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Data Sheet

3dGRO® Human iPSC Derived Colon Organoids

Stem Cell Line

SCC300

Pack Size ≥ 1500 organoids/vial

Store at: Liquid Nitrogen

FOR RESEARCH USE ONLY
Not for use in diagnostic procedures. Not for Human or Animal Consumption.

Background

Human intestinal organoids are self-organizing, 3D structures that can be expanded in long-term culture and differentiate into intestinal epithelial cell types. Epithelial intestinal organoids, often referred to as enteroids or "mini-guts", maintain the physiological characteristics of the gastrointestinal system and have been a useful cell culture tool to model intestinal development and disease including the study of colon cancer, celiac disease, inflammatory bowel diseases and host microbiome interactions. Organoids can be propagated from patient biopsies and from embryonic (ES) and induced pluripotent stem cells (iPS cells).

3dGRO® Human iPSC Derived Colon Organoids are derived from integration-free human iPS cells (SCC271) reprogrammed using the Simplicon® RNA reprogramming technology (SCR550). Human iPS cells were differentiated using a multi-step process from definitive endoderm to posterior hind-gut endoderm and eventually into colon organoids (Figure 1.). The organoids express colon-specific markers including the posterior hindgut marker CDX2, α-carbonic anydrase II (CA-II), α-carbonic anhydrase IV (CA-IV), and goblet cell markers Mucin-2 and Mucin-5B. 3dGRO® Human iPSC Derived Colon Organoids can be propagated long-term by passaging every 10-12 days using 3dGRO® Human Colon Organoid Expansion Medium (SCM304).

Quality Control Testing

- Each vial contains ≥ 1500 organoids
- Mycoplasma: Negative
- Genotyped by STR analysis: Pass
- Human species verified
- Organoids are negative for HPV-16, HPV-18, Hepatitis A, B, C, and HIV-1 and 2 viruses by PCR
- Cell viability test: Pass

Storage and Handling

3dGRO® Human iPSC Derived Colon Organoids should be stored in liquid nitrogen.

MilliporeSigma

Materials Required (Not provided)

Products	Cat. No.	Notes
3dGRO® Human Colon Organoid Expansion Medium	SCM304	Thaw overnight at 2-8 °C or alternatively in an ice bucket filled with room temperature water**. Do not thaw at 37 °C**. Swirl to ensure even mixing as media components may have settled during storage. Inspect to ensure that the color of the medium is homogeneous and that there are no color gradations. Before use, allow medium to warm up to room temperature. Do not warm medium at 37 °C.
ROCK Inhibitor, Y-27632	SCM075	Make a 10 mM or 1000X stock by reconstituting 5 mg with 1.47 mL sterile water. Aliquot and store long-term at –20 °C.
Penicillin-Streptomycin Solution (100X)	TMS-AB2-C	Aliquot & store long-term at –20 °C.
3dGRO® Organoid Dissociation Reagent	SCM300	
3dGRO® Organoid Freeze Medium	SCM301	
Dimethyl Sulfoxide (DMSO)	D2650	
1X D-PBS without Ca^{2+} and Mg^{2+}	BSS-1006-B	
Primary & Secondary Antibodies	User defined	
Triton® X-100	T9284	
Blocking Buffer		
DAPI	D9542	
Nunc Lab-Tek® 8-well chamber slides	C7057	
Matrigel® Growth Factor reduced (GFR) Basement Membrane Matrix	Corning, 356231	Thaw and maintain on ice. Make 1 mL aliquots. Aliquot the amount needed and maintain on ice. Store unused aliquots at –20 °C.
24-well tissue culture treated plates	Thermo Fisher, 142475	
Cryovials	Thermo Fisher, 156570	
8% paraformaldehyde Solution	Electron Microscopy Sciences 157-8-100	

Representative Data

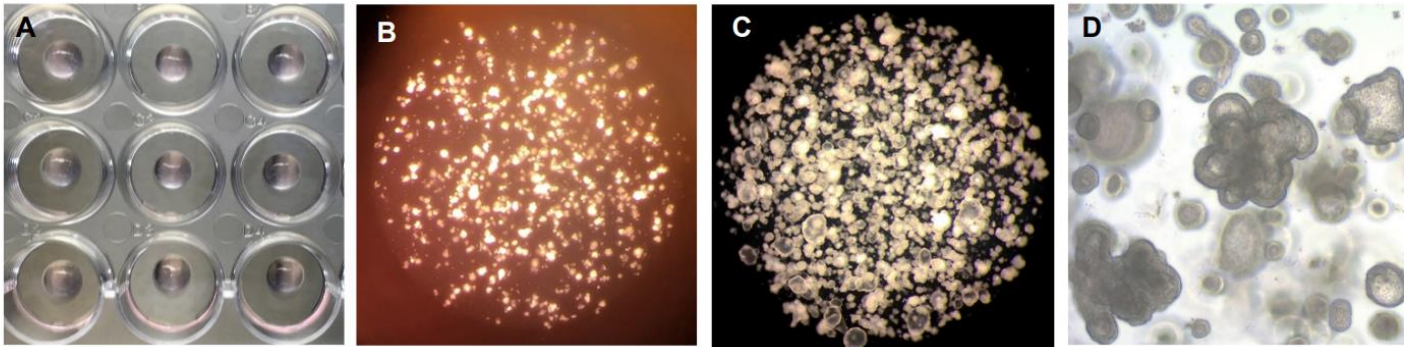

Figure 1. Human iPSC Derived Colon Organoids. (**A**) Colon organoids encapsulated in Matrigel® domes. (**B**) 2 days after thaw. (**C**) By days 10-12 in culture, colon organoids occupy approximately 85-90% of the dome and are ready to be passaged. (**D**) Images in B and C were captured on a dissecting microscope. Bright-field image of colon organoids cultured for 10 days.

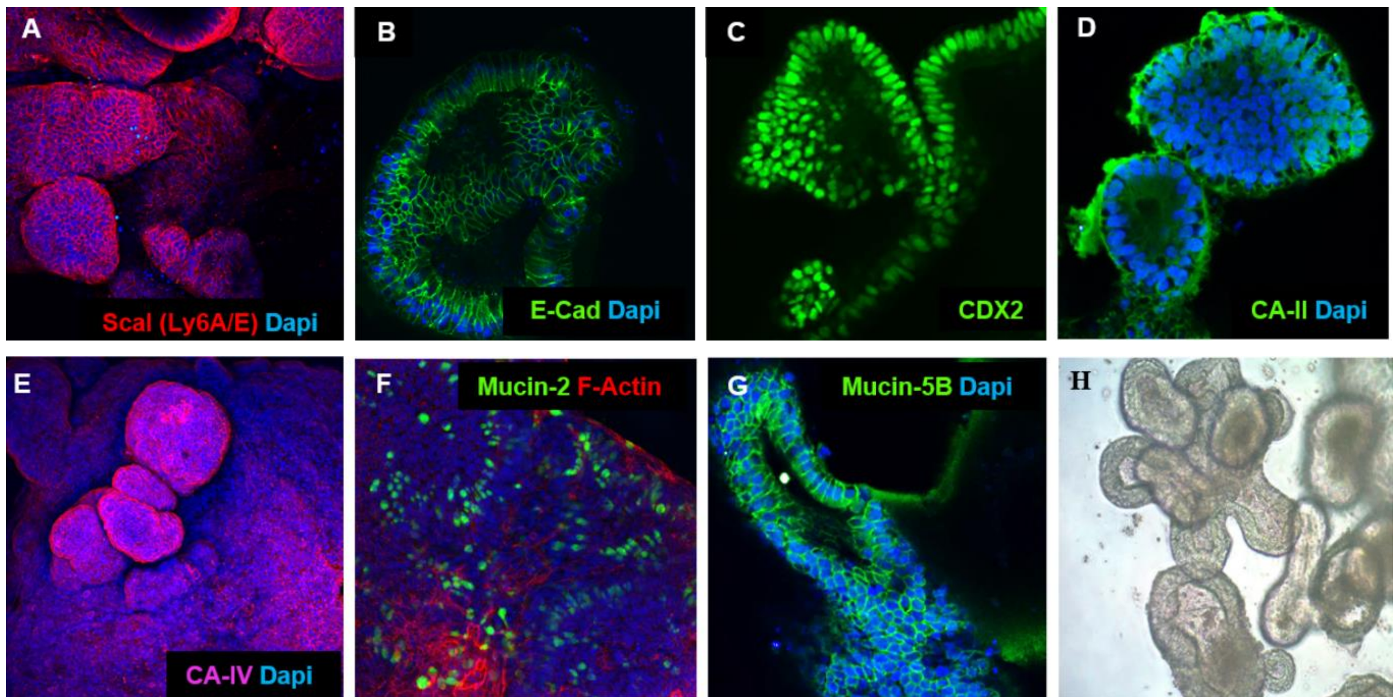

Figure 2. Immunocytochemical Characterization of Human Colon Organoids. (**A**) Organoids express the stem cell marker Sca-1 (Ly6A/E), (**B**) the epithelial marker E-cadherin (**C**) along with colonic markers including the posterior hindgut marker CDX2, (**D**) α-carbonic anhydrase II (CAII), (**E**) α-carbonic anhydrase IV (CA-IV), (**F**) pan-goblet cell marker Mucin-2, (**G**) and the colon-specific goblet cell marker Mucin 5B. (**H**) Phase bright image of colon organoids.

Protocols

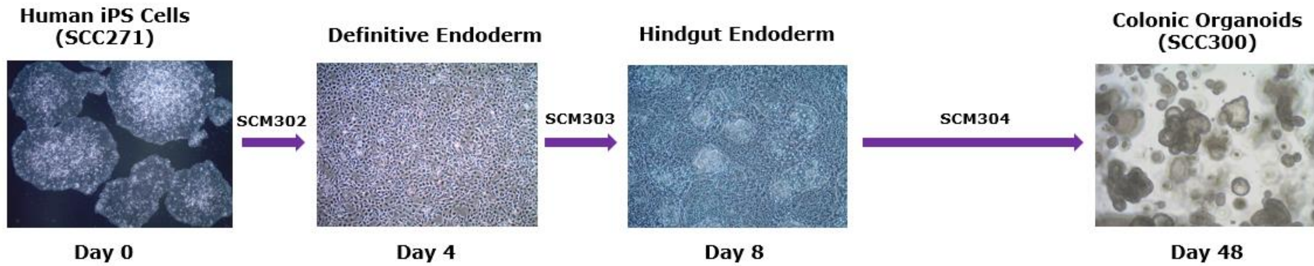

Figure 3. Human Colonic Organoid Differentiation Protocol. Human colonic organoids were generated from human iPS cells using a 3-step differentiation protocol through definitive endoderm, hindgut endoderm and colonic organoid expansion stages. SCM302: Definitive Endoderm Induction Medium, SCM303: Hindgut Induction Medium, SCM304: 3dGRO® Human Colon Organoid Expansion Medium.

Important Notes before Starting

- The term "domes" refer to organoids that are 3D encapsulated in Growth Factor Reduced (GFR) Matrigel® (Corning®, 356231).
- While not necessary, it is highly recommended that organoids are cultured in medium containing penicillin and streptomycin to prevent contamination that may be introduced during the long culture process.

Thawing into 24-well plates

1. Before thawing, prepare sufficient Growth Factor Reduced (GFR) Matrigel® for 12 domes at 25 µL per dome + 5% overage (315 µL total).

   Note: GFR Matrigel® will gel at room temperature; maintain on ice at all times.
2. Prepare 3dGRO® Expansion Medium (SCM304) supplemented with 1X Penicillin/Strep and 10 µM ROCKi. For example, add 250 µL of 100X Pen/Strep solution and 25 µL of 10 mM ROCKi solution (10 µM final) to 25 mL 3dGRO® Expansion Medium. For media containing ROCKi, prepare fresh on the day of media change.
3. Remove the vial of cryopreserved organoids from liquid nitrogen storage and quickly thaw in a 37 °C water bath. Closely monitor until only small ice crystals remain. Quickly remove the vial from the water bath.

   Important: Do not vortex the vial or leave in the water bath for too long. Disinfect the outside of the vial with 70% ethanol or isopropanol.
4. In a laminar flow hood, use a 1- or 2-mL pipette to transfer the organoid suspension to a sterile 15 mL conical tube containing 9 mL 3dGRO® Expansion Medium supplemented with 1X Pen/Strep and 10 µM ROCKi (from step 2) Be careful not to introduce any air bubbles during the transfer process.
5. Centrifuge at 1100 rpm for 5 minutes at 4 °C. Carefully aspirate the supernatant by connecting a P-200 pipette tip to the end of an aspirating pipette. Be careful not to aspirate the organoid pellet.
6. Immediately transfer 315 µL of the ice-cold GFR Matrigel® to the organoid pellet. GFR Matrigel® may be viscous; ensure even mixing of the organoid suspension with GFR Matrigel® by pipetting up and down several times with a P-1000 pipette. Be careful not to introduce air bubbles during pipetting. Place on ice for 3-5 minutes to cool down the organoid suspension.

   Tip: Set the pipette to 290 µL instead of 315 µL and resuspend the organoid pellet as quickly as you can. This will minimize air bubbles during pipetting.
7. Set a P-200 pipette to 25 µL. Swirl the 15 mL conical tube containing the organoid Matrigel® suspension to mix. Dispense 25 µL of the organoid suspension into the center of each well of a 24-well plate. (See Figure 1A).

   Note: Do this as quickly as possible to prevent gelling of the organoid suspension. Total number of domes is equal to 12. Minimize air bubbles during pipetting.
8. Incubate in a 37 °C, 5% CO_2 humidified incubator for 10 minutes. This will allow sufficient time for the organoid suspension to form a solid 3D "dome". (See Figure 1A and 4B).

9. Gently add 1 mL of 3dGRO® Expansion Medium supplemented with 1X Pen/Strep and 10 µM ROCKi into each well containing the organoid domes. To avoid disturbing the domes, dispense the media onto the side of the wells.
10. Incubate in a 37 °C, 5% CO_2 humidified incubator overnight.
11. Next day, inspect the organoids with a bright-field microscope. Live organoids should be rounded in shape and not fragmented (dead). Replace with freshly made 3dGRO® Expansion Medium supplemented with 1X Pen/Strep and 10 µM ROCKi. Incubate at 37 °C overnight.
12. On the second day after thaw, replace with fresh 3dGRO® Expansion Medium supplemented with 1X Pen/Strep and 10 µM ROCKi. Incubate at 37 °C overnight.

    Note: ROCKi is only added for the first 2 days after thaw to enhance cell viability. Thereafter, ROCKi is no longer necessary.
13. Replace the media every other day with 3dGRO® Expansion Medium supplemented with 1X Pen/Strep.

    Note: Media should NOT contain ROCKi.
14. Organoids may be passaged every 10-12 days of culture. Do not exceed 12 days before passaging.

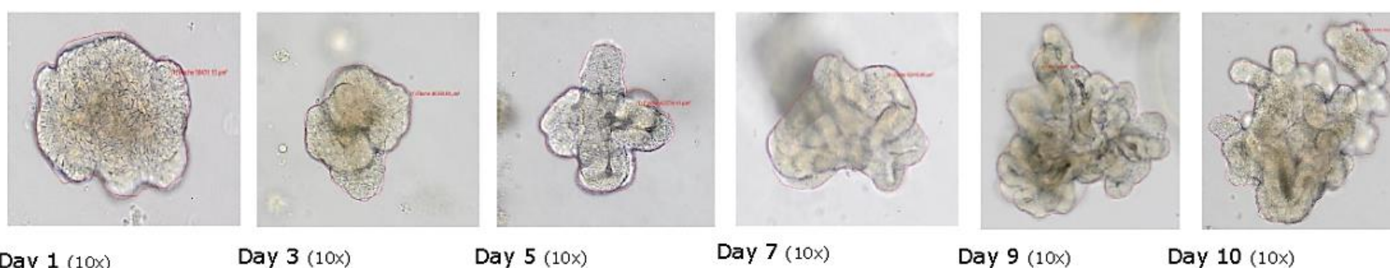

Figure 4. Morphology And Growth of Thawed Human Colonic Organoids. Human colonic organoids were expanded over a 10-day period and an overall increase in mean length, area and villus-like and crypt-like domain formation was observed.

Passaging with 3dGRO® Organoid Dissociation Reagent (SCM300)

Important notes before starting

- At the time of passage, the optimal organoid density inside the domes should be approximately 70-90%. (See Figure 1C). Start with a split ratio of 1:3 for each dome. You can increase the split ratio to 1:4 or higher once you are comfortable with culturing organoids. Organoids should be passaged every 10 days. 12 days is the maximum interval before organoids must be passaged.
- For the first 2 days after every passage, the 3dGRO® Expansion Medium should contain 10 µM ROCKi to enhance cell viability of the organoids. After the second day of passage, ROCKi is no longer necessary.

1. Aspirate the medium from each well containing an organoid dome.
2. Add 1 mL of 1X PBS to each well. Pipette up and down 5-10 times with a P-1000 pipet to break up the organoid domes and release the organoids. Do this for each well containing organoid domes.
3. Combine the organoid suspension from all the wells and transfer to a sterile 15 mL conical tube.
4. Centrifuge at 1100 rpm for 5 minutes at 4 °C.
5. Carefully aspirate the media by connecting a P-200 pipette tip to the end of an aspirating pipette. Be careful not to aspirate the organoid pellet.

   Note: A layer of Matrigel® may be visible on top of the organoid pellet. Carefully remove as much of the Matrigel® as possible using a P-200 pipette tip.
6. Add 1 mL 3dGRO® Organoid Dissociation Reagent (SCM300) to the conical tube. Pipette up and down 10 times with a P-1000 pipette tip.
7. Add an additional 4 mL 3dGRO® Organoid Dissociation Reagent to the conical tube. Gently rock or rotate the conical tube at room temperature for 10 minutes to dissociate the organoids.
8. Centrifuge at 1100 rpm for 5 minutes at 4 °C.

9. Carefully aspirate the media by connecting a P-200 pipette tip to the end of an aspirating pipette. Be careful not to aspirate the organoid pellet.
10. Wash the pellet with 5 mL 1X PBS. Centrifuge at 1100 rpm for 5 minutes at 4 °C.
11. Carefully aspirate the media by connecting a P-200 pipette tip to the end of an aspirating pipette. Be careful to not aspirate the organoid pellet.
12. Quickly add the appropriate amount of the thawed GFR Matrigel® to the organoid pellet with a P-1000 pipette tip and resuspend 10 times. Avoid generating air bubble during pipetting.

    Note: 25 µL GFR Matrigel® is required per dome. Scale up according to the amounts required.
    For example, 12 domes that is passaged at 1:3 split ratio will yield 36 domes at 25 µL + 5% overage = 945 µL of GFR Matrigel® required.
13. Immediately place the conical tube containing the organoid suspension on ice for 3-5 minutes to cool down. Remove the tube from ice and spray the outside of the tube with 70% ethanol or isopropanol before putting it back into the TC hood.
14. Quickly pipette the suspension up and down with a P-1000 pipette set at 100-200 µL below the actual amount. For example, if the actual volume is 1 mL, set the P-1000 to 800-900 µL to mix. This will minimize the generation of air bubbles during pipetting.
15. Quickly dispense 25 µL of the organoid suspension to the center of each well of a 24-well plate. Incubate in a 37 °C, 5% CO_2 humidified incubator for 10 minutes to allow the suspension to solidify into 3D domes.
16. Gently add 1 mL 3dGRO® Expansion Medium supplemented with 1X Pen/Strep and 10 µM ROCKi to each well containing an organoid dome. Incubate in 37 °C, 5% CO_2 humidifier incubator.
17. For the next two days after passage, replace with freshly made 3dGRO® Expansion Medium supplemented with 1X Pen/Strep and 10 µM ROCKi to each well.
18. On the third day after passage, change media every other day with 3dGRO® Expansion Medium that does NOT contain ROCKi.
19. Colon organoids should be monitored daily and passaged every 10-12 days. For the first 2 days after EVERY passage, add 10 µM ROCKi to the 3dGRO® Expansion Medium to enhance cell viability. ROCKi is not necessary from days 3 onwards until the next passage.

Cryopreservation

Important Notes Before Starting

- We recommend freezing 4 domes to 1 cryovial. The average organoid density of each dome should be approximately 90% at the time of freezing. (See Figure 1C). If the density is less than 90%, increase the number of domes frozen per vial.
- Colon organoids may be frozen in 3dGRO® Expansion Medium supplemented with 1X Pen/Strep, 10 µM ROCKi and 10% DMSO or in 3dGRO® Organoid Freeze Medium (SCM301).

1. Prepare appropriate volumes of 3dGRO® Expansion Medium supplemented with 1X Pen/Strep,10 µM ROCKi and 10% DMSO. Alternatively, 3dGRO® Organoid Freeze Medium (SCM301) may be used.
2. Prepare and label the appropriate number of cryovials needed. Prepare Mr. Frosty® containers with the appropriate volume of isopropanol.
3. Aspirate the culture medium from each well containing an organoid dome. Add 1 mL ice-cold 1X PBS to each well. Break the organoid domes into smaller pieces by pipetting up and down 10 times with a P-1000 pipette. Combine and transfer the organoid mixture from each well to a 50 mL conical tube.
4. Rinse the wells with 0.5 mL ice-cold PBS to collect any residual organoid pieces. Transfer the contents to the conical tube. Centrifuge at 1100 rpm for 5-10 minutes at 4 °C.

    Note: For >20 domes, increase centrifugation speed to 1800 rpm.
5. Carefully aspirate the supernatant by connecting a P-200 pipette tip to the end of an aspirating pipette. Be careful to not aspirate the organoid pellet.
6. Add 1 mL ice cold PBS to wash the pellet. Pipette up and down 10 times to further break up the organoids.
7. Add 9 mL ice-cold PBS to the suspension. Centrifuge the suspension at 1100-1800 rpm for five minutes at 4 °C.

    Note: For > 20 domes, increase centrifugation speed to 1800 rpm.

8. Check the pellet to see if there are residual organoid fragments that are floating in the supernatant layer. If fragments are present in the supernatant layer, centrifuge for an additional 5 minutes at 1800 rpm at 4 °C.
9. Carefully aspirate the supernatant by connecting a P-200 pipette tip to the end of an aspirating pipette. Be careful to not aspirate the organoids pellet.
10. Resuspend the organoid pellet in a suitable amount of ice-cold 3dGRO® Expansion Medium containing 10 µM ROCKi and 10% DMSO. Alternatively, 3dGRO® Organoid Freeze Medium (SCM301) may be used. Gently pipette up and down a few times to ensure even mixing.
11. Aliquot 1 mL into labeled cryovials. Place the cryovial(s) in a Mr. Frosty® container with isopropyl alcohol.
12. Transfer the freezing container to a –80 °C freezer; 24 hours later, transfer the cryovial to liquid nitrogen (–135 °C) for long term storage.

Whole Mount Immunocytochemistry

Important Notes before Starting

- The following protocol is meant to serve as a guidance for first time users and is based on organoids cultured in 24-well plates. The protocol may be modified and adapted once users are more familiar with the process.
- We recommend using a pair of scissors that have been sterilized with 70% ethanol or isopropanol to cut the ends of P-1000 tips to enlarge the opening. Modified P-1000 tips are used to transfer fixed organoids without shearing them. Do not use serological pipettes as they are too bulky to handle small volumes and organoids may stick to the side of the pipettes.
- During PBS washes, gravity is used to collect organoids. Do not use centrifugation as the centrifugal force will result in mis-shapened organoids.
- 4% paraformaldehyde performs the dual function of fixing the organoids and to help partially dissolve the Matrigel® and release the organoids. It is important to remove as much of the Matrigel® as possible from the organoids. Matrigel® may result in increased background autofluorescence. The more confluent the organoids are inside the domes at the time of fixing, the more readily the Matrigel® will dissolve.

1. Prepare a 4% paraformaldehyde (PFA) solution by diluting an 8% PFA Solution (Electron Microscopy Sciences,157-8-100) 1:1 with 1X PBS.
2. Prepare modified P-1000 and P-200 pipette tips by cutting the ends with a sterilized scissor.
3. Aspirate the medium from each well containing an organoid dome. Wash each well twice with 1 mL 1X PBS. Aspirate between PBS washes.
4. Add 1 mL of the 4% PFA solution to each well. Incubate 45-60 minutes at room temperature on a gently rocking or shaking platform. The shaker/rocker will help expedite detaching the Matrigel® domes and the release of the organoids from the Matrigel® matrix.

   Note: GFR Matrigel® domes will partially dissolve when fixed in PFA. At the end of the incubation period, you will notice that many (but not all) the domes are dislodged and that some of the organoids (but not all) will have been released from the domes.
5. Using the modified P-1000 pipette tips, collect any released organoids along with the fixative solution and transfer the contents to a 50 mL conical tube. Allow the organoids to settle to the bottom of the conical tube by gravity (approximately 10-15 minutes). DO NOT CENTRIFUGE.
6. In the meantime, add 1 mL 1X PBS per well to the 24-well plate containing the organoid domes. Incubate 10-15 minutes at room temperature. This is done to dilute the PFA in the dome.
7. Carefully aspirate the fixative from the conical tube containing the released organoids (from step 5) and leave a small amount of liquid behind. This will ensure that the organoid pellet will not be aspirated off.
8. Using modified P-1000 pipette tips, collect any released organoids along with the PBS solution from each well (from step 6) and transfer the contents to the 50 mL conical tube. Allow the organoids to settle to the bottom of the conical tube by gravity (approximately 10-15 minutes).
9. Repeat steps 5-8 two more times.
10. Add 0.8 mL of 1X PBS into each well that contains residual organoid domes.
11. Carefully aspirate the supernatant from the conical tube containing released organoids. Leave a small amount of liquid behind. This will ensure that the organoid pellet will not be aspirated off.

12. Add 4.8 mL 1X PBS to the organoid pellet. Swirl the conical tube to resuspend the organoid pellet. Using a modified P-1000 tip, transfer 200 µL of the organoid suspension into each well containing the 0.8 mL volume of residual organoid domes (from step 10).

    Note: Some organoids may stick to the modified P-1000 tip.

13. If staining will not be performed immediately, seal the 24-well plate containing fixed organoids with parafilm and store in the fridge at 2-8 °C for up to 1 month.
14. When ready to perform ICC, transfer the 24-well plate containing the fixed organoids to a dissecting microscope.
15. Using modified P-200 tips (from step 2), pipette 1-4 organoids into each well of an 8-well chamber slide. Remove any residual PBS using an unmodified P-200 pipette tip. Avoid accidentally pipetting up the organoids and shearing them through the P-200 tip.
16. Add 0.4 mL Blocking Buffer (5% horse serum + 0.5% Triton® X-100 in 1X PBS) to each well of an 8-well chamber slide containing the fixed organoids. Block at 2-8 °C overnight or at room temperature for 2-4 hours.

    Note: Use the serum from the same species as the host secondary antibody.

17. Using an unmodified P-200 pipet, remove the blocking buffer while tilting the chamber slide. Avoid pipetting the organoids through the p200 tip.
18. Prepare primary antibodies or directly conjugated antibodies (300-500 µL) in Blocking Buffer.
19. Add primary antibodies. Incubate overnight at 2-8 °C on a gently shaking or rotating platform.
20. Next day, wash 3X with 1X PBS for 10-15 minutes each on the shaking/rotating platform.

    Note: Do not use centrifugation. Remove the PBS with a P-200 pipet after each wash while tilting the chamber slide.

21. Prepare secondary antibodies (300-500 mL) in Blocking Buffer.
22. Add secondary antibodies. Incubate overnight at 2-8 °C on a gently shaking or rotating platform.
23. Next day, wash with 1X PBS for 10-15 minutes on the shaking/rotating platform.

    Note: Do not use centrifugation. Remove the PBS with a P-200 pipet after each wash while tilting the chamber slide.

24. Counterstain with DAPI (5 µg/mL in 1X PBS) for 15-20 minutes.
25. Wash 3X with 1X PBS for 10-15 minutes each on the shaking/rotating platform.

    Note: Do not use centrifugation. Remove the PBS with a P-200 pipet after each wash while tilting the chamber slide.

26. Add 300-400 µL of 1X PBS into each well. Samples are now ready to be imaged on a confocal microscope.

References

1. Clevers H et al. (2011) Long-term expansion of epithelial organoids from human colon, adenoma, adenocarcinoma, and Barrett's epithelium. Gastroenterology 141(5): 1762-1772.
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4. Crespo M et al. (2017) Colonic organoids derived from human induced pluripotent stem cells for modeling colorectal cancer and drug testing. Nat Med. 23(7): 878-884.

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