and after Activin A Day 3 (**E**). Staining of DE with SOX17 (F), FOXA2 (**G**), and SOX17/FOXA2 merged with DAPI (**H**). Images were acquired using a 10x objective of an Olympus IX50 inverted microscope. Scale bars = 100 µm. Abbreviations: hPSCs = human pluripotent stem cells; DE = definitive endoderm; SOX17

= sex-determining region Y (SRY)-box transcription factor 17; FOXA2 = forkhead box A2 protein.

Figure 3: Morphogenesis of mid/hindgut spheroids from the definitive endoderm. Photographs of the DE monolayer following treatment of DE with MHGI medium for 24 h (**A**), 48 h (**B**), 72 h (**C**), and 96 h (**D**).MHGI medium was changed daily. Yellow arrows point to areas of endoderm condensation. White arrows point to emerging mid-hindgut spheroids. Photographs of plated spheroids (**E**), Day 21 HCOs (**F**), and Day 35 HCOs (**G**). Images in **A–D** were acquired using a 10x objective of an Olympus IX50 inverted microscope. Scale bars = 100 µm. Images in **E–F** were acquired using a Leica S9D stereomicroscope using a 1x objective. Abbreviations: DE = definitive endoderm; MHGI = Mid-hindgut Induction.

Figure 4: *HOX* gene expression in human intestinal and colonic organoids at day 21. RT-qPCR using standard methods to determine the relative expression of the anterior HOX gene *HOXD3* and the posterior HOX genes *HOXA13* and *HOXD13*. For NOG HIOs, n=3. For HCOs, n=4. Error bars depict the standard error of the mean while p values are from a two-tailed Student's *t*-test with equal variance. *: $p < 0.05$ *, **: $p < 0.01$,***: $p < 0.001$. Abbreviations: HOX = homeobox protein; NOG = noggin; HIOs = human intestinal organoids; HCOs = human colonic organoids.

Figure 5: Immunofluorescence staining of human intestinal and colonic organoids. Day 35 HIOs (top panels) and HCOs (lower panels) were stained for CDX2 (green), CDH1 (white), SATB2 (red), and DAPI (blue). Images were acquired using a 25x objective on an LSM 880 confocal microscope. Scale bars = 50 µm. Abbreviations: HIOs = human intestinal organoids; HCOs = human colonic organoids; CDH1 = E-cadherin; SATB2 = special AT-rich sequence-binding protein 2; DAPI = 4',6-diamidino-2-phenylindole.

Table 1: Volume of ECM/spheroids required for plating.

Table 2: List of primers and sequences. Abbreviations: HOX = homeobox; CPHA = cyclophilin A; MSX2 = msh homeobox 2; BMP = bone morphogenetic protein.

DISCUSSION:

The differentiation of hPSCs into HIOs and HCOs is a complex process requiring quality controls at each step. The starting hPSCs need to have minimal differentiation before initiating differentiation into DE. Optimizing the density of hPSCs plated for DE differentiation is critical for the success of the protocol. To ensure the quality of DE differentiation, perform IF for FOXA2 and SOX17 to determine the efficiency of DE differentiation. DE differentiation should result in over 80% of the treated cells staining positive for FOXA2 and SOX17. Once the optimal density is established, this same density can be used for multiple experiments with similar success. Following successful DE differentiation, mid/hindgut induction should be highly efficient. After plating in ECM, patterning of mid/hindgut spheroids with BMP2 lowers the efficiency of organoid formation from spheroids (~15%). Therefore, plate 2 to 3 times more spheroids per ECM bubble for HCO generation as compared to HIOs.

The optimal density for DE differentiation will vary from cell line to cell line. However, some cell

lines are difficult to differentiate into DE with Activin A alone. If multiple experiments fail, add 5–15 ng/mL of BMP4 to the day 1 Activin A medium. The addition of BMP4 has been shown to improve DE differentiation through inhibition of the pluripotency factor SOX2[14]. This modification does not affect mid/hindgut induction. If spheroid generation is unsuccessful, the DE monolayer should be checked for CDX2 expression to ensure proper patterning of the DE into mid/hindgut. If the DE is $CDX2^+$ but does not yield any spheroids, the monolayer can be passaged as clumps and plated in ECM[15-18]. Clumps of mid/hindgut monolayer can self-organize, grow, mature, and differentiate similar to spheroid-derived HIOs.

Despite their cellular complexity, HIOs and HCOs lack an enteric nervous system (ENS). In addition, HIOs and HCOs are immature and lack expression of brush border enzymes, limiting their utility. The ENS is derived from vagal neural crest cells (NCCs). The hPSCs that have differentiated into vagal NCCs have been incorporated into HIOs and HCOs to establish an ENS[5,19]. Co-culture of HIOs with T lymphocytes (Jurkat cells) induces HIO maturation *in vitro* resulting in the expression of brush border enzymes, mature intestinal stem cell markers, and increased expression of enteroendocrine cell-expressed hormones[20]. Similar approaches will be needed to increase the cellular complexity of HIOs and HCOs by incorporating other immune cells such as tissue-resident macrophages.

Reprogramming of patient somatic cells into iPSCs has allowed the use HIOs and HCOs for modeling diseases such as dyskeratosis congenita[21], familial adenomatous polyposis[17,22], and ulcerative colitis[10]. Furthermore, clustered regularly interspaced palindromic repeats (CRISPR)-CRISPR-associated protein 9 (Cas9)-mediated gene editing of hPSCs has allowed functional studies of neurogenin3[23,24], paired-like homeobox 2B[19], and CDX2[25] proteins. Further improvements in the incorporation of cell types and the induction of maturation in HIOs and HCOs will lead to better disease models. In addition, CRISPR-Cas9-mediated gene editing of genes involved in regional patterning should provide new insights into the regional specification of the intestines. HIOs and HCOs are an exciting model for studying human fetal intestine and will continue to provide insights into human intestinal development and disease.

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DISCLOSURES:

The authors have no conflicts of interest to disclose.

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Figure 1

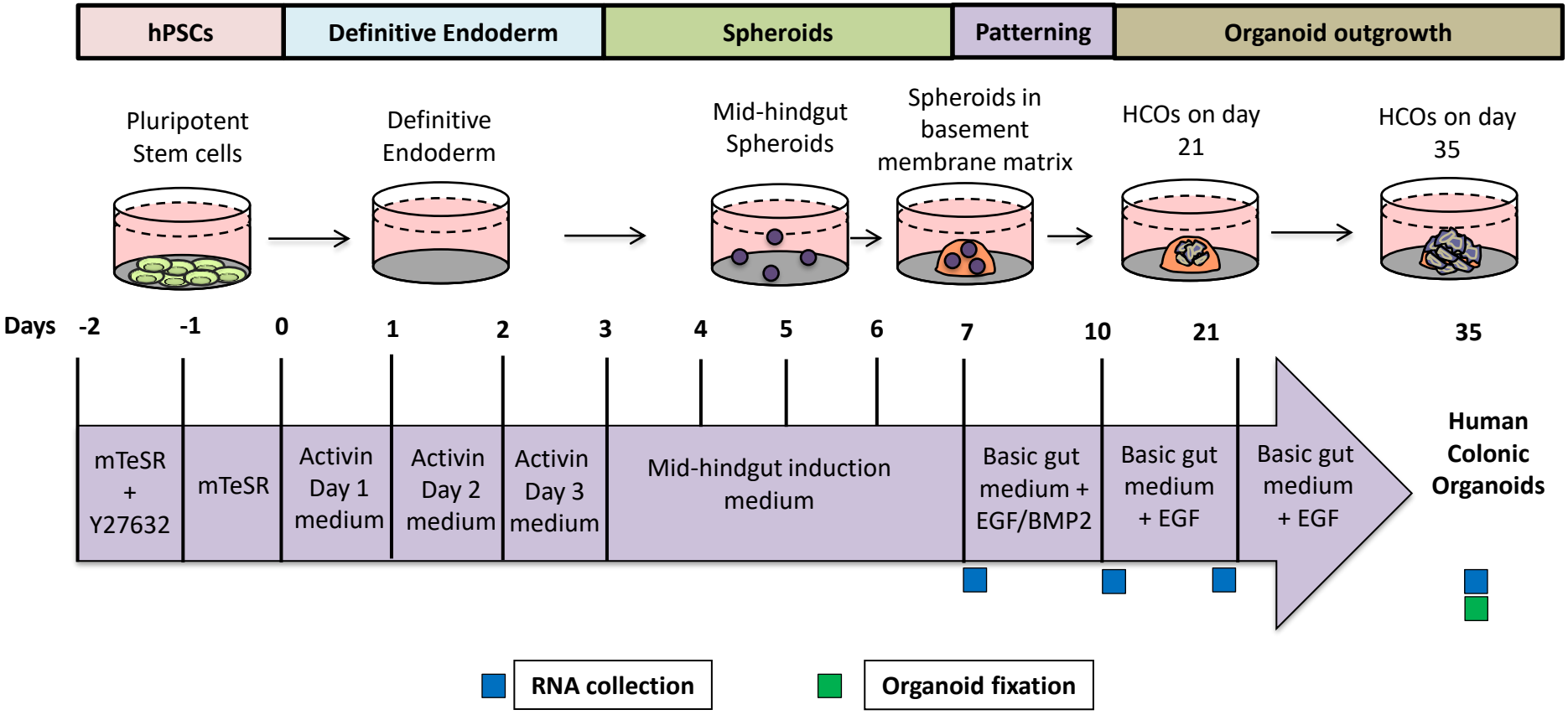

Figure 2

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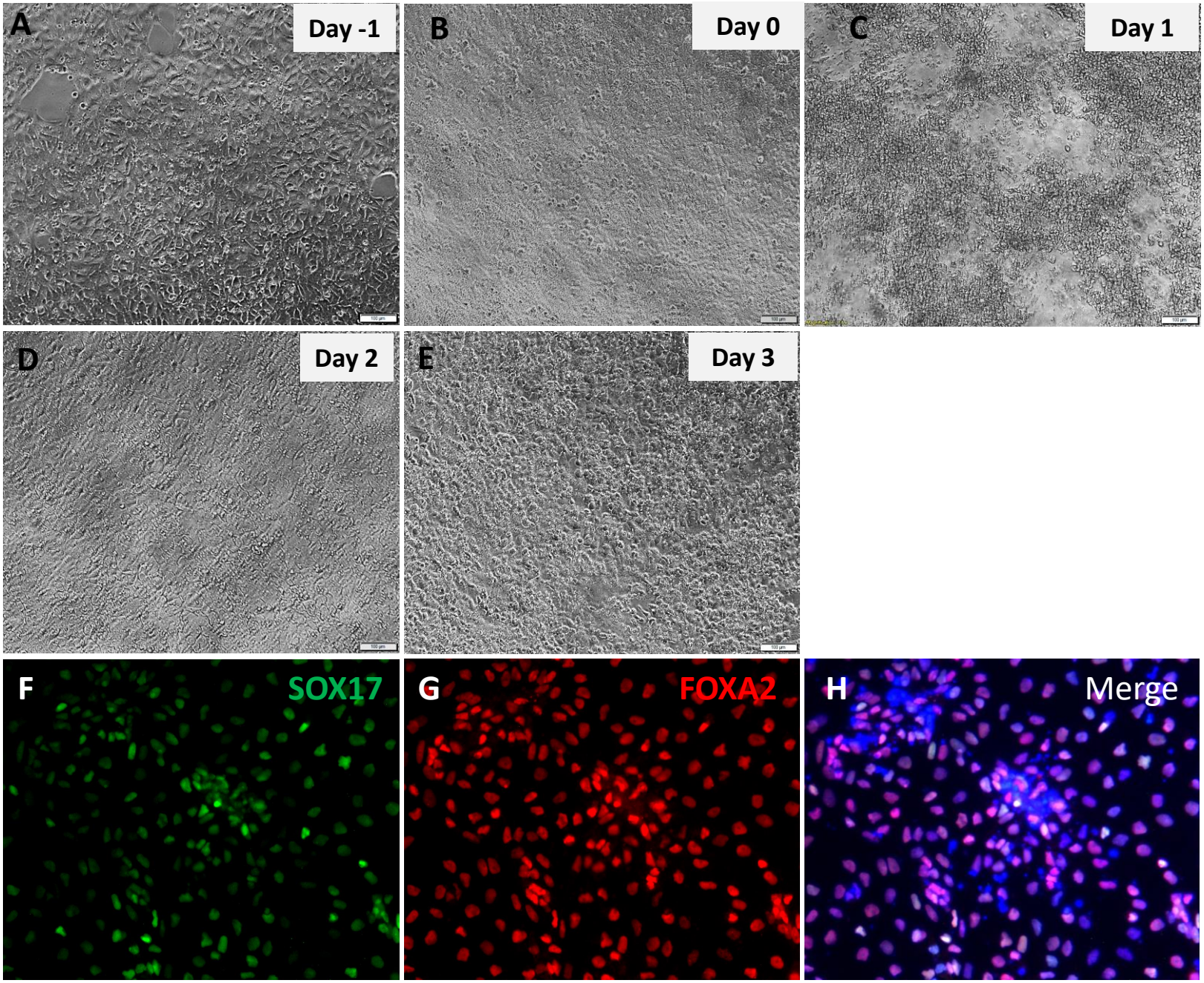
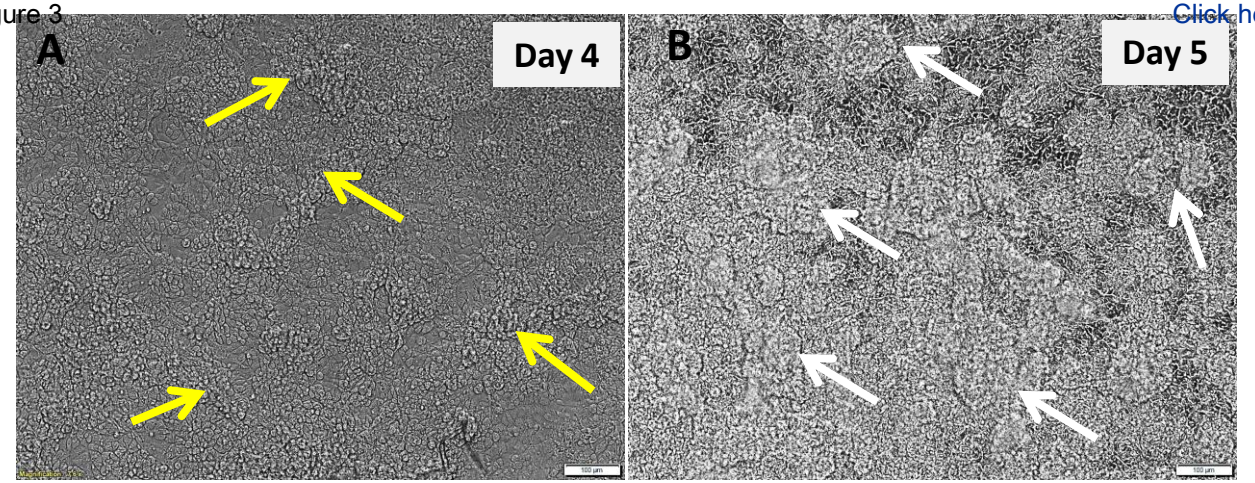
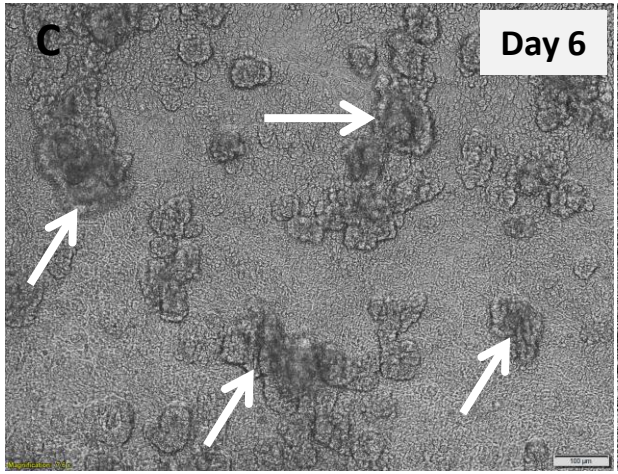
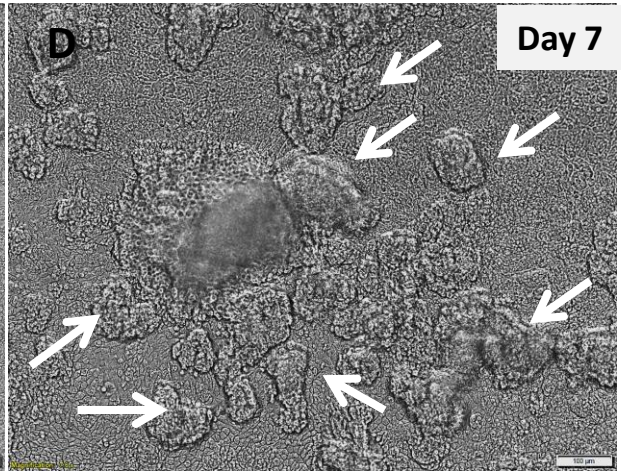
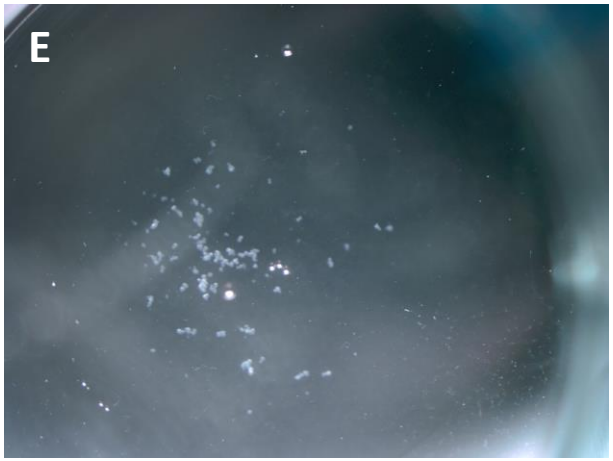


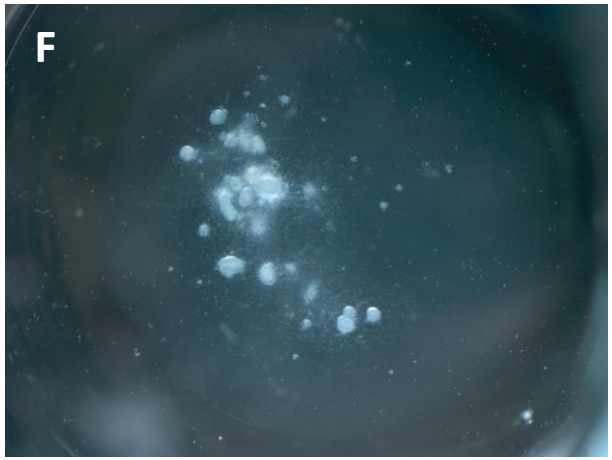
Figure 3











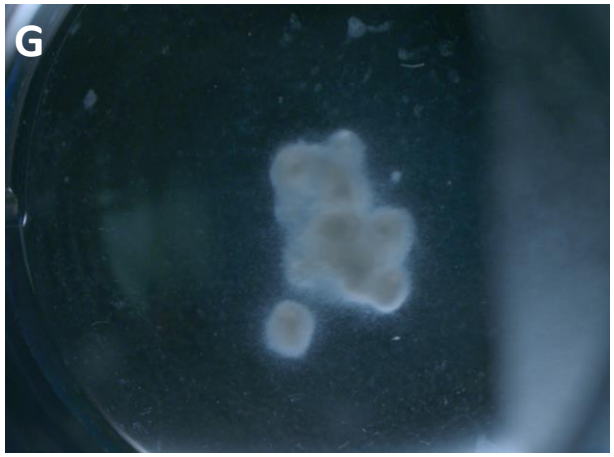


Figure 4

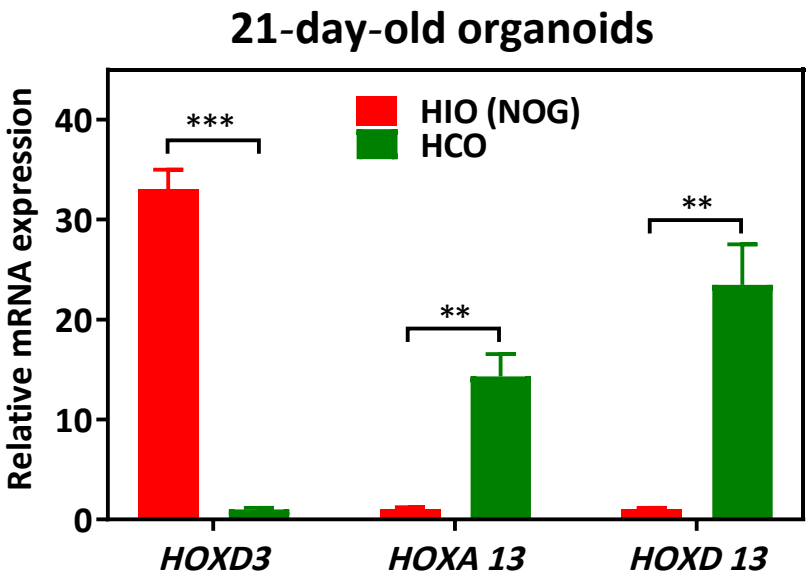


Figure 5

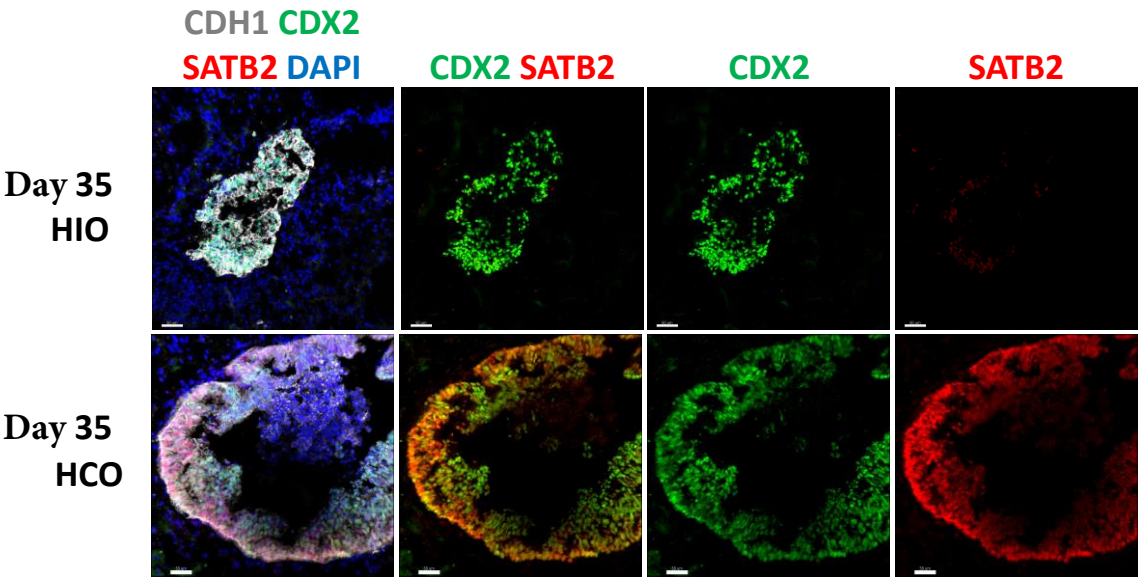


Table 1

	ECM (µL)	Spheroid suspension (µL)
6-Wells	375	120
12-Wells	750	240

Table 2

Gene	Primers	Notes:
CPHA	Forward: CCCACCGTGTTCTTCGACATT Reverse: GGACCCGTATGCTTTAGGATGA	Housekeeping gene
HOXD3	Forward: CACCTCCAATGTCTGCTGAA Reverse: CAAAATTCAAGAAAACACACACA	Anterior HOX gene
HOXA13	Forward: GCACCTTGGTATAAGGCACG Reverse: CCTCTGGAAGTCCACTCTGC	Posterior HOX gene
HOXD13	Forward: CCTCTTCGGTAGACGCACAT Reverse: CAGGTGTACTGCACCAAGGA	Posterior HOX gene
MSX2	Forward: GGTCTTGTGTTTCCTCAGGG Reverse: AAATTCAGAAGATGGAGCGG	Direct BMP target

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Table of Materials

Table 3 Jove_Material_Equipment List new JM.xlsx

Editorial comments: **We have addressed all the comments below.**

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.
2. Please provide an institutional email address for each author.
3. Please revise the following lines to avoid previously published work: 44-46, 123-124, 172-175,184-186, 267-269, 353-356, 364-367.
4. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).
5. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials.
For example: Matrigel, Falcon, Nunclon, kimwipes, MYFUGE, Fluoromount-G, etc.
6. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets or dashes.
7. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.
8. For time units, please use abbreviated forms for durations of less than one day when the unit is preceded by a numeral. Do not abbreviate day, week, month, and year. Examples: 5 h, 10 min, 100 s, 8 days, 10 weeks (Lines: 80, 84,87, 102, etc.)
9. Line 325: Please specify the parameters used for imaging (e.g., magnification)
10. Please include a one-line space between each protocol step and then highlight up to 3 pages of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.
11. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique
12. Please do not use the &-sign or the word “and” when listing authors in the references. Authors should be listed as last name author 1, initials author 1, last name author 2, initials author 2, etc. Title case and italicize journal titles and book titles. Do not use any abbreviations. Article titles should start with a capital letter and end with a period and should appear exactly as they were published in the original work, without any abbreviations or truncations.
13. Figure 1: Please remove commercial terms (Matrigel) from the figure and replace them with generic terms.
14. Please remove trademark (™) and registered (®) symbols from the Table of Equipment and Materials. Sort the table in alphabetical order.

_____

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

The is a review of the manuscript "Generation, maintenance, and characterization of human pluripotent stem cell derived intestinal and colonic organoids." In this manuscript, the authors are attempting to do what they suggest in the title.

Major Concerns:

- The authors should have three distinct sections of the paper that reflect the title. **We feel that this is difficult to do since the generation and maintenance overlap. We have streamlined section 3 to make the manuscript more succinct.**
- I don't agree with the use of the term hPSC as this is not used in the literature - the authors can abbreviate as they wish, but it must be representative of Human iPSC. **We use hPSC as an all-inclusive term for human embryonic stem cells and induced pluripotent stem cells. We have revised the text to reflect this.**

- It may be a formatting issue but it would be more helpful to have tables describing the different media used. **We have added media composition to the materials table**
- An image of how the iPSCs should look before DE is begun should be given. In my experience, its best to have iPSCs that are only 75% confluent. **The confluence for starting differentiation can vary depending on the iPSC line used. We've included the confluency that has worked for the cell line used in this manuscript.**

The authors may disagree but at the least they should show the confluence of iPSC in 24 well plates just before DE day 1 medium is added **Figure 2B shows that.**

- The timing is confusing to new readers attempting to enter the field. Regarding the term "HCOs on day 21"- some readers may read this as organoids being cultured for 21 days in Matrigel. Technically, these HCOs would be 14 days old and people don't characterize iPSC-derived organoids from time of initial iPSC development (ie endoderm day 1), its more when they add hindgut spheroids to Matrigel **Although timing from Matrigel plating is used in Spence et al. 2011 and Munera et al. 2017, other organoid papers from the same lab have used the same timing we used in this manuscript (McCracken et al. 2014 and 2017, Sinagoga et al. 2018). We feel that this is more straightforward.**
- Given the nature of this manuscript, images of organoids should be shown as they develop. There should be images of organoids after just being seeded in Matrigel, organoids at time of passage and at time of harvest. **We have added pictures of organoids at the different time points in figure 3.**
- While I understand that you cannot get HIOs or HCOs if you don't have endoderm, I think it would be useful if it was shown how to verify DE induction. It would be easy to show upregulation of FOXA2 or SOX17 or something along those lines after day 3 endoderm via qPCR. **We have included FOXA2 SOX17 IF to the protocol and figure 2.**
- I think a lot of space is taken up with sections 3.2 and 3.3. This is not specifically related to organoid culture and perhaps should be shortened. **We have shortened section 3.**
- The abstract needs to be more reflective of the manuscript. Starting off with statements about differences in tight junctions/cell populations/villi is not appropriate for this manuscript when none of these factors are discussed. It would be much more appropriate to state that organoids are useful but challenging to use etc and here is how you can make this technique more robust. **We thank the**

reviewer for their recommendation and we have edited the abstract accordingly.
- The name and equipment section needs more detail in the correct areas. The authors list the company and catalogue number for the hydrophobic pen but don't include the catalogue number for EGF, noggin and BMP2 which is crucial for colonic organoid generation. Also what is the value of adding in "HIO patterning medium" etc when company and catalogue number are N/A? **We thank the reviewer for pointing out this oversight and we have now edited the materials section. N/A is used for the HIO patterning medium since this medium is made up of components that are already have catalog numbers listed.**
- There is no legend for table 1. **We have added a legend.**

Minor Concerns:
- References typically aren't included in an abstract. Why were they added here? **We've removed references from the abstract.**
- Is REF 7 correct in the introduction? it appears REF 10 should be in its place **We've fixed this.**
- Remove "place fig 1 here" at end of first paragraph **This is for the journal editors to know where figures should go.**
- The writing needs to be more sophisticated - remove "just to name a few" in introduction **We've removed non-technical language from the manuscript.**
- Why is there the term "instructions" underlined in blue next to all the titles? **We've removed these.**
- Why are some parts of the manuscript highlighted in yellow? **This is for the journal editors to know which parts of the manuscript should be recorded.**

Reviewer #2:
Manuscript Summay:
In this manuscript, Munera et al described a detailed protocol to generate, maintain and characterize intestinal and colonic organoids from human pluripotent stem cells. Overall, the protocol is very clear and well written. It is highly appreciated that the authors include a lot of technical tips throughout the protocol, making it easier to be reproduced.

Major comments
The reviewer has only one major comment regarding to the abstract. The author presented an overall description of the human intestinal organoids and colonic organoid in the abstract, yet some of the information is not provided in the protocol. For example, the authors mentioned "HCOs containing all of the main epithelial cell types present in human colon as well as co-developing mesenchymal cells" and "HIOs and HCOs morphologically and molecularly resemble human developing small intestine and colon respectively", yet the manuscript did not provide relevant information to support the claims. Although these claims are supported by the previous publication of the author, they should be described in the Introduction but not abstract. **We have provided further details in the introduction.**

On the other hand, it might be helpful to provide some images of human developing small intestine and colon in the protocol for better comparison if possible. **Unfortunately, South Carolina does not allow use of fetal tissue for research.**

Minor comments
Title: a bar should be added between "cell" and "derived". **Done**

Line 12: the last common should be omitted. **Done**

Line 21: "the colon lacks villi and Paneth cells, has a higher tight junction number and integrity". **Done**

Line 43: reference 7 is inappropriate. **We have fixed this issue.**
Section 1.2: it would be helpful to separate this section into Day -1, Day 0, D1, etc.

Line 102: please provide the temperature for centrifugation. **Done**

Line 174: it is confusing what it means by "the Matrigel concentration is at least 75% in the droplet". **We've clarified this by adding a legend to table 1.**

Line 202: it is confusing what it means by "it is best to change the medium when it becomes yellow". Do the authors mean that the medium should not be changed until it is yellow? **We have changed this.**

Line 222: "less" should be changed to "fewer". **Done**

Line 226-227: it is hard to understand how to take up the organoids first then the Matrigel, as the Matrigel and the organoids should be mixed well according to line 224. **We have no provided a better description of how to do this.**

Line 295 and 309: the dilution factors of antibodies cannot be found in Table 3. **Dilution factors are present.**

The authors should improve the format of Comments/description section, as it is hard to read now. **We have rewritten the comments/description section.**

The authors should provide the storage time of each reconstituted reagents and media. **We thank the reviewer for the suggestion and have included storage time.**

Reviewer #3:
Manuscript Summary:
The authors have published a paper for generating human colon organoid from iPSC in cell stem cell, 2017. As acknowledged by authors, obtaining renewable and reproducible sources of HCO from PSC will benefit future in vitro human colonic disease modeling. Overall, the content is timely and intriguing but there are some concerns that needs to be addressed by additional minor revision before being published. The followings are some suggestions.

Major Concerns:
None

Minor Concerns:
*Line28, 53, 395: The authors mention the importance of mesenchymal cells, yet, no characterization protocols are provided. Adding representative proportions and any QC metrics at earlier stage will be helpful. **Although we agree that characterization of mesenchymal cells is important, this characterization has been done previously. We have revised the text to include an explanation that HOX factors are generally expressed in mesenchyme and therefore the protocol now at least addresses mesenchymal patterning.**

*Line37: Species differences are certainly of interest but need to be explicit. It would be great if the authors can expand how different between rodent and human. **We thank the reviewer for this suggestion. We've added text highlighting major differences between human and mouse intestinal development and cellular composition.**
*Line83 and onwards: this seems pre-differentiation of hPSC. What would be a routine passage/maintenance condition? **Routine passaging can vary from lab to lab which is why we start at the differentiation stage.**
*Line160: Any trouble shooting when no budding spheroids are observed? **We have now added passaging the HG monolayer as clumps as and alternative when spheroid generation is not possible.**
*Line170: What happens if one use hESC qualified Matrigel? If this is critical, please mention this. Also as Matrigel has variety of products, it would be helpful to elaborate the other critical factors: e.g., hESC qualified, Phenol red, growth factor reduced. **I'm not sure I understand the question. Does the reviewer mean what happens if one uses hESC qualified Matrigel for plating of spheroids? hESC qualified Matrigel is much more expensive then regular Matrigel which is why we don't use hESC qualified Matrigel for plating spheroids. We have not tested any of the critical factors but we provide the catalog number for each of the Matrigel types used.**