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Protocol for the establishment and characterization of South African patientderived intestinal organoids

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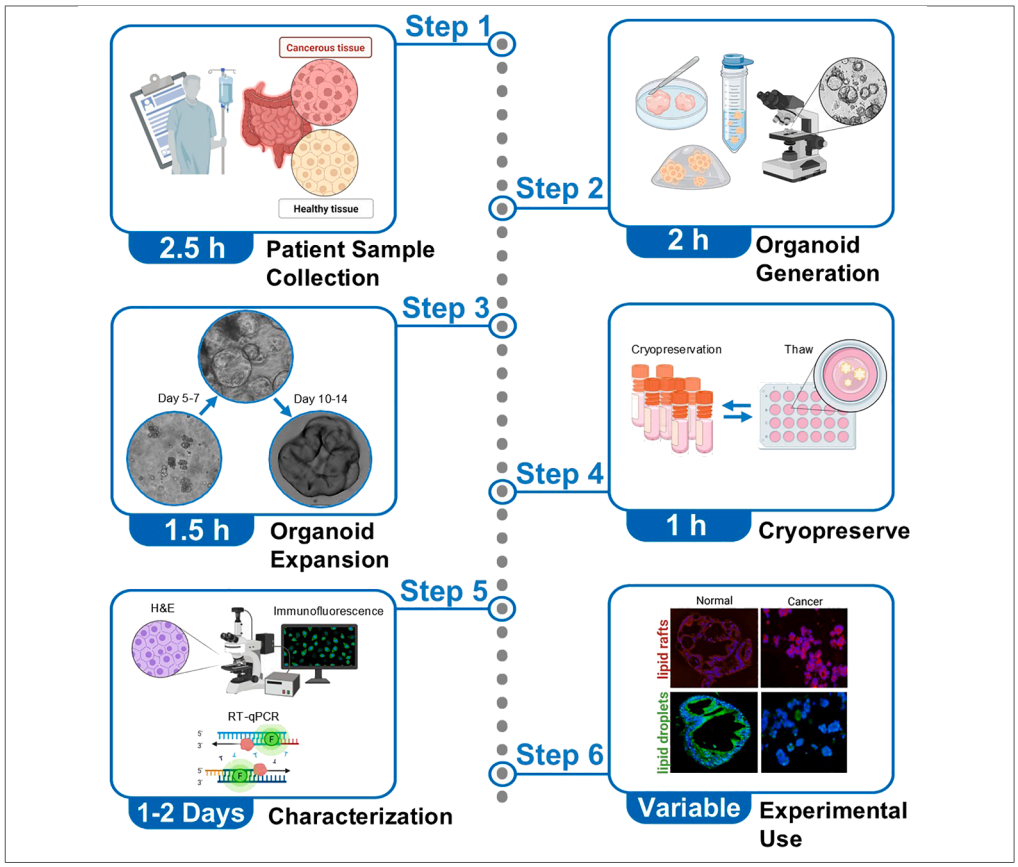
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Protocol

Protocol for the establishment and characterization of South African patient-derived intestinal organoids



Patient-derived organoids are valuable for modeling disease pathogenesis, therapeutic screening, and advancing personalized medicine. Here, we present a protocol for generating and characterizing intestinal organoids from South African patients, using both healthy and cancerous tissues. We describe steps for generating organoid cultures, quantitative reverse-transcription polymerase chain reaction (RT-qPCR), immunofluorescence microscopy, and histological staining. Notably, we provide a detailed method for cholesterol profiling in organoids and tissues, offering a powerful tool for researchers exploring lipid biology in organoid systems.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

Naaziyah Abdulla, Ruth Aronson, Thea-Leonie du Plessis, Brendan Bebington, Mandeep Kaur

1348887@students.wits.ac.za (N.A.)
mandeep.kaur@wits.ac.za (M.K.)

Highlights

Establishment and maintenance of intestinal organoids from South African patients

Characterizing tissue and organoids using microscopy and transcriptional analysis

Processing of intestinal tissue and organoid cultures for histological analysis

Profiling cholesterol content in intestinal tissue and organoid cultures

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Protocol for the establishment and characterization of South African patient-derived intestinal organoids

Naaziyah Abdulla,[1,2,4,*] Ruth Aronson,[1] Thea-Leonie du Plessis,[1] Brendan Bebington,[3] and Mandeep Kaur[1,2,5,*]

[1]School of Molecular and Cell Biology, Faculty of Science, University of the Witwatersrand, Private Bag 3, Johannesburg, South Africa

[2]Infectious Diseases and Oncology Research Institute (IDORI), Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, South Africa

[3]Wits Donald Gordon Medical Centre, Parktown, Johannesburg, South Africa

[4]Technical contact

[5]Lead contact

*Correspondence: 1348887@students.wits.ac.za (N.A.), mandeep.kaur@wits.ac.za (M.K.)
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SUMMARY

Patient-derived organoids are valuable for modeling disease pathogenesis, therapeutic screening, and advancing personalized medicine. Here, we present a protocol for generating and characterizing intestinal organoids from South African patients, using both healthy and cancerous tissues. We describe steps for generating organoid cultures, quantitative reverse-transcription polymerase chain reaction (RT-qPCR), immunofluorescence microscopy, and histological staining. Notably, we provide a detailed method for cholesterol profiling in organoids and tissues, offering a powerful tool for researchers exploring lipid biology in organoid systems.

BEFORE YOU BEGIN

Human intestinal organoids (HIOs) are defined as miniature ex-vivo versions of the human intestinal epithelium that develop from multipotent stem cells.[1–5] The crypt can be defined as the smallest functional unit of colonic mucosa which house the stem cells that are crucial to facilitate the culturing of HIOs.[6] Upon crypt isolation, crypts embedded in an extracellular matrix are exposed to a growth factor cocktail facilitating the derivation of organoids, its proliferation as well as long-term expansion.[5] Following the withdrawal of stem cell promoting factors, organoids adopt budding cyst-like structures with distinct crypt-villus axis comprising proliferative intestinal stem cells (ISCs), progenitor cells as well as multiple differentiated epithelial cell types.[4] This in turn serves as a powerful platform to elucidate the underlying biological mechanisms that dictate intestinal epithelial physiology.[7] Here, we detail a protocol for the successful generation of human intestinal organoids from colonic crypts isolated from South African patient-derived healthy and cancerous tissue fragments. Organoids grown in this manner successfully recapitulate characteristics of patient-derived tissue enabling these models to serve as a promising platform to study South African disease pathogenesis and in the prediction of patient response to various therapeutics. This is crucial based on emerging evidence indicating that individuals of African descent are more often diagnosed at earlier age with CRC and present with a more advanced disease.[8] Furthermore, increased African ancestry is also associated with higher odds of *APC*, *KRAS* and *PI3KCA* mutations and CMS3 tumors with a lower odd of *BRAF* mutations.[9] This is in contrast to European populations that display significantly higher levels of *BRAF* mutations.[10] Based on the data obtained we anticipate that this protocol could serve as a robust means to generate patient-derived organoids from a wide array of intestinal cancers with varying molecular characteristics. It is important to emphasize that the success of this protocol is

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largely attributed to the quality of the initial specimen obtained. The protocol requires rapid and meticulous processing of the initial specimen and consistent and timeous maintenance of cultures to ensure a successful outcome. Successful establishment of these lines potentiates their use in a range of translational applications thus paving the way for innovative population-specific therapeutic approaches.

The required transportation and digestion buffers (see 'buffers and media composition') should be prepared prior to collection of the patient specimens so that the specimens may be processed as rapidly as possible. Some buffers are stable for storage and hence may be prepared in advance whereas others need to be prepared fresh and used on the same day.

Institutional permissions

Studies conducted on patient – derived specimens require reviewing and approval by the designated Human Medical Research Ethics Committee. Following approval, patients at the listed sites need to be consented to participate in the study. Patients should be well – informed as to the procedures that will be undertaken following collection of the specimens emphasizing that confidentiality of patient information is crucial and that specimens will be processed in accordance with the approved protocols as listed in the relevant ethics application.

The current project has been reviewed and accepted by the Wits University Human Medical Research Ethics Committee with the ethics clearance number: M210233. All specimens employed in this study have been obtained following informed patient consent. The inclusion criteria for this study were South African individuals between the ages of 18–85 diagnosed with colon or rectal cancer. A thorough standard operating procedure is in practice to ensure that patient confidentiality is always maintained, and under all circumstances. All specimens are processed in accordance with the approved protocols listed in the ethics application.

Treat all tissue as potentially infectious. Personal protective equipment (PPE) must be worn throughout the procedure, including a lab coat and disposable gloves. All protocols outlined in this document should be conducted in a Biosafety Level II Facility, where human tissue is strictly processed under a laminar flow cabinet employing routine aseptic technique. Solid and liquid waste as well as empty containers must be disposed of as per Institutional Biohazard Waste Management Policy and local regulations. Institutional policies regarding the handling and disposal of chemicals and sharps must be adhered to. All equipment must be sterile and standard sterile techniques employed.

Sample collection preparation

Timing: 30 min

This section describes the procedure for preparing the sample collection box for specimen retrieval.

⚠ CRITICAL: Components required for collection and transportation of specimens should be prepared on the day before the surgery.

1. Print project information leaflet, patient consent forms, and medical questionnaire.
2. Label the appropriate falcon tubes, cryovials and petri-plates with the internal patient file reference number and the date.
3. Prepare the tissue transport medium (See 'buffers and media composition', Tissue Transport Medium) and aliquot 20 mL of medium per falcon to two labeled falcons corresponding to sample type, namely tumor core (C) and a normal healthy matched control sample (N).

Note: In the event that additional samples are being collected, prepare the respective tubes accordingly.

4. Prepare tissue wash buffer solution (See 'buffers and media composition', Tissue Wash Buffer Solution).

Note: All reagents, buffers, media and aliquots thereof should be prepared in a sterile environment using a laminar flow hood.

5. Store both the tissue transport medium and tissue wash buffer solution (See 'buffers and media composition') at 4°C until ready to use.
6. Prepare a polystyrene icebox by lining the base and the sides of the box with ice packs.
7. Place a falcon rack in the icebox and fill it with ice.
8. Place the two falcon-tubes with tissue transport medium (See 'buffers and media composition', Tissue Transport Medium) in the falcon rack and immerse falcon tubes in ice.
9. Attach a document containing surgeon's instructions to the ice box.

Note: This includes details of the type of specimens to be resected, as well as appropriate storage of the specimen for downstream processing. Tissue specimens are isolated from the tumor core as well as at a distant site 10–15 cm away from the tumor periphery termed "normal tissue". The region of resection is defined by the location of the tumor. For a right sided colonic lesion, this is defined as a right hemicolectomy, whereas for a left sided colonic lesion this is defined as a left hemicolectomy. For rectal tumors, that are positioned higher up a "lower anterior resection" is completed. For lesions close to the anus an "abdominoperineal resection" procedure is employed.

10. Once patient consent is obtained, the sample collection icebox is handed over to the clinical associate who facilitates sample collection and notifies the researchers as to a suitable time to collect samples.
11. Prior to the collection of samples, the researcher should ensure the following has been completed:
    a. All plasticware and dissection kits are placed under UV light for a minimum of one hour prior to use.
    b. Pre-labeled cryogenic specimen storage containers for each specimen are appropriately labeled and organized.
    c. Clean disposable scalpels and forceps should be used for processing different anatomical sites of the tissue.
    d. Contact with absorbent materials which may contaminate dissected research tissues or where capillary action may draw fluid from tissue samples should be avoided.
    e. All reagents should be prepared and ready to use as is. See 'sample processing preparation'.
12. Following collection, samples should be transported immediately to the laboratory for downstream processing.
13. Timing is critical. Ideally, no more than 30 min should elapse between the time of biopsy/resection and time for processing of a given sample.

Sample processing preparation

Timing: 45 min

This section describes the procedure for preparing buffers and reagents required for the downstream processing of the sample once obtained.

Note: All reagents, buffers, media and aliquots thereof should be prepared in a sterile environment using a laminar flow hood and stored appropriately as and when needed.

14. Prepare the following reagents and incubate on ice:
    a. Tissue Wash Buffer Solution (See 'buffers and media composition', Tissue Wash Buffer Solution).
    b. 25% Bovine Serum Albumin (BSA).
15. Reconstitute the following reagents according to manufacturers' instructions and incubate on ice:
    a. 2200 U/mL Collagenase II.
    b. 18 U/mL Dispase.
    c. 10 mM Y-27632 (ROCKi).
16. Aliquot the following reagent and incubate at 37°C:
    a. Advanced DMEM/F12.
17. Aliquot the following reagent and incubate on ice:
    a. Cultrex RGF Basement Membrane Extract, Type 2.
18. Prepare the following reagents and equilibrate to room temperature (15°C–25°C):
    a. Human IntestiCult Organoid Growth Medium (OGM Human Basal Medium and Organoid Supplement).
    b. Organoid Cryopreservation Media (See 'buffers and media composition', Organoid Cryopreservation Media).
19. Acclimate the following reagents to room temperature (15°C–25°C):
    a. Primocin.
    b. Glutamax.
    c. HEPES.
    d. ACK Lysing Buffer.
20. Turn on the following instruments and equilibrate to the required temperatures:
    a. Centrifuge (swing-out rotor) at 4°C.
    b. Water bath at 37°C.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-human EpCAM (1:50 dilution)	R&D Biosystems, USA	Cat# AF960, RRID: AB_355745
Anti-human Mucin 2 (F-2) (1:50 dilution)	Santa Cruz Biotechnology. USA	Cat# SC -515032, RRID: AB_2815005
Anti-human Ki-67 (1:50 dilution)	Novus Biologicals, CA	Cat# NB500-170
Anti-human Villin (1D2C3) (1:50 dilution)	Santa Cruz Biotechnology, USA	Cat# SC-58897, RRID: AB_2304475
Anti-human ChgA (C-12) (1:50 dilution)	Santa Cruz Biotechnology, USA	Cat# SC-393941, RRID: AB_2801371
Texas Red goat anti-rabbit IgG (1:100 dilution)	Thermo Fisher Scientific, USA	Cat# 2015320
Alexa Fluor 488 donkey anti-mouse IgG (1:100 dilution)	Thermo Fisher Scientific, USA	Cat# 2017408
Alexa Fluor 488 donkey anti-goat IgG (1:100 dilution)	Thermo Fisher Scientific, USA	Cat# 2059218
Biological samples		
Resected healthy intestinal tissue and patient matched tumor tissue specimens	Wits Donald Gordon Medical Centre	N/A
Chemicals, peptides, and recombinant proteins		
Advanced DMEM/F12	Thermo Fisher Scientific, USA	Cat# 12634010
HEPES (1 M solution)	Thermo Fisher Scientific, USA	Cat# 15630056
GlutaMAX Supplement (100×)	Thermo Fisher Scientific, USA	Cat# 35050061
Collagenase II	Thermo Fisher Scientific, USA	Cat# 17101015
Dispase	Thermo Fisher Scientific, USA	Cat# 17105041
Fetal bovine serum	BioWest, USA	Cat# S181G

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Cultrex RGF Basement Membrane Extract, Type 2 Select	R&D Biosystems, USA	Cat# 3536-005-02
IntestiCult Organoid Growth Medium (Human)	STEMCELL Technologies, Canada	Cat# 06010
Y-27632 (ROCK inhibitor)	Sigma-Aldrich, UK	Cat# Y0503-1MG
TrypLE Express Enzyme	Thermo Fisher Scientific, USA	Cat# 12604-021
β-mercaptoethanol	Sisco Research Laboratories (SRL), IN	Cat# 83759
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich. UK	Cat# D8418
Trypan blue	Thermo Fisher Scientific, USA	Cat# T10282
Paraformaldehyde solution 4%, buffered, pH 6.9	Sigma-Aldrich, UK	Cat# 1004965000
Triton X-100	Sigma-Aldrich, UK	Cat# T8787
Tween 20	Sigma-Aldrich, UK	Cat# P9416
4′,6-diamidino-2-phenylindole (DAPI) stain	Sigma-Aldrich, UK	Cat# 075M4010V
NucRed Dead 647 ReadyProbes Reagent	Thermo Fisher Scientific, USA	Cat# R37113
Molecular grade ethanol (>99%)	ACE Chemicals, RSA	Cat# E0805FF02500
Xylene	Sigma-Aldrich, UK	Cat# 534056
Bovine serum albumin (BSA)	VWR, USA	Cat# 9048-46-8
Primocin	InvivoGen, USA	Cat# ant-pm-1
Agarose powder	Accuris Reagents, CA	Cat# A1701
RNALater	Thermo Fisher Scientific, USA	Cat# AM7924
DPBS	Thermo Fisher Scientific, USA	Cat# 14190144
PBS	Thermo Fisher Scientific, USA	Cat# 10010023
Nuclease-free water	Thermo Fisher Scientific, USA	Cat# R0582
Citric acid	ACE Chemicals, ZA	Cat# C0539NN00500
Differentiation solution	Sigma-Aldrich, UK	Cat# A3179-1L
Scotts tap water	Sigma-Aldrich, UK	Cat# S5134
DPX mounting solution	Sigma-Aldrich, UK	Cat# 06522
Paraffin wax solid	Multi Success Supplies Pty Ltd, ZA	Cat# P0250
Hematoxylin	Sigma-Aldrich, UK	Cat# H3136-25G
Eosin Y	Sigma-Aldrich, UK	Cat# 1041340100
1% Periodic acid	Sigma-Aldrich, UK	Cat# 3951
Schiff's reagent	Sigma-Aldrich, UK	Cat# 3952016
6% Nitric acid	Sigma-Aldrich, UK	Cat# SAAR4465085LC
0.5% Toluidine blue	Sigma-Aldrich, UK	Cat# 89640
Picrosirius	Abcam, UK	Cat# ab246832
ACK lysing buffer	Thermo Fisher Scientific, USA	Cat# A1049201
MACS Tissue storage solution	Miltenyi Biotec, GER	Cat# 130-100-008
Fluoromount Aqueous mounting solution	Sigma-Aldrich, UK	Cat# F4680
Entellan mounting medium	Sigma-Aldrich, UK	Cat# 1079600500
Filipin complex	Sigma-Aldrich, UK	Cat# F9765
Vybrant Alexa Fluor 594 Lipid Raft Labeling Kit	Thermo Fisher Scientific, USA	Cat# V34405
BODIPY 493/503	Thermo Fisher Scientific, USA	Cat# D3922
Critical commercial assays		
E.Z.N.A H. P Total RNA Kit	Omega Bio-Tek, USA	Cat# R6812-02
RevertAid First Strand cDNA Synthesis Kit	Thermo Fisher Scientific, USA	Cat# K1621
SensiFAST SYBR No-ROX Kit	Meridian Biosciences, USA	Cat# BIO-98005
Oligonucleotides		
Human *EPCAM* primer (Table S1)	Integrated DNA Technologies Inc., USA	N/A
Human *VIL1* primer (Table S1)	Integrated DNA Technologies Inc., USA	N/A
Human *LGR5* primer (Table S1)	Integrated DNA Technologies Inc., USA	N/A
Human *LYZ* primer (Table S1)	Integrated DNA Technologies Inc., USA	N/A
Human *MUC2* primer (Table S1)	Integrated DNA Technologies Inc., USA	N/A
Human *CHGA* primer (Table S1)	Integrated DNA Technologies Inc., USA	N/A
Human *MKI67* primer (Table S1)	Integrated DNA Technologies Inc., USA	N/A
Software and algorithms		
Celleste Image Analysis Software	Thermo Fisher Scientific, USA	AMEP4941
GraphPad Prism 9	GraphPad	www.graphpad.com

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
BioRender	BioRender	www.biorender.com
Other		
Costar 24-well TC-treated multiple well plates, individually wrapped, sterile	Sigma-Aldrich, UK	Cat# 3526
15 mL centrifuge tubes (conical polypropylene centrifuge tube sterile)	Corning, USA	Cat# 430791
70 μm cell strainers	Corning, USA	Cat# 352350
0.22 μm Filter	Merck, USA	Cat# SLMP025SS
Pasteur pipettes (3 mL)	Sigma-Aldrich, UK	Cat# HS206373B-500EA
25-Gauge needles	Lasec, RSA	Cat# HLSCINSULINIS-0.5ml
Nalgene cryo freezing container	Sigma-Aldrich, UK	Cat# C1562
Cryogenic vials	NEST Biotec, CN	CAT# 607101
50 mL Falcon tubes	VWR, USA	Cat# 10025-698
Invitrogen Countess cell counting chamber slides and holder - disposable	Thermo Fisher Scientific, USA	Cat# C10283
Invitrogen Cell Counter Countess II	Thermo Fisher Scientific, USA	Cat# AMQAX1000
Vortex-Genie 2	Sigma Aldrich, UK	Cat# Z258423
Zigma centrifuge (fixed angle rotor)	Zigma Zentrifugen – model 1-14, GER	Cat# 10014
Allegra X-30R centrifuge (swing bucket rotor)	Beckman Coulter, USA	Cat# SX-4400
Leica DMiL inverted light microscope	Leica Technologies, GER	Cat# LEI-11521265
EVOS M7000 microscope	Thermo Fisher Scientific, USA	Cat# AMF7000HCA3D
Sterile scissors	Lasec, RSA	Cat# HRRE6027
Sterile forceps	Lasec, RSA	Cat# HRRE6027
37°C Humidified incubator	ESCO Lifesciences Group, SG	Cat# 2170002
37°C Water bath	Nuve, TR	Cat# ST30
Fridge (4°C)	KIC, RSA	N/A
Freezer (−20°C)	Defy, RSA	N/A
Freezer (−80°C)	ESCO Lifesciences Group, SG	Cat# 2180098
Liquid nitrogen storage tank	Thermo Fisher Scientific, USA	Cat# CK509X3
Nanodrop One spectrophotometer	Thermo Fisher Scientific, USA	Cat# ND-ONE-W4
MultiGene PCR machine	Bio-Rad, USA	Cat# T1000
QuantStudio 5	Thermo Fisher Scientific, USA	Cat# A28134
Microtome	Leica Biosystems, Germany	Cat# RM2125 RTS
Water bath	Leica Biosystems, Germany	Cat# HI1210

Where, RRDI: Research Resource Identifiers Project; and Cat#: Catalog Number.

MATERIALS AND EQUIPMENT

Buffers and media composition

Tissue transport medium

Reagent	Final concentration	Amount
Advanced DMEM/F12	N/A	15.6 mL
HEPES (1 M Solution)	10 mM	200 μL
GlutaMAX Supplement (100×)	1×	200 μL
Primocin (50 mg/ml)	100 μg/ml	40 μL
Total	N/A	20 mL

Prepare tissue collection and transport media in a sterile environment and store at 4°C for up to 1 month. Keep the media pre-chilled on ice prior to use.

Tissue wash buffer solution		
Reagent	Final concentration	Amount
DPBS	1×	49.9 mL
Primocin (50 mg/mL)	100 μg/ml	100 μL
Total	**N/A**	**50 mL**

Prepare tissue wash buffer solution in a sterile environment and store at 4°C for up to 1 month. Keep the wash buffer pre-chilled on ice prior to use.

Tissue digestion media		
Reagent	Final concentration	Amount
Advanced DMEM/F12	N/A	10 mL (final volume)
HEPES (1 M Solution)	10 mM	100 μL
GlutaMAX Supplement (100×)	1×	100 μL
Primocin (50 mg/mL)	100 μg/mL	20 μL
Collagenase II	75 U/mL	variable (dependent on enzyme activity of stock solution)
Dispase	1.25 U/mL	variable (dependent on enzyme activity of stock solution)
Y-27632 (ROCKi)	10 μM	variable (dependent on concentration of stock solution)
Total	**N/A**	**10 mL**

Prepare tissue digestion media in a sterile environment, sterile filter solution prior to use employing a 0.22 μm syringe filter and use on the day. Aliquot required volume of tissue digestion buffer and incubate at 37°C prior to use.

Tissue seeding media		
Reagent	Final concentration	Amount
Advanced DMEM/F12	N/A	10 mL (final volume)
HEPES (1 M Solution)	10 mM	100 μL
GlutaMAX Supplement (100×)	1×	100 μL
BSA	0.1%	variable (dependent on concentration of stock solution)
Total	**N/A**	**10 mL**

Prepare tissue seeding media in a sterile environment, sterile filter solution prior to use employing a 0.22 μm syringe filter and store at 4°C for up to 1 month. Keep the tissue seeding media pre-chilled on ice prior to use.

Complete IntestiCult Organoid Growth Medium		
Reagent	Final concentration	Amount
Intesticult OGM Human Basal Medium	1×	2 mL
Organoid Supplement	1×	2 mL
Primocin (50 mg/mL)	100 μg/mL	8 μL
Y-27632 (ROCKi)	10 μM	variable (dependent on concentration of stock solution)
Total	**N/A**	**4 mL**

Equilibrate to room temperature (15°C–25°C) prior to use. Excess media can be stored at 4°C for 1 week.

Organoid cryopreservation media		
Reagent	Final concentration	Amount
FBS	80%	800 μL
DMSO	20%	200 μL
Total	**N/A**	**1 mL**

Equilibrate to room temperature (15°C–25°C) prior to use. Excess media can be stored at 4°C for 1 week.

Antigen retrieval buffer (pH 6.0)		
Reagent	Final concentration	Amount
Citric acid	10 mM	1.92 g
Tween-20	0.05%	50 μL
dH_2O	N/A	1 L
Total	**N/A**	**1 L**

Reconstitute citric acid in 900 mL of dH_2O and pH to 6.0. Add Tween-20 and make up to 1 L. Store at 4°C for up to 1 month.

Immunofluorescence wash buffer		
Reagent	Final concentration	Amount
DPBS	N/A	14.985 mL
Tween-20	0.1%	15 μL
Total	**N/A**	**15 mL**

Store at 4°C for up to 1 month.

Immunofluorescence permeabilization buffer		
Reagent	Final concentration	Amount
DPBS	N/A	9.975 mL
Triton-X-100	0.25%	25 μL
Total	**N/A**	**10 mL**

Store at 4°C for up to 1 month.

Immunofluorescence blocking buffer		
Reagent	Final concentration	Amount
DPBS	N/A	12 mL
BSA	5%	3 mL
Total	**N/A**	**15 mL**

Store at 4°C for up to 1 month.

Antibody dilution buffer		
Reagent	Final concentration	Amount
DPBS	N/A	5 mL
Tween-20	0.1%	5 μL
Total	**N/A**	**5 mL**

Store at 4°C for up to 1 month.

STEP-BY-STEP METHOD DETAILS

Tissue processing for intestinal organoid establishment

Timing: 2.5 h

This section describes the procedure for the preparation and establishment of human intestinal organoids derived from patient tissue. The protocol presented is an adaptation of a protocol published by several groups (Figure 1).[2,11,12]

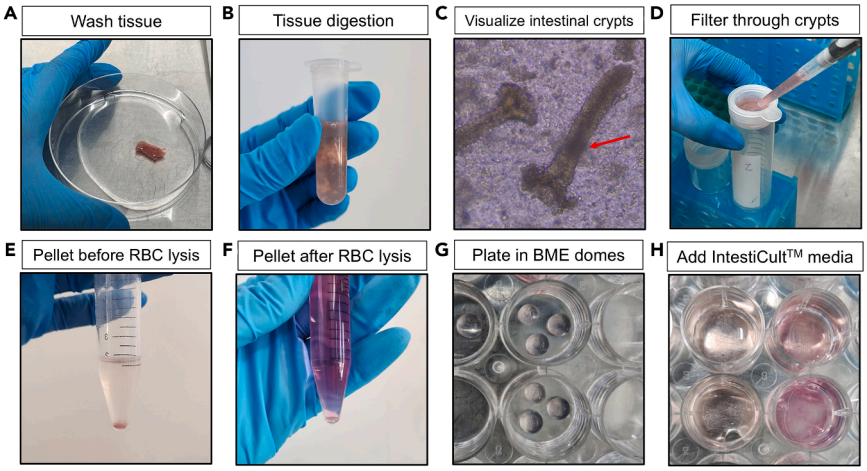


Figure 1. Workflow of generating primary organoid cultures from fresh tissue

(A) The tissue is washed.

(B) The tissue subsequently undergoes mechanical and enzymatic digestion. Digestion time varies between tissue specimens.

(C) Sufficient digestion is confirmed with the visualization of intestinal crypts.

(D) Crypts are filtered through a 70 μM strainer and pelleted.

(E and F) Red blood cell (RBC) lysis is required if the pellet is red in color (indicative of contaminating red blood cells).

(G) The pellet is then resuspended in a mixture of tissue seeding media and BME and plated in a pre-warmed 24-well plate.

(H) Following a maximum of 30 min, 500 μL of IntestiCult organoid media is added.

⚠ CRITICAL: All steps in this protocol should be conducted under sterile conditions using a laminar flow hood. The following protocol has been optimized to work best on fresh tissue. However, it has also proved successful in culturing organoids from tissue that has been stored in MACS Tissue Storage Solution at 4°C overnight (16–24 h).

1. Before you begin this protocol, ensure that the following steps have been completed:
   a. Ensure that 24-well plates have been prewarmed in the 37°C incubator, overnight (16–24 h).
   b. Prepare complete IntestiCult Organoid Growth Medium (See 'buffers and media composition', Complete IntestiCult Organoid Growth Medium) and equilibrate to room temperature (15°C–25°C).
   c. Prepare tissue digestion media (See 'buffers and media composition', Tissue Digestion Media) and pre-warm at 37°C.
   d. Thaw sufficient aliquots of Cultrex on ice.
   e. Prepare and pre-chill tissue wash buffer solution (See 'buffers and media composition', Tissue Wash Buffer Solution) on ice.
2. Wash the tissue specimens 3× in pre-chilled (4°C) tissue wash buffer solution (See 'buffers and media composition', Tissue Wash Buffer Solution):
   a. Using a sterile forceps transfer the tissue sample from the transport media to a petri plate containing pre-chilled (4°C) wash buffer solution.
   b. Completely immerse the sample in the wash solution and wash thoroughly by gently swirling the tissue in circular motions within the petri plate. This ensures the wash solution permeates the sample effectively.

⚠ CRITICAL: A separate set of forceps and petri plates should be used when processing normal vs cancerous tissue. The following steps should be completed on ice to maintain the viability of the specimen.

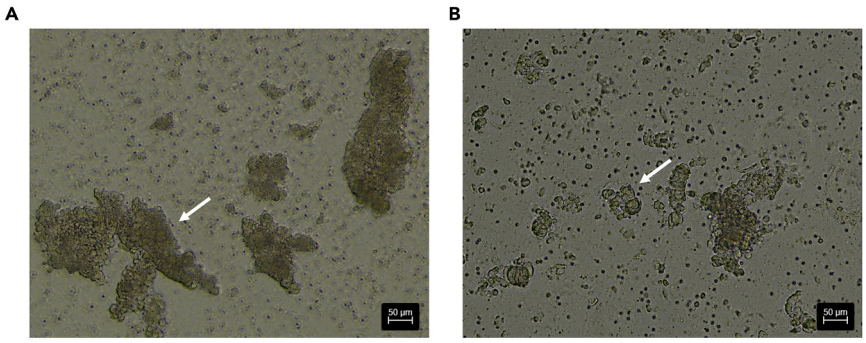


Figure 2. Representative images of cancerous tissue digestion for organoid generation

(A) Tissue digestion after 10 min incubation shows the presence of large cell clusters, which requires further digestion.
(B) Tissue digestion after 20 min incubation shows sufficiently digested cell clusters, with the ability to pass through a 70 µm strainer.
Images acquired using the Leica DmiL Inverted Light Microscope at 10× magnification with the representative scale bar indicating 50 µm.

3. Transfer the specimen to a petri dish containing the appropriate volume (~1–2 mL) of tissue digestion media (See 'buffers and media composition', Tissue Digestion Media).
4. Using a sterile scalpel, thoroughly cut the tissue into the smallest possible pieces (<10 mm^3).

Note: The volume of the digestion buffer is dictated by the size of the specimen, where the entire specimen should be submerged completely in the digestion buffer for efficient digestion.

5. Transfer the specimen into a 15 mL falcon containing 2 mL of pre-warmed (37°C) tissue digestion media (See 'buffers and media composition', Tissue Digestion Media).
6. Resuspend the tissue fragments in the additional tissue digestion media (See 'buffers and media composition', Tissue Digestion Media) and incubate at 37°C in the water bath.

⚠ CRITICAL: Pipette tips should be pre-coated with FBS for all downstream steps to prevent the adherence and loss of crypts to plasticware.

7. Complete regular trituration of the sample at 15 min intervals using p1000 pipette to liberate crypts.
8. Following the initial 15 min incubation, assess morphological digestion of the sample every 5 min through bright-field microscopy to validate the success of the digestion.

⚠ CRITICAL: Digestion time at 37°C is dependent on the sample size, where bigger fragments will take a longer time to digest compared to smaller chunks. Incubation time can vary but should not exceed 90 min.

Note: For normal samples, the digestion buffer should liberate villi and crypts which would be visible under the microscope. For cancer tissue, distinct crypt villi structures will not be visible but instead one should expect to see cell clusters. Smaller clumps are preferred over large fragments of tissue (Figure 2).

⚠ CRITICAL: Avoid the over digestion of samples, which is indicated by the presence of single cells. If some tissue fragments are apparent, strain the digested fragments and dilute the filtrate with an equal volume of FBS, to inhibit the enzymatic activity of the Collagenase and Dispase. Add fresh tissue digestion media to the undigested fragments and continue with digestion.

9. Strain homogenate/suspension using a 70 μm strainer to filter the crypt fragments from the villi and tissue associated debris into a 50 mL tube.
10. Rinse the strainer with 10 mL of pre-chilled (4°C) DPBS, to ensure complete filtration of the crypts and to inhibit the enzymatic activity of the Collagenase and Dispase.
11. Transfer the filtrate to a 15 mL tube and centrifuge the flow through at 500 × *g* for 5 min (4°C).

Note: At this step the sample may or may not contain contaminating red blood cells (RBCs). It is crucial to remove these RBCs prior to downstream processing as they would interfere with growth of the organoid cultures.

12. In the presence of RBCs complete the following steps:
    a. Resuspend the pellet in 3 mL of ACK Lysing Buffer and incubate at room temperature (15°C–25°C) for 5–15 min.

Note: Incubation time in ACK Lysing Buffer is dependent on the extent of RBC contamination in the specimen of interest. Once the tissue fragments display a uniformly distributed white pinkish hue, the incubation may be terminated.

    b. Following this incubation, neutralize the ACK Lysing Buffer with 5 mL of Advanced DMEM/F12.
    c. Centrifuge the crypts at 500 × *g* for 5 min (4°C).
    d. Wash the crypt pellet two additional times using Advanced DMEM/F12 by centrifuging each time at 500 × *g* for 5 min (4°C) and aspirating the supernatant.
13. Resuspend the crypt pellet in 1 mL of tissue seeding media (See 'buffers and media composition', Tissue Seeding Media) to complete the crypt count.

Note: An increased amount of starting material may result in an increase in the number of crypts obtained. The volume of tissue seeding media may be adjusted to complete a feasible crypt count however the resulting dilution factor should be taken into account when calculating the number of crypts to be seeded.

14. Determine the total number of crypts in the sample.
    a. Place 3 × 10 μL aliquots on an appropriate counting surface (glass slide).
    b. Count the number of crypts per aliquot.
    c. Determine the average number of crypts in the 3 aliquots and multiply by 100 to obtain the total number of crypts in the 1 mL sample.
    d. Determine how many culture domes can be plated at 1000 crypts/ 50 μL dome.
       Example:
       i. Aliquot 1: 15 crypts.
       ii. Aliquot 2: 25 crypts.
       iii. Aliquot 3: 20 crypts.
       iv. Average: 20 crypts x 100 = 2000 crypts (total) which is sufficient for culturing 2 culture domes.

Note: 1000 crypts/dome results in 150–200 mature organoids.

15. Centrifuge the sample at 500 × *g* for 5 min (4°C).
16. Add an appropriate volume of Cultrex to yield a final concentration of 10 mg/mL Cultrex diluted with pre-chilled (4°C) tissue seeding media (See 'buffers and media composition', Tissue Seeding Media).

⚠ CRITICAL: Resuspend the pelleted crypts by gently aspirating to the first stop on the pipette to avoid the formation of bubbles.

17. Using a 200 μL pipette tip, pre-coated with FBS, draw up 50 μL of the Cultrex-crypt suspension and add to 1 of 8 central wells of a pre-warmed (37°C) 24-well tissue culture plate as follows:
    a. Hold the pipette vertically over the center of the well and bring the pipette tip near to but not in contact with the floor of the well.
    b. Slightly depress the plunger until a droplet is visible at the end of the pipette tip.
    c. Slowly lower the pipette until the droplet touches the floor of the well.
    d. Gently dispense (first stop) the remaining volume while lifting the pipette away from the well. Avoid introducing bubbles.
    e. Work quickly to plate the Cultrex-crypt suspension upon removing it from ice.
    f. Allow plated domes to solidify at room temperature (15°C–25°C) in the hood for 5 min.
18. Carefully transfer the plate to a 37°C incubator. Incubate at 37°C for 15–30 min to allow the domes to solidify.

⚠ CRITICAL: Do not leave organoids for longer than 30 min without supplementing them with media.

19. Prepare the required volume of complete IntestiCult Organoid Growth Medium (See 'buffers and media composition', Complete Intesticult Organoid Growth Medium) at room temperature (15°C–25°C).

Note: Each culture dome requires a minimum 500 μL of medium per 24-well plate.

20. Supplement cultures with media and incubate at 37°C and 5% CO_2.
21. Add sterile PBS to surrounding wells to prevent evaporation of media.
22. Replace media every alternate day (refer to section "organoid maintenance") and passage organoids once confluency is reached (refer to section "organoid passaging").

Organoid maintenance

Timing: 15 min

This section describes the procedure for routine maintenance of human intestinal organoids derived from human intestinal crypts.

Note: Media is refreshed every alternate day.

⚠ CRITICAL: Ensure organoid media has been prepared and warmed to room temperature (15°C–25°C).

23. Assess organoids under the microscope to infer consensus on organoid health and size/phenotype.

Note: Organoids may present as varying sized pre-swollen proliferative structures, displaying a clear lumen with no debris or dying cells (Figure 3). Media changes without passage are permissible between Days 3–7 depending on the expansion and growth rate of each organoid line.

24. Remove organoid media from each well of the culture vessel by gentle aspiration.
25. Supplement organoids with 500 μL fresh complete IntestiCult Organoid Growth Medium (See 'buffers and media composition', Complete Intesticult Organoid Growth Medium).
26. Return organoids to the humidified CO_2 incubator (5%).

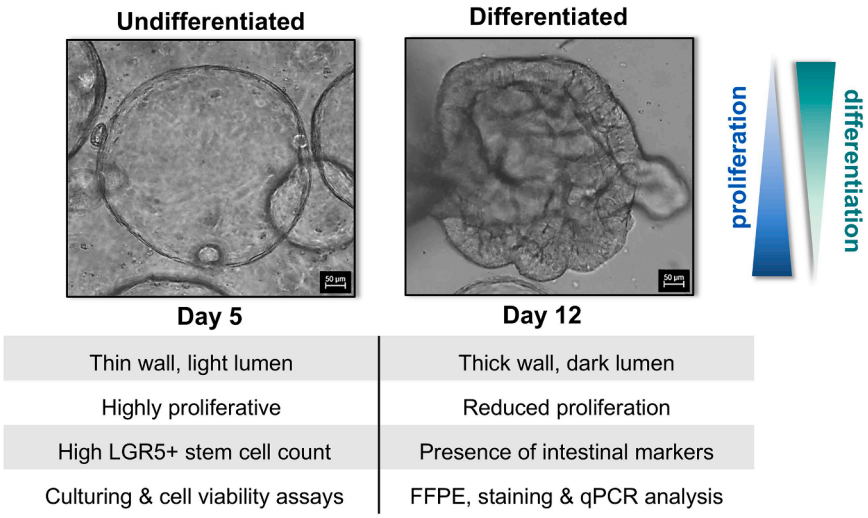


Figure 3. Insights into organoid morphology

Undifferentiated organoids exhibit a thin-walled structure with a clear lumen, indicative of a proliferative state suitable for maintenance and passaging. Differentiated organoids display a thicker wall and a dark lumen, suggesting organoid maturation and suitability for downstream analysis of differentiated cell types. Images acquired using the Leica DmiL Inverted Light Microscope at 10× magnification with the representative scale bar indicating 50 μm.

Organoid passaging

Timing: 1.5 h

This section describes the procedure for passaging human intestinal organoids derived from human intestinal crypts (Figure 4). In summary, organoids are harvested from the basement membrane

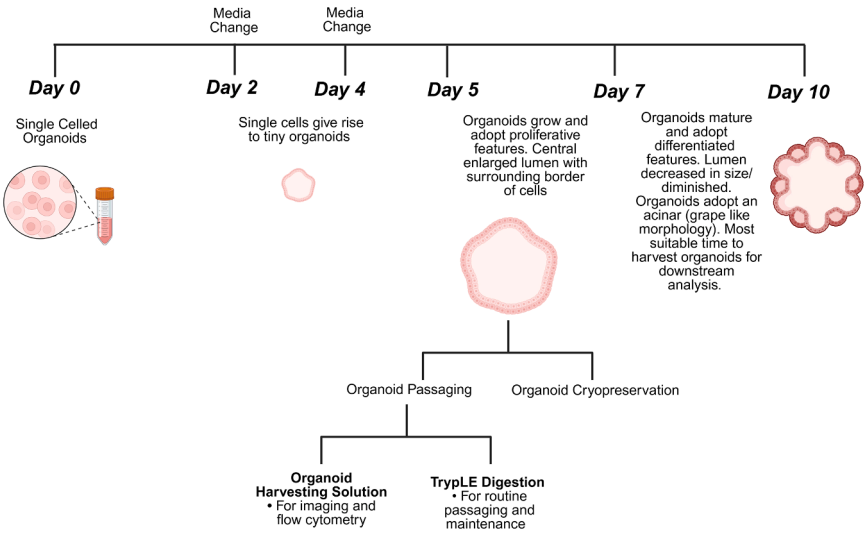


Figure 4. Timeline of organoid development over 10 days

Organoids are digested down to single cells and resuspended in BME (D0). Organoids are monitored for growth and differentiation where they will increase in size as they progress through culture. At Day 7 organoids should be passaged or may be left to differentiate post Day 10 for experimental use. Organoids left for longer than 10 days without passaging will be susceptible to cell death compromising the viability of the line. Figure created using BioRender.com.

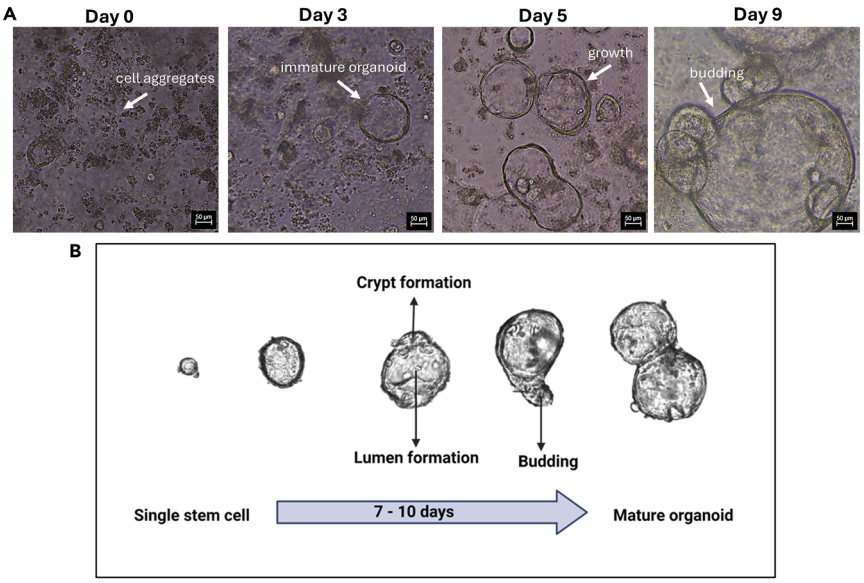


Figure 5. Monitoring human intestinal organoid growth dynamics

(A and B) Organoid growth dynamics. At day 0, the initial growth of the organoid presents as a single stem cell that will progress to form a cyst-like structure. The cyst-like structure is comprised of proliferating progenitor cells and a central lumen that will increase in size. Between days 3 and 4, budding of the cyst-like structure will appear, which will give rise to more cyst-like organoids. These cyst-like structures will increase in size and will reach maturity between days 7 and 10 in culture, where the structure of the organoid resembles that of the intestine. The lumen will also progress to become darker as this is the region in which the villi would develop, and dead cells are shed towards the lumen. Images acquired using the Leica DmiL Inverted Light Microscope at 10× magnification with the representative scale bar indicating 50 μm.

matrix (BME) and mechanically and enzymatically dissociated into single cells. These single cells are resuspended in BME to facilitate stem cell mediated expansion of organoid lines for downstream experimentation. For primary cultures at P0, passage after 7–14 days. For previously passaged organoids, passage every 7–10 days. Larger cystic or budded organoids will result in higher yields of viable fragments than smaller, dark, collapsed or overly budded organoids. The outlined protocol is an adaptation of a protocol published by several groups[2,11,12] and has proved successful in expanding viable and functional organoids until P20.

27. Before you begin this protocol, ensure that the following steps have been completed:
    a. Ensure that 24-well plates have been prewarmed in the 37°C incubator, overnight (16–24 h).
    b. Prepare complete IntestiCult Organoid Growth Medium (See 'buffers and media composition', Complete Intesticult Organoid Growth Medium) and warm to room temperature (15°C–25°C).
    c. Pre-warm the desired volume of TrypLE supplemented with 10 μM Y-27632 (ROCKi) to 37°C.
    d. For each well to be passaged, 1 mL of TrypLE and 500 μL of medium is required.
    e. Thaw sufficient aliquots of Cultrex on ice.
28. Using a bright-field microscope, assess all wells that are to be passaged to determine the split ratio.

Note: Routine microscopic observation of organoid cultures is crucial to monitor growth dynamics to ensure that organoids are passaged at the most optimal time point (Figure 5). The time to be passaged and the split ratio are dictated by the proliferation rate of each patient-derived organoid line. On average organoids that have reached 80% confluency are passaged. For newly established lines, a 1:2 split ratio is advised whereas well established

lines may even be split 1:3–1:5 depending on how robust the line grows and according to the desired confluency. One should work in a conservative manner, opting to plate the domes dense (with a low split ratio) and diluting further if needed, rather than seeding organoids too sparse which may result in compromising the organoid line.

Note: The size of the organoids will dictate the time needed to digest in TrypLE. Increased organoid size will lead to increased incubation time.

⚠ CRITICAL: Pipette tips should be pre-coated with FBS for all downstream steps to prevent the adherence and loss of organoids to plasticware.

29. Carefully remove and discard medium from each well to be passaged, without disturbing the Cultrex dome.
30. Gently wash each well (by swirling) with 500 μL (volume should be sufficient to cover the domes plated) of pre-chilled (4°C) PBS.
31. Using a 3 mL Pasteur pipette pre-coated with FBS, gently scrape domes in a circular motion to dislodge domes within the PBS.

Note: Be careful not to introduce bubbles in the PBS when completing this. Do not compress the stopper on the Pasteur pipette. Simply use the tip of the Pasteur pipette to scrape and dislodge domes. You do not have to be gentle at this step as you want to dislodge the organoids from the BME. More PBS can be added to the wells if the BME is not sufficiently removed.

32. Using the Pasteur pipette, transfer dislodged organoids in PBS to a falcon tube and aspirate vigorously 3–4 times for further dislodging of organoids from BME.

Note: A maximum of 3–4 wells of organoids should be dispensed to one falcon tube to facilitate sufficient action of TrypLE. This restriction is due to the upscale of organoids and the high volume of BME added when upscaling, which might risk solidification if you are upscaling by a significant degree.

33. Centrifuge dislodged organoids at 500 × *g* for 5 min (4°C).

Note: The centrifugation step is completed using a swing bucket rotor to ensure distinct pelleting of the organoids. If multiple falcons are being centrifuged it is permissible to keep the organoids on ice while waiting to use the centrifuge.

34. Once the centrifugation is complete, organoids pelleted within the BME should be visible.

Note: The BME/organoid suspension should be a solid pellet and should not be easily dislodged.

35. Gently remove PBS and add 1 mL of pre-warmed TrypLE (37°C) (per well of organoids).
36. Stack a p1000 pre-coated pipette tip, first using a p200 pipette and then a p10 pipette, and aspirate 10–20 times to dislodge the pellet containing organoids in BME.

Note: For an unestablished or recently established line it is permissible to supplement the TrypLE with 10 μM ROCKi, to enhance cell survival and minimize apoptosis during digestion.

a. It is advisable to incubate in TrypLE for 5–30 min (37°C) to dislodge organoids to single cells. Assess organoid suspension after 5 min have elapsed. If organoids or cell aggregates are present, double stack a p10 tip onto a p1000 tip and aspirate the mixture 10–15 times to ensure dissociation to single cells (Figure 6).

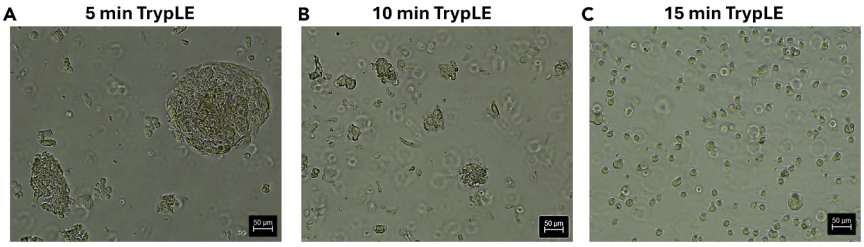


Figure 6. Organoid digestion to single cells

(A) Organoids incubated in TrypLE for 5 min exhibit partial dissociation, with the presence of large cell clusters indicative of incomplete enzymatic digestion.

(B) Continued incubation with aspiration facilitates further dissociation of organoids into smaller cell clusters.

(C) Following 15 min of TrypLE incubation and aspiration, organoids are sufficiently dissociated into single cells and the reaction is terminated.

Images acquired using the Leica DmiL Inverted Light Microscope at 10× magnification with the representative scale bar indicating 50 μm.

b. Assess organoid suspension under the microscope to view single cells. If cell clusters or clumps are present, incubate in TrypLE (37°C) for longer (maximum 30 min), with continuous aspiration.
c. Following incubation, you should see a population of ~90% of single cells and no cell clumps.

Note: Pay attention to incubation with TrypLE as over digestion may affect cell surface antigens, especially if you intend to use organoids for flow cytometry as a downstream assay.

37. To inactivate the TrypLE add 9 mL of pre-chilled (4°C) PBS per 1 mL of TrypLE.

Note: Tubes can be stored on ice for multiple digestions that are taking place concurrently.

38. Centrifuge single celled organoids at 500 × *g* for 5 min (4°C) and resuspend in 1 mL of pre-chilled (4°C) PBS.
39. Complete cell counts and assess viability by resuspending 10 μL of single cells in 10 μL of Trypan Blue.

Note: It is preferred to plate 70 000–80 0000 single cells per 50 μL dome of organoids.

40. Centrifuge single cells at 500 × *g* for 5 min (4°C) for pelleting and resuspension in the appropriate volume of tissue seeding media (See 'buffers and media composition', Tissue Seeding Media) and BME.
41. Using a pre-coated 200 μL pipette tip draw up 50 μL of the Cultrex-crypt suspension and add to 1 of 8 central wells of a 24-well tissue culture plate as outlined in Step 17.
    a. Label the 24-well plate with the organoid line, passage number and date.

⚠ CRITICAL: Do not leave organoids for longer than 30 min without supplementing them with media.

42. Supplement organoids with 500 μL of Complete IntestiCult Organoid Growth Medium (See 'buffers and media composition', Complete Intesticult Organoid Growth Medium) to each well by pipetting the medium gently down the wall of the well. Do not pipette directly onto the domes.

Note: Supplement media with 10 μM ROCKi for the first three days after passaging only, to support viability of single cells.

43. Add sterile PBS to the unused wells to prevent media evaporation.
44. Incubate at 37°C in a humidified 5% CO_2 incubator.

Organoid cryopreservation

Timing: 1 h

This section describes the procedure for cryopreservation of human intestinal organoids. Human intestinal organoids are typically cryopreserved in the highly proliferative state when organoids present as small cystic structures (Day 3–5). Cryopreservation is crucial to preserve the derived organoid lines for upscaling and expansion of experiments and to also provide suitable reserves if the organoid culture is compromised.

45. Before you begin this protocol ensure that the following steps have been completed:
    a. Prepare Organoid Cryopreservation Media (See 'buffers and media composition', Organoid Cryopreservation Media).
    b. Equilibrate Nalgene Cryo Freezing Container to room temperature (15°C–25°C).
46. Aspirate media from wells.
47. Add 500 μL of pre-chilled (4°C) PBS per well.

Note: A maximum of 3–4 wells are combined in 1 falcon for cryopreservation.

⚠ CRITICAL: Pipette tips should be pre-coated with FBS for all downstream steps to prevent the adherence and loss of crypts to plasticware.

48. Using a 3 mL pre-coated Pasteur pipette, detach organoids from the appropriate number of wells of a 24-well plate (~1000 organoids per cryovial).
49. Pipette vigorously to dislodge the organoids from BME (first using a p1000 and then a p200 pipette).

Note: If multiple wells are stocked it is permissible to store subsequent falcons on ice.

50. Centrifuge dislodged organoids at 500 × *g* for 5 min (4°C).
51. Discard supernatant and add freshly prepared organoid cryopreservation media (See 'buffers and media composition', Organoid Cryopreservation Media).
52. Gently resuspend the organoid pellet containing BME fragments in an appropriate volume of freezing media.

Note: Aspiration of organoids should result in fragmentation of organoids, as insufficient aspiration would result in cryopreservation of intact organoids whereas excessive aspiration would result in single cells and debris. This would prove detrimental to re-establishment of the cryopreserved line when needed.

Note: A maximum of 3–4 wells are combined per vial to be cryopreserved (~1000 organoids/vial).

53. Transfer the suspension to a cryovial and label cryovial appropriately, including name of organoid line, date of stock creation, passage number and number of wells combined for the generation of organoid stock.
54. Place the cryotubes into Nalgene Cryo Freezing Container and place into −80°C freezer.

Note: This will facilitate 1°C/min cooling rate required for successful cryopreservation of organoids.

55. Within the following days cryovials are to be transferred to liquid N_2 stores.

Organoid thawing

Timing: 1 h

This section describes the procedure for thawing human intestinal organoids for downstream experimentation.

56. Before you begin this protocol ensure that the following steps have been completed:
    a. Ensure that 24-well plates have been placed in the 37°C incubator overnight (16–24 h).
    b. Prepare complete IntestiCult organoid growth medium (See 'buffers and media composition', Complete Intesticult Organoid Growth Medium) and equilibrate to room temperature (15°C–25°C).
    c. For each well to be passaged 500 μL of media is required.
    d. Thaw sufficient aliquots of Cultrex on ice.
    e. Prepare a 10 mL aliquot of Advanced DMEM/F12 and pre-warm to 37°C.
57. A cryovial of the selected organoid line is taken from the liquid nitrogen tank using the appropriate safety measures and transferred to a container with sufficient dry ice.

    Note: The maximum number of wells that are combined in a vial is 4 wells (1 mL of organoid freezing media, See 'buffers and media composition', Organoid Cryopreservation Media). The high concentration of DMSO is to account for the freezing of organoids with BME. It is preferable to freeze organoids with BME to protect organoids from ice crystal perforation upon freezing.

58. Quickly, thaw the vial of organoid stock in a 37°C water bath until 50% of the 1 mL volume has melted.
59. Following thawing add the 1 mL of organoid stock to 10 mL of pre-warmed Advanced DMEM/F12.
60. Gently invert the tube to ensure that organoids are homogenously distributed in the solution.
61. Centrifuge the sample at 500 × *g* for 5 min at room temperature (15°C–25°C).

    Note: Orientation of the falcon tube is critical if the pellet is small and may help with removal of supernatant if sample is not visible to the naked eye. Falcon tubes that are placed with the white label facing the outer side of the rotor will lead to a pellet forming on the side of the label guiding the experimenter when removing media.

62. Label a 24-well plate with the organoid line, passage number and date.

    Note: When thawing an organoid line and introducing into culture, this constitutes a new passage. e.g., if the stock was p14 prior to thawing after thawing and plating the stock is now p15.

63. Aspirate the supernatant and re-suspend the pellet in the appropriate volume of BME (50 μL per well of a 24-well plate).
64. Refer to Step 17 for plating of organoids.

    Note: Ensure that the resuspension is done thoroughly to prevent clumping of the organoids which could limit growth of cultures once seeded.

65. Supplement organoids with media as detailed in Steps 19–21.

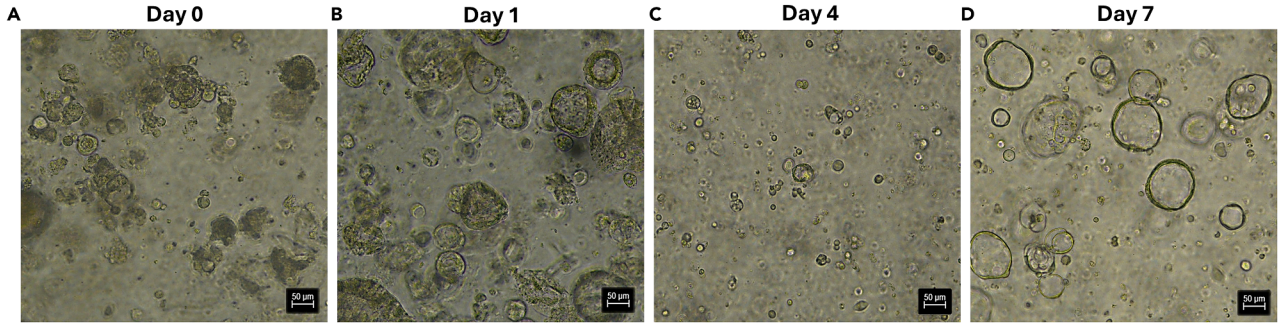


Figure 7. Representative images of organoid morphology following organoid thawing

(A) Organoids on the day of thawing exhibit a slightly darkened, granular morphology.

(B) Following 1-day post-thaw, organoids show an increase in size and at this point are ready for digestion to single cells.

(C) Single cells give rise to many organoids and should adopt a proliferative morphology with thin walls and clear lumen.

(D) Organoids progressively increase in size and should subsequently be cultured as per the standard timeline.

Images acquired using the Leica DmiL Inverted Light Microscope at 10× magnification with the representative scale bar indicating 50 µm.

Note: A day after thawing, organoids should be exposed to TrypLE to digest organoids down to single cells to facilitate expansion of newly thawed organoids (Figure 7). (Refer to Organoid Passaging Protocol).

RNA extraction from organoid cultures

Timing: 1.5 h

This section describes the procedure for extracting RNA from human intestinal organoids. RNA can be used for a range of downstream molecular analysis techniques including reverse transcription quantitative polymerase chain reaction (RT-qPCR) approaches for characterizing organoid cultures.

Note: An adaptation of the E.Z.N.A H.P Total RNA Kit (https://omegabiotek.com/product/e-z-n-a-hp-total-rna-kit/) proves most efficient in the extraction of RNA from organoids.

66. Before you begin this protocol ensure that the following steps have been completed:
    a. Equilibrate all samples and reagents to room temperature (15°C–25°C) except for Nuclease free water which is pre-chilled at 4°C.
    b. All steps should be completed at room temperature (15°C–25°C) except for RNA elution which is completed on ice.
    c. Prepare GTC Lysis Buffer and RNA Wash Buffer II according to the manufacturer's instructions on the day of extraction.

⚠ CRITICAL: GTC lysis buffer supplemented with β-mercaptoethanol is stable for 4 weeks at room temperature (15°C–25°C).

67. View organoids under the microscope to make an informed decision as to the number of wells to be used for extraction.

Note: This will be dictated by the confluency of the organoids in the wells. Typically, organoids that have reached 80% confluency (~1 × 10^6 single cells) will result in suitable yields of RNA (Figure 8).

Note: Make decision as to which time point organoids should be extracted. If you would like to analyze proliferative and/or stem cell markers, extract organoids by Day 7. If you would like to analyze differentiation markers, extract organoids at Day 10–14.

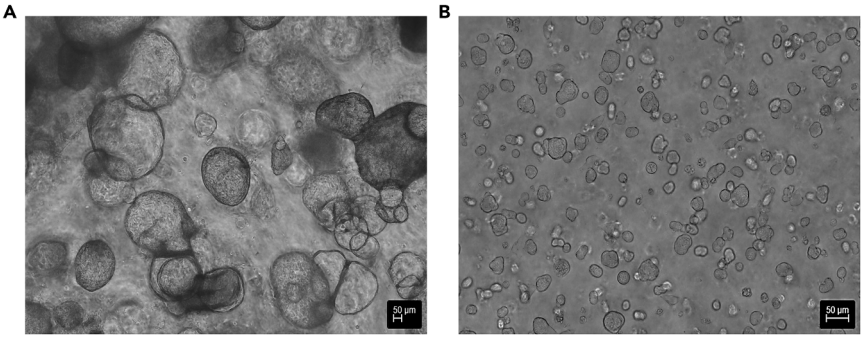


Figure 8. Visual representation of sufficient organoid confluency for RNA extraction

(A and B) Organoids that have reached a confluency of ~80% per 100 µL dome are suitable for RNA extraction. Representative images for appropriate confluency of (A) normal organoids that grow to form large cystic structures and (B) cancer organoids that form smaller acinar structures.

Images acquired using the Leica DmiL Inverted Light Microscope at 4× and 10× magnification, respectively, with the representative scale bar indicating 50 µm.

Note: The number of organoids to be used is dependent on the kit and the binding capacity of the columns in the kit. The maximum binding capacity of the HiBind RNA Mini Column is 100 µg. The maximum number of cells that can be efficiently lysed employing GTC Lysis Buffer is 1×10^7 cells.

68. Gently aspirate media from the selected wells in the 24-well plate containing organoids.
69. Wash organoids with 500 µL of pre-chilled (4°C) D-PBS.
70. Dispense 500 µL of GTC lysis buffer into each well of organoids.
71. Aspirate, using FBS pre-coated tips, to dislodge the organoids from the domes contained in the Cultrex matrix.

Note: At this point, the Cultrex should be completely dissolved.

72. Transfer the suspension to an Eppendorf tube and vortex tubes for 30 s at maximum speed to maximize lysis of organoids.
73. Centrifuge the lysate at 12,000 × *g* for 5 min. Discard the pellet (if present).
74. Transfer the lysate to the RNA homogenizer mini column contained in a 2 mL collection tube and centrifuge at 12,000 × *g* for 1 min.
75. Save the filtrate and discard the RNA homogenizer mini column.
76. Transfer the filtrate to an Eppendorf tube and supplement the filtrate with an equal volume of 70% ethanol.
77. The resulting mixture should be vortexed to mix thoroughly (30 s).
78. Transfer up to 700 µL of the sample mixture including any precipitate that may have formed to the HiBind RNA Mini Column contained within a 2 mL collection tube.
79. Centrifuge at 12 000 × *g* for 1 min. Discard the filtrate and reuse the collection tube.
80. Repeat this step until all the sample has been transferred to the column.
81. Add 500 µL RNA Wash Buffer I to the HiBind RNA Mini Column.
82. Centrifuge at 10,000 × *g* for 30 s. Discard the filtrate and reuse the collection tube.
83. Add 500 µL RNA Wash Buffer II to the HiBind RNA Mini Column.
84. Centrifuge at 10,000 × *g* for 1 min. Discard the filtrate and reuse the collection tube.
85. Repeat to Step 83 for a second RNA Wash Buffer step.
86. Centrifuge the empty HiBind RNA Mini Column at maximum speed for 8 min to dry the column. This step is critical for removal of trace ethanol that may interfere with downstream applications.
87. Transfer the HiBind RNA Mini Column to a clean 1.5 mL microcentrifuge tube.
88. Add 30–60 µL pre-chilled (4°C) Nuclease-Free Water and incubate on ice for 2 min to facilitate permeation of nuclease free water.

Table 1. RevertAid first strand cDNA synthesis reaction mix

Reagent	Volume (μL)
Template RNA	volume dependent on RNA concentration (1 μg)
oligo(dT)$_{18}$ primer	1
Nuclease-free water	to make a volume of up to 12 μl after template RNA added
Master mix	
5× Reaction Buffer	4
RiboLock RNase Inhibitor	1
10 mM dNTP Mix	2
RevertAid M-MulV RT	1
Volume of Master mix	8
Total volume for reaction	20

89. Centrifuge at maximum speed for 2 min to elute the RNA. Store eluted RNA at −80°C for downstream experiments.
90. Quantify total RNA extracted and validate RNA quality (purity and integrity) prior to proceeding to downstream experiments.

Note: The concentration and quality of RNA may be quantified using the Nanodrop One spectrophotometer and denaturing agarose gel electrophoresis to assess the integrity of the 28S and 18S ribosomal RNA. An $A_{260/280}$ ratio of ~2.0 and $A_{260/230}$ ratio of ~2.0–2.2 is indicative of pure RNA. Extracted RNA with an $A_{260/A280}$ and $A_{260/230}$ ratio of ~2.0 may be utilized for cDNA synthesis. For RNA integrity analysis, the 18S and 28S rRNA should appear as sharp bands, with the 28S band displaying twice the intensity of the 18S rRNA band.

Characterization of organoid cultures: RT-qPCR

Timing: 4.5 h

This section describes the procedure for completing first strand cDNA synthesis and reverse transcription quantitative polymerase chain reaction (RT-qPCR) to characterize organoid cultures. The genes of interest included *CHGA* (enteroendocrine cells), *EPCAM* (epithelial cells), *LGR5* (adult intestinal stem cells), *LYZ* (Paneth cells), *MKI67* (actively proliferating cells), *MUC2* (goblet cells) and *VIL1* (enterocytes).

Note: The RevertAid First Strand cDNA Synthesis Kit (https://www.thermofisher.com/order/catalog/product/K1621) is employed for the conversion of mRNA to cDNA.

91. Before you begin this protocol, ensure that the following steps have been completed:
    a. Ensure that the RNA has been quantified and that the quality and integrity of RNA has been validated prior to completing cDNA synthesis (as detailed in the above section).
    b. All reagents are thawed on ice, centrifuged and gently mixed prior to use.
92. Reagents to be added to the tubes and their corresponding volumes are listed in Table 1 (RevertAid First Strand cDNA Synthesis Reaction Mix).

Note: 1 μg of total RNA is employed for the first strand cDNA synthesis reaction.

93. Following the addition of all reaction components, mix gently and centrifuge briefly.
94. For oligo(dT)$_{18}$ or gene specific primed cDNA synthesis, the reaction mixture should be incubated at 42°C for 60 min using a Thermocycler. The reaction should subsequently be terminated by incubating at 70°C for 5 min (Refer to Table 2, 'Cycling Parameters for cDNA Synthesis').

Table 2. Cycling parameters for cDNA synthesis

Steps	Temperature (°C)	Time
Reaction Initiation & Amplification	42	60 min
Reaction Termination	70	5 min

Note: It is crucial to include a no template control (primer dimer and PCR contaminant amplification) and reverse transcriptase control (gDNA contamination).

95. cDNA products may be stored at −20°C for less than a week or at −80°C for long-term storage.

Note: An adaptation of the SensiFAST SYBR No-ROX Kit (https://www.bioline.com/sensifast-sybr-no-rox-kit.html) is employed to amplify target cDNA that was previously synthesized. The SensiFAST SYBR No-ROX kit is supplied as a ready-to-use master mix containing the SYBR Green I dye, dNTPs, stabilizers and enhancers.

96. Before you begin this protocol ensure that the following steps have been completed:
    a. Ensure that primers have been designed with the acceptable guidelines (GC content, melting temperature, amplicon length, self-complementarity, intron spanning) stipulated and that primers accurately target the gene of interest.

Note: For primer sequences and the corresponding annealing temperatures please refer to Table S1.

    b. All reagents should be thawed on ice and should be centrifuged and gently mixed prior to use.
97. Reagents to be added to the tubes and their corresponding volumes are listed in Table 3 (SensiFast SYBR No-ROX qPCR Reaction Mix) .
98. Following the addition of all reaction components, mix gently and centrifuge briefly (Quick Spin Function–10 s).
99. A 3-step cycling system should be set up on a Thermocycler (Table 4, 'RT-qPCR Cycling Parameters') as per manufacturers' guidelines.
100. For the validation of primer efficiency, a standard curve is created with a 10-fold serial dilution of cDNA by plotting Ct values against log(cDNA amount) and calculating the slope.

Note: A slope of −3.1 and −3.6, yielding reaction efficiencies between 90 and 110%, are typically acceptable. Thereafter, the Livak ($2^{-\Delta\Delta Cq}$) method may be used to quantify the relative gene expression of the PCR products.

Processing of human intestinal tissue and organoid cultures for formalin fixation and paraffin embedding

Timing: 2 days

Table 3. SensiFast SYBR No-ROX qPCR reaction mix

Reagent	Volume (µL)	Final concentration
2× SensiFast SYBR No-ROX mix	5	1×
10 µM Forward Primer	0.4	400 nM
10 µM Reverse Primer	0.4	400 nM
cDNA Template	2	–
dH_2O	2.2	–
Final volume	10	–

Table 4. RT-qPCR cycling parameters

Steps	Temperature (°C)	Time	Cycles
Polymerase activation	95	2 min	1
Denaturation	95	5 s	39
Annealing & extension	[a]	10 s	
Termination	72	20 s	

[a]Annealing temperatures are primer dependent

This section describes the procedure for processing human intestinal tissue and organoids for formalin fixation and paraffin embedding (FFPE) to facilitate histological and immunocytochemistry staining. In this protocol, it is important to ensure that all steps are conducted gently to maintain the fidelity of the organoid in terms of morphology and structure. A general workflow of organoid processing for FFPE is shown in Figure 9.

Note: For processing of tissue, ensure tissue has previously been stored in 10% neutral buffered formalin at 4°C for at least 48 h. Wash with pre-chilled (4°C) DPBS and proceed with step 113.

Note: Steps 101–112 describe the preparation of organoids for FFPE.

101. Before you begin this protocol ensure that the following steps have been completed:
    a. Pre-chill (4°C) DPBS and 4% paraformaldehyde (PFA).
    b. Acclimate the remainder of the freshly prepared reagents to room temperature (15°C–25°C).
    c. Organoids are seeded at a reasonable confluency (50 000–60 0000 single cells per 50 µL dome) to allow fully differentiated structures to form prior to fixation and staining.

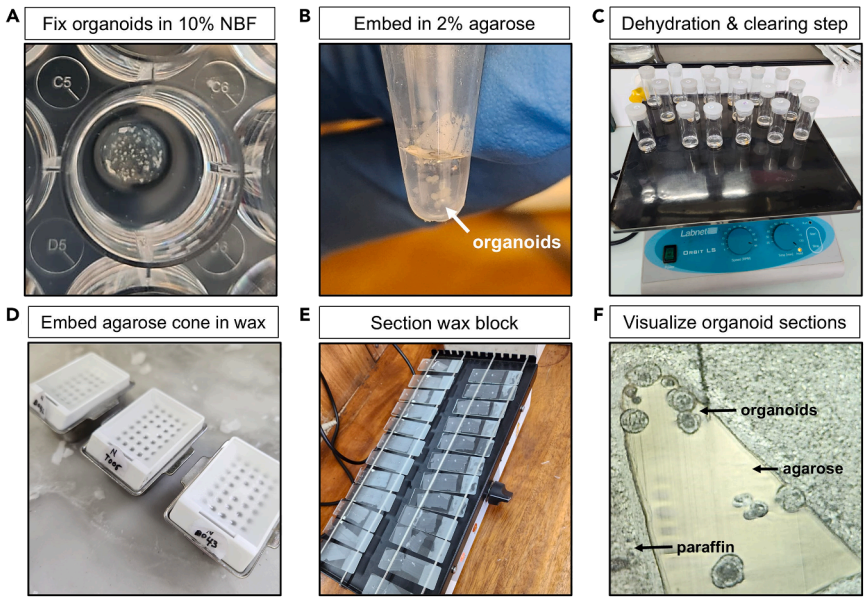


Figure 9. Organoid processing for FFPE workflow

(A) Following organoid differentiation (post day 10 in culture) organoids are fixed in 10% neutral buffered formalin (NBF) for 30 min.

(B) Organoids are washed and resuspended in 2% agarose.

(C) The agarose cone is subjected to ethanol dehydration and clearing with xylene.

(D) Thereafter, the agarose cone is embedded in paraffin wax and allowed to set in a square mold.

(E) The wax block is sectioned with a microtome, where slides are left to dry on a drying rack (~60°C) for at least 2 h.

(F) The presence of organoids on each section should be validated before beginning downstream assays.

Note: This should also be completed to prevent the merging of organoids if densely seeded which will affect the fidelity of the sections generated and impact the staining procedure.

102. Carefully remove and discard spent medium from selected wells to be fixed.
103. Gently wash each well using 500 μL of pre-chilled (4°C) DPBS.

⚠ CRITICAL: Be careful not to dislodge the BME containing organoids at this stage.

104. Discard DPBS and supplement wells with 500 μL of PFA.
105. Incubate organoids with PFA for 30 min at room temperature (15°C–25°C).
106. Prepare a 2% agarose solution for organoid embedding.
    a. Keep the solution at 95°C (melting point) until ready to use. This can be done with either a heating block or a water bath.
107. Following the incubation period gently collect the PFA-organoid suspension by scraping the dome off the surface of the plate using a Pasteur pipette and transferring the organoids to a 1.5 mL Eppendorf tube.
108. Pellet the organoids 600 × *g* for 3 min and complete a wash step by supplementing organoids with 500 μL of pre-chilled (4°C) DPBS.
109. Pellet the organoids and repeat the wash step one more time. Remove the supernatant.
110. Resuspend the organoids in 2% agarose in the 1.5 mL Eppendorf tube.
    a. Allow the agarose to cool to 50°C as indicated on the display of the heating block.
    b. Cut the tip of a p200 pipette tip to allow for easy aspiration of the agarose solution.
    c. Add 50–100 μL of warm agarose to the pellet and gently resuspend the organoid pellet by aspiration.
    d. You may use a 25-gauge needle attached to a syringe to assist with organoid resuspension and positioning of the organoids in the agarose.

⚠ CRITICAL: Bubbles in the agarose solution will disrupt the integrity of your sections. Do not depress the pipette to the second stop.

111. Store organoids embedded in agarose at room temperature (15°C–25°C) for approximately 1 h to facilitate solidification of agarose.

Note: Upon solidification of agarose, organoids will be embedded in a cone-like structure.

112. Using a long needle tip, gently lift the dome away and out of the Eppendorf tube and transfer to a glass vial.

Note: Be careful not to disrupt the cone-like structure.

113. Complete dehydration of the tissue and organoids in a glass vial by exposing them to an ethanol gradient as shown below:

Note: Embedded organoids may be stored in 70% ethanol at 4°C, for a maximum of 48 h. A longer storage time may affect the integrity of the organoid cone, causing it to be unusable.

Step	Reagent	Tissue (h)	Organoids (min)	Temperature
Dehydration	70% ethanol	1	30	room temperature (15°C–25°C)
	80% ethanol	–	30	
	90% ethanol	–	30	
	95% ethanol	1	30	
	100% ethanol	1	30	
	100% ethanol	1	30	

114. Complete clearing and embedding as follows:

Step	Reagent	Tissue (h)	Time (min)	Temperature
Clearing	Xylene	1	30	room temperature (15°C–25°C)
	Xylene	–	30	
Embedding	100% Wax	Overnight (16–24 h)	60	60°C

115. Following the wax incubation, samples should be embedded in paraffin wax in a square mold and left to solidify at room temperature (15°C–25°C) overnight (16–24 h).
    a. Place samples into a tissue mold and fill mold with molten paraffin wax.
    b. Ensure the sample is completely submerged in the wax and that no air bubbles are present around the sample.
    c. Carefully position a plastic cassette over the mold and press gently to ensure the cassette adheres well to the paraffin.
    d. Leave the paraffin to solidify at room temperature (15°C–25°C) for at least 2 h.
    e. Gently remove the solidified wax block from the mold.

Note: Embedded blocks can be stored at room temperature (15°C–25°C) or at 4°C until needed.

116. Before you begin sectioning ensure the following:
    a. Paraffin blocks are pre-chilled at 4°C overnight (16–24 h) to minimize tearing.
    b. The microtome is set up with an unused and pre-chilled (4°C) blade.
    c. A flotation bath is prepared with water at 42°C.
    d. A slide drying rack is set up at 60°C.
117. Prepare organoid/tissue sections as follows:
    a. Section the paraffin blocks at 4 μm thickness.
    b. Carefully transfer paraffin ribbons into the pre-warmed flotation bath, using a fine-tipped brush.
    c. Submerge a clean glass slide into the flotation bath and position the slide under the section.
    d. Slowly lift the section out of the water and place onto a drying rack for at least 2 h, to ensure the paraffin adheres firmly to the glass slide.

Note: Slides can be stored in a slide box at room temperature (15°C–25°C) for as long as necessary.

Hematoxylin and eosin staining of human intestinal tissue and organoids

Timing: 1 h

This section describes the procedure for completing Hematoxylin and Eosin staining on human intestinal tissue and organoids. In this protocol, it is important to ensure that all steps are conducted gently to maintain the fidelity of the organoids.

118. Complete H&E staining of organoid and tissue sections as follows:

Step	Reagent	Tissue (min)	Organoids (min)
Deparaffinization	100% Xylene	3	3
	100% Xylene	3	3

(*Continued on next page*)

Continued

Step	Reagent	Tissue (min)	Organoids (min)
Rehydration	100% Ethanol	5	5
	100% Ethanol	5	5
	95% Ethanol	2	2
	70% Ethanol	2	2
	Water	>30 s	>30 s
Hematoxylin Staining	Hematoxylin	3	3
	Running stream of tap water	1	1
Differentiation	Differentiation Solution	30 s	10 s
	Running stream of tap water	1	1
Bluing	Scotts Tap Water	1	1
	Running stream of tap water	5	2
Eosin Staining	95% Ethanol	10 s	10 s
	0.1% Eosin Y	1	1
Dehydration	95% Ethanol	5	5
	100% Ethanol	5	3
	100% Ethanol	5	3
Clearing	100% Xylene	5	3
	100% Xylene	5	3

119. Following this, sections should be subsequently mounted using DPX mounting medium.

Periodic acid-Schiff staining of human intestinal tissue and organoids

Timing: 1 h

This section describes the procedure for completing Periodic acid–Schiff (PAS) staining on human intestinal tissue organoids. In this protocol, it is important to ensure that all steps are conducted gently to maintain the fidelity of the organoids.

120. Complete PAS staining of organoid and tissue sections as follows:

Step	Reagent	Tissue (min)	Organoids (min)
Deparaffinization	100% Xylene	10	10
	100% Xylene	10	10
Rehydration	100% Ethanol	10 s	10 s
	100% Ethanol	10 s	10 s
	95% Ethanol	10 s	10 s
	Water	>30 s	>30 s
Oxidation	1% Periodic Acid	5	5
	Rinse Distilled Water	1	1
Schiff Reagent Staining	Schiff's Reagent	15	15
	Distilled Water	1	1
	Running stream of tap water	2	2
Hematoxylin Counter Staining & Bluing	Hematoxylin	30 s	30 s
	Running stream of tap water	2	2
Dehydration	95% Ethanol	10 s	10 s
	100% Ethanol	10 s	10 s
	100% Ethanol	10 s	10 s
Clearing	100% Xylene	10 s	10 s
	100% Xylene	10 s	10 s

121. Following this, sections should be subsequently mounted using Entellan mounting medium.

New Pentachrome staining of human intestinal tissue and organoids

Timing: 1 h

This section describes the procedure for completing New Pentachrome staining on human intestinal tissue organoids. In this protocol, it is important to ensure that all steps are conducted gently to maintain the fidelity of the organoids.

122. Complete New Pentachrome staining of organoid and tissue sections as follows:

Step	Reagent	Tissue (min)	Organoids (min)
Deparaffinization	100% Xylene	10	10
	100% Xylene	10	10
Rehydration	100% Ethanol	10 s	10 s
	100% Ethanol	10 s	10 s
	95% Ethanol	10 s	10 s
	Water	>30 s	>30 s
Nitric Acid Pre-Treatment	6% Nitric Acid	5	5
Toluidine Blue	0.5% Toluidine Blue	5	5
	Distilled Water	1	1
Picrosirius	Picrosirius	5	5
	Rinse Distilled Water	1	1
Dehydration	95% Ethanol	10 s	10 s
	100% Ethanol	10 s	10 s
	100% Ethanol	10 s	10 s
Clearing	100% Xylene	10 s	10 s
	100% Xylene	10 s	10 s

123. Following this, sections should be subsequently mounted using Entellan mounting medium.

Immunofluorescence staining of human intestinal tissue and organoid sections

Timing: 1 h

This section describes the procedure for completing immunofluorescence staining on human intestinal tissue and organoid sections. In this protocol, it is important to ensure that all steps are conducted gently to maintain the fidelity of the sections.

Note: A heat induced epitope retrieval (HIER) step is required to expose epitopes that have been altered as a result of tissue fixation with formalin. Organoids do not require HIER as the short exposure to formalin does not cause antigen crosslinking. Furthermore, HIER is detrimental to organoid sections as it dissolves the agarose.

124. Perform deparaffinization and rehydration of tissue and organoid sections as follows:

Step	Reagent	Time (min)	Temperature
Deparaffinization	100% Xylene	3	room temperature (15°C–25°C)
	100% Xylene	3	
Rehydration	100% Ethanol	5	
	100% Ethanol	5	
	95% Ethanol	2	
	70% Ethanol	2	
	Water	>30 s	

125. Perform HIER on tissue sections only.
    a. Place the slides in pre-warmed Antigen Retrieval Buffer (pH 6.0) (See 'buffers and media composition', Antigen Retrieval Buffer (pH 6.0) and microwave for 10 min.
126. Place the slides in pre-warmed Antigen Retrieval Buffer and microwave for 10 min.

Note: Leave the container lid open and ensure that the buffer does not boil over. If the buffer begins to boil over, stop the microwave, allow for the buffer to slightly cool and continue boiling.

⚠ CRITICAL: Do not allow the slides to dry out, this will destroy the tissue. If too much evaporation has occurred, top up the container with Antigen Retrieval Buffer and continue boiling.

127. Remove the container from the heat source and allow for slides to cool in the buffer at room temperature (15°C–25°C) for 15–20 min.

Note: Heating time can be optimized for individual samples.

128. Complete staining of tissue and organoid sections as follows:

Equilibration	PBS	>30 s	room temperature (15°C–25°C)
	PBS	>30 s	
Permeabilization	Immunofluorescence Permeabilization buffer	15 min	
Blocking	Immunofluorescence blocking buffer	60 min	
Primary Antibody Reaction	Primary Antibody[a]	Overnight (16–24 h)	4°C
Secondary Antibody Reaction	Immunofluorescence Wash Buffer	0.5	room temperature (15°C–25°C)
	Immunofluorescence Wash Buffer	0.5	
	Secondary Antibody[a,b]	60 min	
	PBS	>30 s	
	PBS	>30 s	
	PBS	>30 s	
Nuclear Staining	DAPI[b]	10	
	PBS	>30 s	
	PBS	>30 s	
	PBS	>30 s	

[a]These reagents are reconstituted as detailed in (See 'buffers and media composition', Antibody Dilution Buffer).
[b]These reagents are fluorescently labeled and hence light sensitive and slides should be incubated in the dark during these steps.

129. Following this, sections should be subsequently mounted using Fluoromount Aqueous Mounting Solution.

Filipin staining of human intestinal tissue and organoids to quantify free cholesterol

Timing: 3 h

This section describes the procedure for completing Filipin staining on human intestinal tissue organoids to quantify levels of free cholesterol. In this protocol, it is important to ensure that all steps are conducted gently to maintain the fidelity of the organoids.

130. Complete Filipin staining of organoid and tissue sections as follows:

Step	Reagent	Tissue (min)	Organoids (min)
Deparaffinization	100% Xylene	10	10
	100% Xylene	10	10
Rehydration	100% Ethanol	10 s	10 s
	100% Ethanol	10 s	10 s
	95% Ethanol	10 s	10 s
	Water	>30 s	>30 s
Filipin Stain	0.01 mg/ mL Filipin Stain[a]	120	120
	PBS	1	1
	PBS	1	1
NucRed Dead Counterstain	NucRed Dead[a]	10	10
	PBS	1	1
	PBS	1	1

[a]These reagents are light sensitive and slides should be incubated in the dark during these steps.

131. Following this, sections should be subsequently mounted using Fluoromount Aqueous Mounting Solution.

Vybrant lipid raft staining of human intestinal tissue and organoids

Timing: 1.5 h

This section describes the procedure for completing Vybrant lipid raft staining on human intestinal tissue organoids to quantify lipid raft cholesterol content. In this protocol, it is important to ensure that all steps are conducted gently to maintain the fidelity of the organoids.

132. Complete Vybrant lipid raft staining of organoid and tissue sections as follows:

Step	Reagent	Tissue (min)	Organoids (min)	
Deparaffinization	100% Xylene	10	10	room temperature (15°C–25°C)
	100% Xylene	10	10	
Rehydration	100% Ethanol	10 s	10 s	
	100% Ethanol	10 s	10 s	
	95% Ethanol	10 s	10 s	
	Water	>30 s	>30 s	

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Step	Reagent	Tissue (min)	Organoids (min)	
Permeabilization	Immunofluorescence Permeabilization Buffer	15	15	–
	PBS	1	1	
	PBS	1	1	
Vybrant Lipid Raft Stain	1 μg/mL Component A (Alexa Fluor 594 CT-B conjugate)	30	30	37°C
	PBS	1	1	room temperature (15°C–25°C)
	PBS	1	1	
	200-fold dilution Component B (Anti-cholera toxin subunit B antibody)[a]	30	30	37°C
	PBS	1	1	room temperature (15°C–25°C)
	PBS	1	1	
DAPI Counterstain	DAPI[a]	10	10	room temperature (15°C–25°C)
	PBS	1	1	
	PBS	1	1	

[a]These reagents are light sensitive and slides should be incubated in the dark during these steps.

133. Following this, sections should be subsequently mounted using Fluoromount Aqueous Mounting Solution.

BODIPY staining of human intestinal tissue and organoids to assess lipid droplet cholesterol content

Timing: 1.5 h

This section describes the procedure for completing BODIPY staining on human intestinal tissue organoids to quantify lipid droplet cholesterol content. In this protocol, it is important to ensure that all steps are conducted gently to maintain the fidelity of the organoids.

134. Complete BODIPY staining of organoid and tissue sections as follows:

Step	Reagent	Tissue (min)	Organoids (min)	
Deparaffinization	100% Xylene	10	10	room temperature (15°C–25°C)
	100% Xylene	10	10	
Rehydration	100% Ethanol	10 s	10 s	
	100% Ethanol	10 s	10 s	
	95% Ethanol	10 s	10 s	
	Water	>30 s	>30 s	
Permeabilization	Immunofluorescence Permeabilization Buffer	15	15	–
	PBS	1	1	
	PBS	1	1	
BODIPY Stain	1 μg/mL BODIPY Stain[a]	30	30	37°C
	PBS	1	1	room temperature (15°C–25°C)
	PBS	1	1	
DAPI Counterstain	DAPI[a]	10	10	room temperature (15°C–25°C)
	PBS	1	1	
	PBS	1	1	

[a]These reagents are light sensitive and slides should be incubated in the dark during these steps.

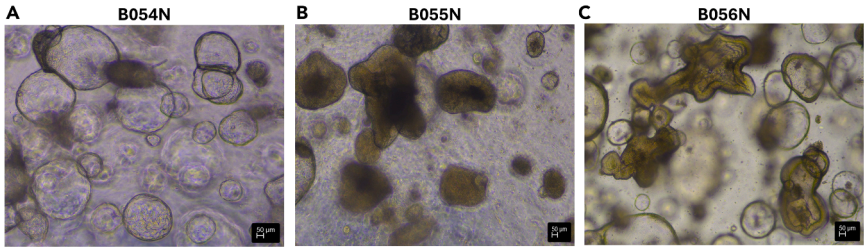


Figure 10. Organoid morphology variation between patient samples

(A) Patient B054N exhibits predominantly cystic like organoid structures.

(B) Patient B055N exhibits predominantly extensively budded (acinar) like organoid morphology.

(C) Patient B056N exhibits heterogeneous morphology of both cystic and budded structures. All images were captured at P1, Day 10.

Images acquired using the Leica DmiL Inverted Light Microscope at 10× magnification with the representative scale bar indicating 50 μm.

135. Following this, sections should be subsequently mounted using Fluoromount Aqueous Mounting Solution.

EXPECTED OUTCOMES

Organoid derivation and maintenance

Given the inherent biological differences between individuals of African and European ancestry, studying cancer progression across these populations is essential for improving therapeutic outcomes. To our knowledge, this is the first protocol paper that details the establishment and maintenance of intestinal organoids derived from South African patients. The rate of organoid formation and morphology is dictated by inter-patient heterogeneity, where organoids typically form between Days 3–14 (Figure 10). Figure 5 illustrates the trajectory of organoid growth kinetics and formation. Following successful organoid formation, organoids may be subjected to passaging, employing a 1:2 or 1:3 split ratio for the initial passage. Seeding the organoids too sparsely at the initial stage could compromise the expansion capacity of the organoid line; hence, it is crucial to maintain the organoids at a reasonably high seeding density to facilitate paracrine interactions which will stimulate robust growth of the organoids. Once the organoids have been expanded to a reasonable number of wells, it is crucial to cryopreserve the line at a reasonably early passage number for future experimental use. Following on from passage 2, organoids may be passaged on a weekly basis employing a more liberal split ratio of 1:3 to 1:5 for lines that are growing robustly. Data presented in this section included for healthy organoids are denoted with the suffix "N" whereas cancerous organoids are denoted with the suffix "C".

Organoid morphology is further influenced by inter-patient heterogeneity where they may either adopt a cystic structure characterized by a single polarized layer of epithelial cells surrounding a large central lumen or they may adopt an acinar structure characterized by a dense grape-like morphology with several branches and involutions (Figure 10). Additionally, organoid morphology tends to differ between normal and cancerous tissue (Figure 11).

Organoid morphology

Hematoxylin and Eosin (H&E) staining is the most well employed staining method to distinguish histological features of a specimen.[13] It works on the basis of differential staining of the nucleus and cytoplasm which facilitates the identification of cellular structures.[14] The basic dye Hematoxylin stains acidic structures such as the nuclei of cells a purplish hue, due to its high content of nucleic acids.[13] Conversely, Eosin is an acidic dye with high affinity for basic structures which are typically found in the cytoplasm, staining these proteins a pink-reddish hue.[13] The structure of the tissue is crucial to dictating tissue function. The principal function of the colon is water absorption and lubrication which consequently dictates the segmentation of cellular composition into two main

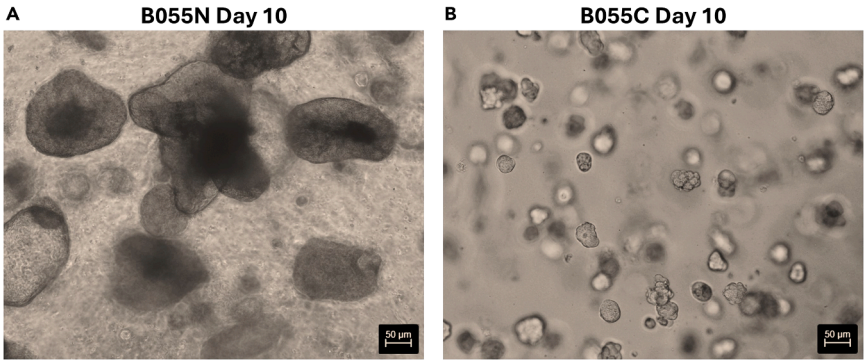


Figure 11. Morphological differences in normal versus cancer organoid morphology

(A) Healthy organoids tend to grow very large prior to exhibiting detailed differentiated structures while (B) cancer organoids typically form smaller acinar structures when fully differentiated.

Images acquired using the Leica DmiL Inverted Light Microscope at 10× magnification with the representative scale bar indicating 50 µm.

categories, absorptive cells, and secretory cells. The main absorptive cells in the colon are the enterocytes, primarily involved in nutrient absorption and digestion.[15] Goblet cells, Paneth cells, tuft cells and enteroendocrine cells constitute the four main secretory cell types, with goblet cells being the most abundant secretory cell type in the colonic mucosa.[15] In the colon, the absorptive and secretory cell types arrange to form tightly packed crypts that extend to the base of the muscularis mucosae.[7] Absorptive cells typically associate with the luminal surface of the colon while goblet cells

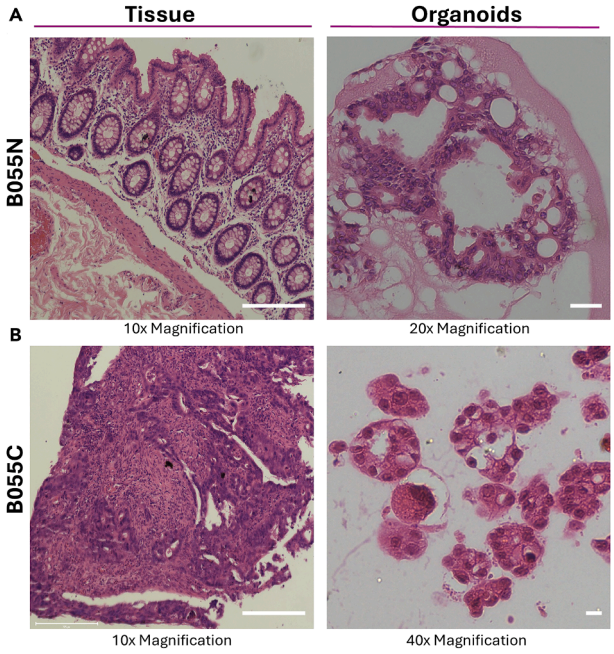


Figure 12. H&E staining of organoids and patient-matched tissue

(A) Human colon transverse section indicating crypt and villus axis (epithelial cells and goblet cells) as well colonic lumen as well as associated stroma, and patient-matched normal organoid with an accurate representation of columnar epithelial and goblet cells surrounding a central lumen.

(B) Human cancerous colonic tissue displaying a highly dysmorphic structure mainly depicting cancer associated stroma, and patient-matched cancerous organoids present as aberrant crypt-like structures recapitulating the malignant crypts present in the tumor tissue.

All images were acquired using the EVOS M700 Imaging System (Thermo Fisher Scientific, USA). Scale bars represent 200 µm.

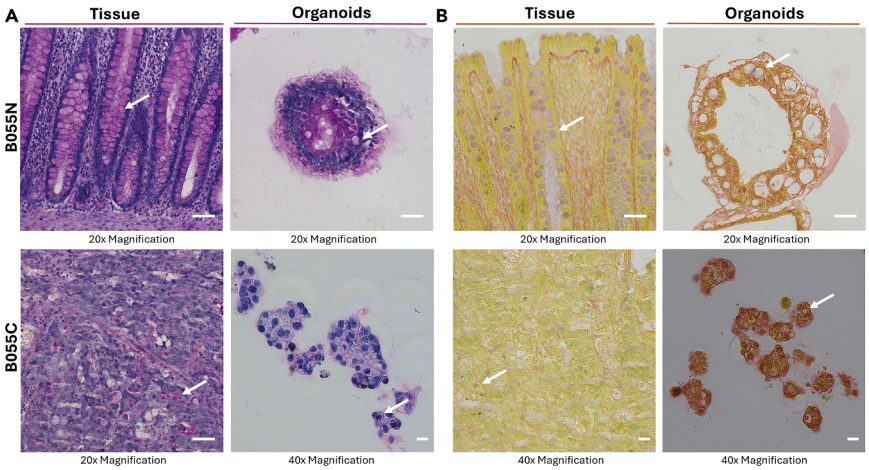


Figure 13. PAS and NPS staining of organoids and patient-matched tissue

(A) PAS staining of monosaccharide units to characterize goblet cell population within normal and cancer tissue and organoids.

(B) NPS staining of sulfated mucopolysaccharides to characterize goblet cell population within normal and cancer tissue and organoids. Arrows indicate the presence of goblet cells.

All images were acquired using the EVOS M700 Imaging System (Thermo Fisher Scientific, USA). Scale bars represent 200 μm.

are typically found lining the interior of crypts.[7] This is illustrated in Figure 12. Importantly, patient-derived organoid morphology recapitulates the cellular architecture and characteristics of patient-derived tissue consisting of columnar epithelial cells and goblet cells surrounding a central lumen. Moreover, patient-derived organoids accurately model patient-specific heterogeneity presenting as either thin-walled cystic structures or dense acinar like structures.

The goblet cell population was identified by employing PAS and NPS technique. PAS displays great versatility in the identification of carbohydrates, particularly, mucins which are a characteristic feature of goblet cells.[16] It is reliant on the reactivity of the free aldehyde groups of the monosaccharide units with the Schiff's reagent producing a bright red magenta color.[17] The NPS technique facilitates simultaneous staining of collagen and sulfated mucopolysaccharides in the same section. The sulfated groups in mucopolysaccharides stain violet, due to the characteristic of metachromasia where tolonium adopts a violet color upon binding to compounds with increased levels of negative charges such as glycosaminoglycans.[18] Collagen and RBCs stain red and yellow respectively, as Sirius Red contains sulfonic acid groups which react with hydroxyl and amide groups present in collagen.[19] Contrarily, the cytoplasm of RBCs bind to picric acid displaying an intense yellow color[18] (Figure 13).

Organoid characterization

Following successful establishment of organoids, the expression of characteristic makers may be studied to classify various epithelial cell types that constitute human intestinal organoids. The heterogeneous epithelial cell composition is attributed to the intestinal stem cells housed in the crypt base which terminally differentiate to give rise to various epithelial cell types, including enterocytes, goblet cells, Paneth cells, enteroendocrine cells, tuft cells and microfold cells (Table 5). In the following protocol, RT-qPCR was employed to assess characteristic marker gene expression that corresponds to each cell type (Figure 14) and immunocytochemistry staining was utilized to validate the gene expression signatures, to assess the protein expression and spatial localization of the cell types present in human intestinal organoids (Figure 15).

Table 5. Characteristic epithelial cell types in intestinal organoids

Cell type	Gene of interest	Description
Intestinal Stem Cells (ISCs)	*LGR5, MKI67*	Colonic ISCs exclusively express *LGR5* whereas *MKI67* is a marker of actively proliferating cells.[20]
Epithelial Cells	*EPCAM*	Epithelial cell adhesion molecule (EPCAM) serves as a transmembrane glycoprotein localized at tight junctions and the basolateral membrane to regulate intercellular adhesion of epithelial cells.[21]
Enterocytes	*VIL1*	Villin 1 (VIL1) is a microfilament associated actin-binding protein localized at the luminal border of the colon. It is conventionally expressed in highly specialized cells such as enterocytes.[22]
Paneth Cells	*LYZ*	Paneth cells constitute secretory cells of the intestinal epithelium responsible for the production and secretion of antimicrobial peptides, with Lysozyme (LYZ) classified as one of the most expressed antimicrobial peptides by these cells.[23]
Goblet Cells	*MUC2*	Goblet cells are secretory cells of the intestinal epithelium chiefly responsible for the production of mucins, with mucin 2 (MUC2) being the primary mucin secreted in the colon.[24]
Enteroendocrine Cells	*CHGA*	Chromogranin A (CHGA) is a neuroendocrine secretory protein which is used to conventionally classify enteroendocrine cells. These cells secrete various peptides and hormones that regulate satiety, digestion and intestinal motility.[25]

Organoid characterization (immunocytochemistry)

To validate the gene expression signatures obtained through RT-qPCR, immunocytochemistry staining was employed to assess the protein expression and spatial localization of the various cell types present in human intestinal organoids (Figure 15). Organoids grown within a basement membrane

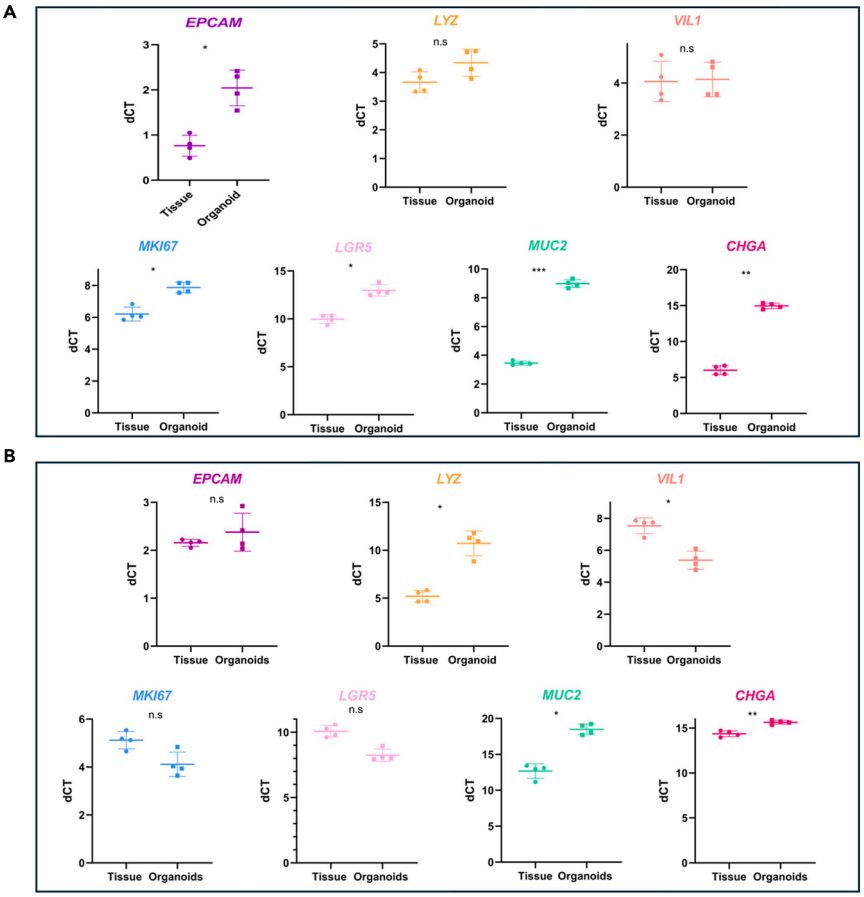


Figure 14. Characterizing organoid cell composition

RT-qPCR analysis of the gene expression markers utilized to characterize organoid cultures in (A) normal and (B) patient-matched cancer organoid lines. Data is represented as dCT values. Gene expression is indicated relative to β-actin (characterization validated in n=4 organoid lines (P2) at Day 10 in culture). A Student's T-Test was performed for statistical analysis, where * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and ns = not significant at 95% CI. Error bars are representative of the standard deviation relative to the mean.

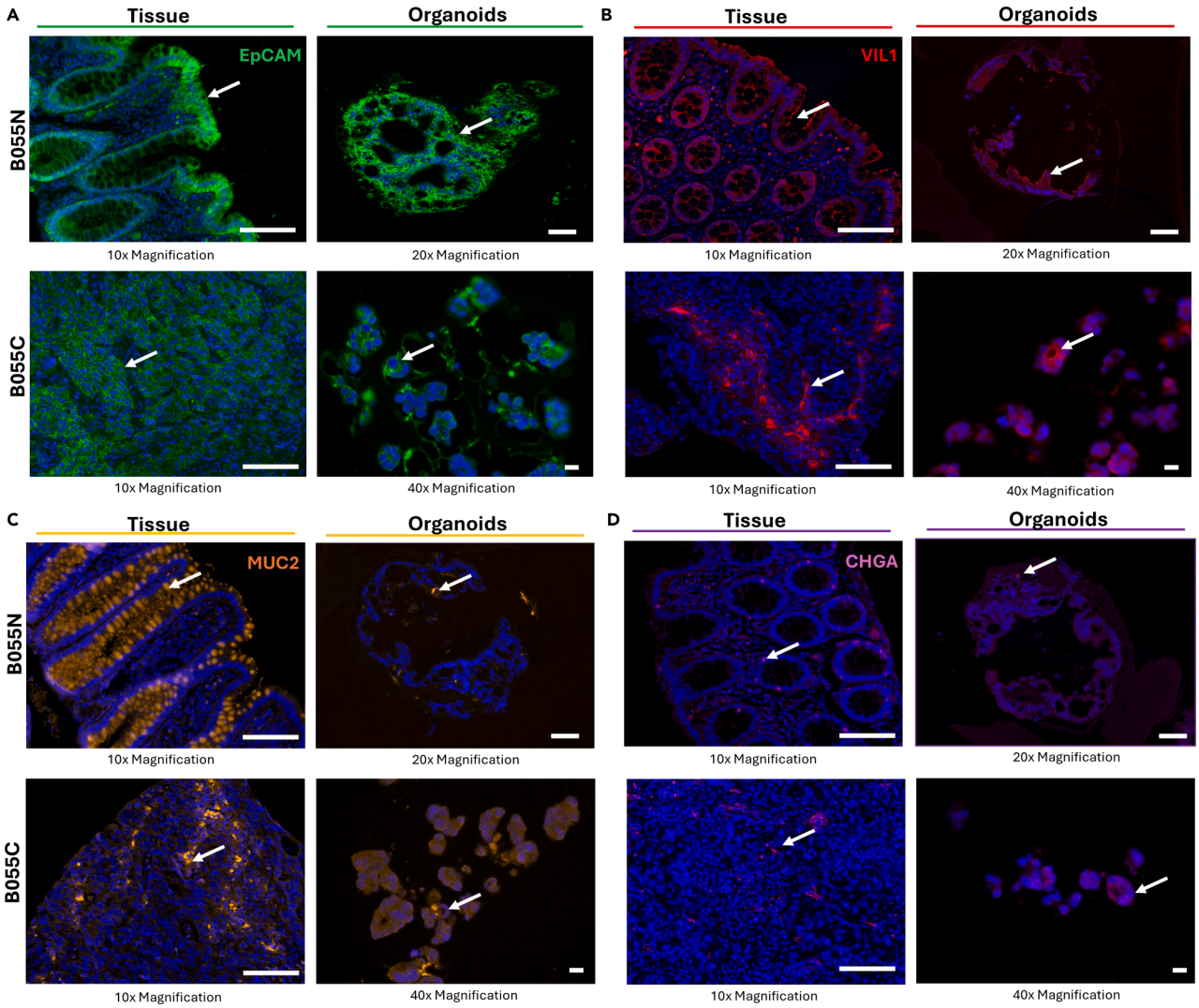


Figure 15. Visual representation of patient matched tissue and organoid, normal and cancer, immunofluorescence staining

(A) EPCAM staining (green) is indicative of epithelial cells. Nuclei are stained blue (DAPI).

(B) VIL1 staining (red) is indicative of enterocytes. Nuclei are stained blue (DAPI).

(C) MUC2 staining (orange) is indicative of goblet cells. Nuclei are stained blue (DAPI).

(D) CHGA staining (pink) is indicative of enteroendocrine cells. Nuclei are stained blue (DAPI).

All images were acquired using the EVOS M700 Imaging System (Thermo Fisher Scientific, USA). (characterization validated in n=6 organoid lines (P2) at Day 10 in culture. Scale bars represent 200 μm.

matrix (as described in this protocol) present with the apical (luminal surface) facing inwards and the basolateral surface in contact with the basement membrane matrix.[26]

Tissue and organoid cholesterol staining

Filipin is a naturally fluorescent compound that specifically binds to free (unesterified) cholesterol but not to esterified cholesterol. It is widely used for the labeling and quantification of free cholesterol within cells. Filipin forms a complex with cholesterol through a hydrophobic interaction which is facilitated by the 3β-OH group present in the chemical structure of cholesterol.[27] Both intestinal tissue and organoids exhibit strong filipin staining, consistent with the known role of cholesterol as a mitogen for intestinal stem cells.[28] Furthermore, Vybrant Alexa Fluor enables fluorescent labeling of lipid rafts in cells. This assay uses cholera toxin subunit B (CT-B), conjugated to the fluorescent dye Alexa Fluor 594.

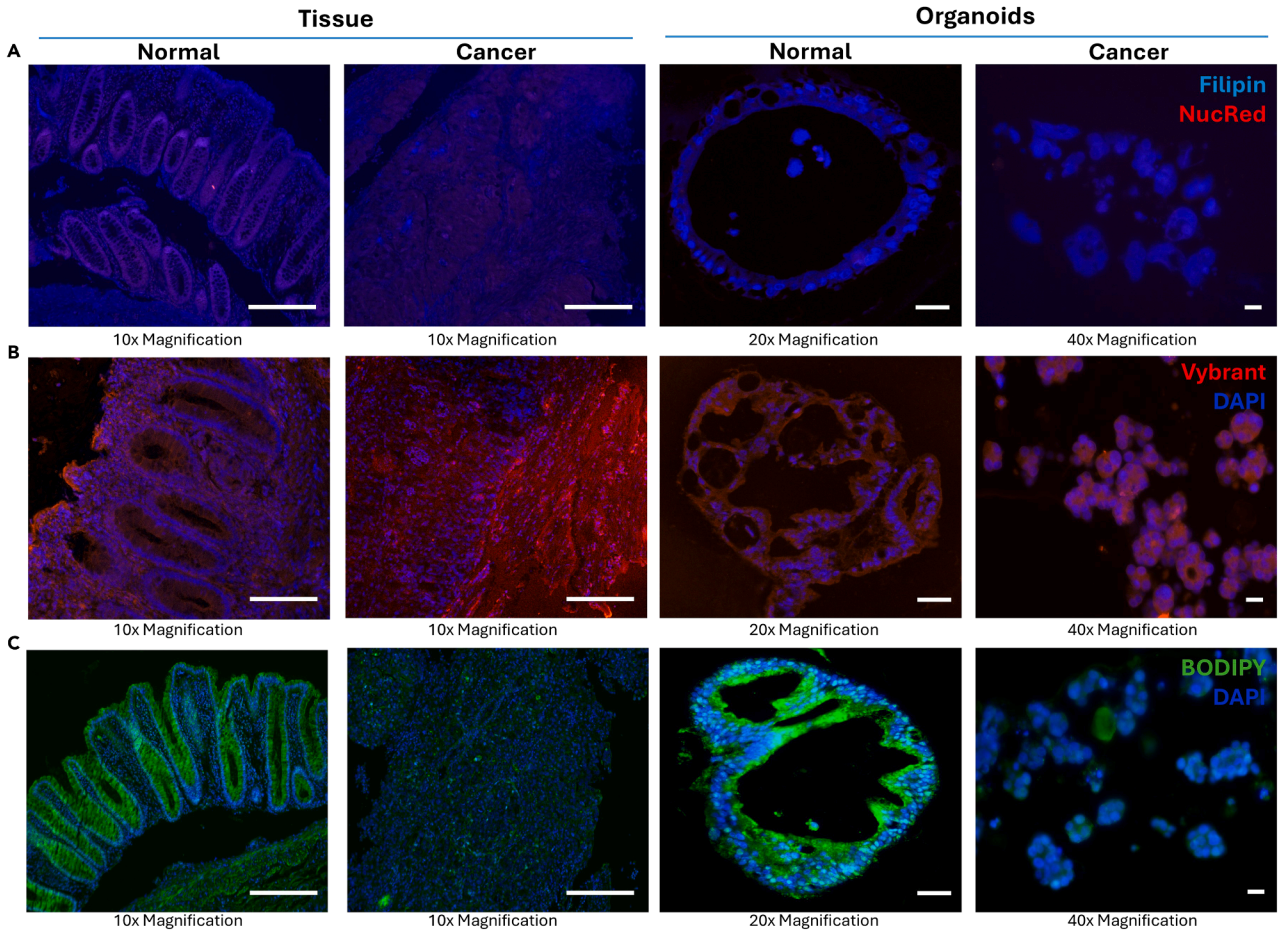


Figure 16. Cholesterol staining of B055 organoid and patient matched tissue

(A) Filipin staining (blue) indicative of free cholesterol (B) Vybrant Alexa Fluor (red) indicative of lipid raft cholesterol content and (C) BODIPY (green) staining indicative of lipid droplets present in normal and cancer tissue and patient-derived organoids respectively.

All images were acquired using the EVOS M700 Imaging System (Thermo Fisher Scientific, USA) (Cholesterol profiles validated in n=6 organoid lines (P2) at Day 10 in culture). Scale bars represent 200 μm.

CT-B binds specifically to the pentasaccharide chain of the ganglioside GM1, a lipid raft marker found in the plasma membrane. This interaction allows for the localization and visualization of cholesterol-rich lipid rafts, which appear as intense red fluorescence, typically counterstained with DAPI to highlight nuclei in blue. Proteins localized in lipid rafts participate in key signaling pathways, including those involved in apoptosis, cell cycle regulation, and cell adhesion. Moreover, cholesterol is a critical modulator of pathways that sustain cancer stemness, promote tumor progression, and confer drug resistance.[29] BODIPY is a lipophilic fluorescent dye characterized by a highly conjugated aromatic structure that favors accumulation within the hydrophobic core of lipid droplets. It preferentially binds to neutral cholesteryl esters,[30] with the BODIPY fluorophore known to conjugate at the C-24 position of the sterol ring. Upon excitation by blue light, BODIPY emits green fluorescence.[31] In colorectal cancer, lipid droplets are essential organelles that protect cells from oxidative stress by buffering reactive oxygen species (ROS) and act as energy reservoirs that support drug resistance and cancer cell survival.[32] For representative cholesterol profiles of organoids and patient-derived tissue, the reader is referred to Figure 16.

LIMITATIONS

The manuscript includes protocols for organoid derivation, maintenance, expansion, cryopreservation, and thawing. It also outlines methods for characterizing organoids through microscopy,

transcriptional profiling, immunocytochemical and histological comparisons with patient-derived tissue. Notably, we introduce a detailed protocol for cholesterol profiling in both patient-derived organoids and tissues, an important tool for researchers interested in lipid biology within organoid systems.

There are several limitations to consider when establishing organoids from fresh tissue. The most important consideration is the availability and viability of fresh tissue. The quality of tissue can be affected greatly by the time elapsed between tissue resection and processing, as well as handling conditions during transport. Additionally, to maximize success of initial organoid generation it is ideal to acquire a tissue section of greater than 10 mm^3. The organoid culture medium enriches the $LGR5^+$ stem cell population. It is noted, that the $LGR5^+$ expression profile declines at the tumor invasive fronts.[33] Therefore, the success of culturing cancer organoids is largely dependent on the location of tumor resection. It is recommended that organoids are generated from the colon epithelium as IntestiCult organoid growth media selectively enriches for the epithelial cell populations.

The downstream applications of organoids, such as qPCR and FFPE, are highly sensitive and it is important to standardize all steps of the protocol to ensure it works per your laboratory workflow. The quality and integrity of RNA needs to be validated before performing qPCR. Improper fixation or handling during FFPE can lead to poor preservation of organoid architecture compromising the validity of histological and molecular analyses.

TROUBLESHOOTING

Problem 1

Insufficient/ incorrect starting material which could compromise the protocol (related to Step 9).

Potential solution

Critical to ensure that specimens that are resected are an appropriate size and type for organoid generation and within the ethical boundaries accepted. This step is also crucial as it should inform surgeons as to where and how the samples should be stored following resection to ensure the viability of the tissue for organoid generation.

Problem 2

Between sample cross-contamination (Related to step 11c and 11d).

Potential solution

Critical to ensure that there is no sample-sample cross-contamination as this would affect the validity of the organoids generated from the patient tissue.

Problem 3

Bacterial contamination of cultures post seeding (related to Step 2).

Potential solution

This step is critical to remove any contaminating debris and feces which could potentially compromise the culture and should be completed meticulously and thoroughly. If contamination is a recurring problem in cultures the number of wash steps with the wash buffer solution may be increased.

Problem 4

Single cells are present when visualizing under the microscope, but majority of the tissue is not yet digested (related to Step 8).

Potential solution

If the tissue requires further digestion and single cells are visible under the microscope, remove the buffer without disturbing the tissue and dilute the buffer with DPBS, to inhibit the reaction. Add fresh pre-warmed tissue digestion media to the sample and continue with digestion.

Problem 5

Tissue slurry does not strain well (related to Step 9).

Potential solution

This could be due to insufficient digestion of primary tissue in the initial processing steps. Cutting the tissue into smaller pieces and incubating for a longer period should facilitate a more efficient digestion of the sample. This could also be due to contaminating adipose and muscular tissue that is potentially blocking the strainer. These contaminating tissue pieces should be resected prior to immersing the sample into the tissue digestion buffer to enhance the efficiency of the digestion.

Problem 6

Contaminating RBCs in organoid culture (related to Step 12).

Potential solution

This could be due to insufficient incubation of tissue slurry with RBC lysis buffer. Contaminating RBCs will interfere with viability of organoids and further affect matrix stability.

Problem 7

Excessive air bubble formation when pipetting BME (related to Step 16).

Potential solution

This could be due to harsh pipetting or insufficient thawing of BME prior to use. BME should be gradually thawed on ice in small volume aliquots (500 μL) for at least an hour prior to use. BME and tissue seeding media should be slowly but thoroughly aspirated to ensure a homogenous mix prior to plating organoids.

Problem 8

BME polymerizes in tip while pipetting (related to Step 16).

Potential solution

This could be due to incorrect handling of BME under temperatures that exceed (2°C–8°C) where BME will polymerize. It is recommended to work on ice when handling BME. If large volume aliquots of BME are being seeded, pipette tips can be pre-chilled to prevent BME from polymerizing.

Problem 9

Bubble formation when seeding organoids (related to Step 17).

Potential solution

This could be due to rapid dispensing of organoids when seeding. The plunger of the pipette should only be depressed to the first stop to avoid the formation of bubbles when seeding. Excessive number of bubbles in BME will affect the growth of organoids and further influence the stability of the dome which may lead to premature dome collapse.

Problem 10

BME flattens out or collapses upon dispensing to plate (related to Step 17).

Potential solution

This could be due to insufficient pre-warming of the culture plate prior to the seeding of organoids. A minimum overnight (16–24 h) period of pre-warming culture plates is recommended to maintain the

fidelity of organoid cultures. Additionally, the plate may be inverted for 5 min while the BME solidifies employing the hanging-drop technique to ensure that crypts settle and aggregate within the drop without risking the fidelity of the BME.

Problem 11

BME collapses upon the addition of media to the plate (related to Step 20).

Potential solution

BME was too diluted upon seeding thereby affecting the integrity of the dome. Ensure that BME concentration is not below 10 mg/mL following dilution with tissue seeding media. BME was not permitted sufficient time to solidify prior to the addition of media. Ensure that domes are incubated for at least 15–30 min prior to the addition of media. Otherwise, media was added harshly/directly on to the BME droplet affecting the fidelity of the domes. Ensure that the media is dispensed gently along the wall of the well to maintain the fidelity of organoid domes.

Problem 12

Organoids do not form post seeding (related to Step 28).

Potential solution

Organoid formation efficiency is dictated by several factors including tissue viability, time taken to process the sample, digestion time of the tissue, stem cell count per tissue fragment, seeding density as well as organoid media fidelity. Samples should be processed as soon as possible to maintain the integrity of stem cells that will give rise to organoids. The digestion time of the tissue should not exceed 90 min and should be terminated as soon as crypt/cell clumps are visible. Seeding density is also crucial as organoids rely on paracrine signaling for optimal growth. Organoid media should be stored under the correct conditions (−20°C for long term storage; 4°C for short term storage). It is important to note that patient to patient variability is expected due to sample dependent intrinsic growth capacity.

Problem 13

Organoids do not form a pellet (related to Step 38).

Potential solution

In some instances, organoids may be present but might not be visible to the naked eye. This could be due to the small sample size. If an insufficient number of organoids are pelleted (confluency is low or the presence of small cystic structures) organoids may not be visible to the naked eye. When pelleting small sample sizes, pay attention to the orientation of the tube when placed in the centrifuge. This will assist the individual to remove supernatant from the opposite side of the tube, reducing the possibility of disturbing the organoid pellet. For the most part when organoids are present in low numbers it is advisable to pool the samples across wells to obtain a more distinct pellet. Additionally, the type of centrifuge used could also influence the presence of a distinct pellet where in our instance the use of a swing bucket rotor facilitates efficient pelleting of organoids.

Problem 14

Organoids present with low viability (related to Step 39).

Potential solution

This could be due to excessive digestion in TrypLE. Reduce TrypLE digestion time to maintain viability of the organoids. Instead, organoids should be vigorously aspirated to enhance dissociation which will facilitate a reduced incubation time in TrypLE while maintaining viability of organoids.

Problem 15

Organoids present with different sizes following passaging (related to Step 44).

Potential solution

This could be due to insufficient digestion of some organoids in the population. To facilitate homogenous growth of the organoids, ensure that 90% of the population is digested to single cells prior to seeding.

Problem 16

Organoids do not expand well following passaging (related to Step 44).

Potential solution

Organoids may not expand well due to the apoptosis of cells upon dissociation to a single-celled state. It is important to supplement TrypLE with Y-27632 (ROCKi) upon dissociation and to also supplement organoid media with Y-27632 (ROCKi) for three days following dissociation to a single celled state. Rho kinase inhibitors such as Y-27632 ameliorate dissociation-induced apoptosis by modulating gap junctions and strengthening the adhesive potential of single cells. It is important to note that patient to patient variability is expected due to sample dependent intrinsic growth capacity which may contribute to variable expansion rates between organoid lines.

Problem 17

Organoids do not expand well post thawing and single cell dissociation (related to Step 65).

Potential solution

This could be due to inappropriate handling of organoids upon the completion of cryopreservation. Incorrect or insufficient freezing media utilized, incorrect use of the Nalgene Cryofreezing container, excessive dissociation of organoids to single cells which renders them labile to expansion.

Problem 18

RNA yield is low (related to Step 90).

Potential solution

This could be due to insufficient starting material. Organoids should be grown to a differentiated state to increase the yield of RNA obtained from the starting material. If RNA is extracted from organoids in the highly proliferative state (cystic structures with large clear lumens) subsequent wells may be pooled to increase the yield of RNA obtained.

Problem 19

RNA is contaminated with other molecular and chemical components (related to Step 90).

Potential solution

For contamination with gDNA, RNA should be treated with an appropriate RNase-free DNase. Contamination with phenols could be due to the presence of a clogged column due to sample overloading which would interfere with buffer elution in subsequent steps that could lead to phenol contamination of the sample. Additionally, insufficient drying of the column, prior to the elution of RNA could also lead to phenol contamination.

Problem 20

RNA is degraded (related to Step 90).

Potential solution

Meticulous handling of the RNA is crucial to maintain its fidelity for downstream experimentation. Ensure that all reagents are free from RNase contamination. For highly sensitive samples, pre-chilling nuclease free water (elution) as well as eluting the RNA on ice could prevent the degradation of RNA.

Problem 21
No RT -PCR product (related to Step 100).

Potential solution
RNA could potentially be degraded prior to cDNA synthesis, poor primer design, low concentration of primers utilized, or primers are degraded. cDNA concentration or cycling conditions are suboptimal.

Problem 22
Non-specific amplification products (related to Step 100).

Potential solution
RNA could be potentially contaminated with gDNA leading to non-specific amplification. Suboptimal primer design could lead to the formation of primer dimers; to avoid this, primers should be designed to span exon-intron boundaries. Suboptimal primer and template concentrations as well as incorrect cycling conditions could also lead to non-specific amplification in the PCR reaction.

Problem 23
Agarose solidifies in the tip before pipetting (related to Step 110).

Potential solution
A 2% agarose solution is highly viscous and polymerizes very quickly when below its melting temperature. Work quickly at this step, over a heating block. Ensure to cut the p200 tip for a fast and easy aspiration of the agarose.

Problem 24
Bubbles form in the Eppendorf tube when resuspending organoids in agarose (related to Step 110).

Potential solution
Bubbles in the agarose solution will disrupt the integrity of your sections. Do not depress the pipette to the second stop, resuspend very gently.

Problem 25
H&E staining looks uneven, hazy and/or poor contrast between the nuclear and cytoplasmic stain (related to Step 118).

Potential solution
Poor quality staining may mean that steps such as dehydration and clearing need to be optimized for your specimen. Ensure reagents are freshly prepared for every use. If nuclei are not staining distinctly this may be due to incomplete fixation or inadequate dehydration of the sample. You can adjust the hematoxylin and eosin staining times to improve contrast between the nuclear (hematoxylin) and cytoplasmic (eosin) stains.

Problem 26
There are no organoids visible following H&E staining (related to Step 118).

Potential solution
It is possible that organoids are not sectioned on every slide, therefore, it is recommended to visualize and confirm the presence of organoids embedded in the agarose before proceeding with the staining.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Mandeep Kaur (mandeep.kaur@wits.ac.za).

Technical contact

Technical questions on executing this protocol should be directed to and will be answered by the technical contact, Naaziyah Abdulla (1348887@students.wits.ac.za).

Materials availability

This study did not generate new unique reagents.

Data and code availability

The published article includes all information generated or analyzed during this study.

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AUTHOR CONTRIBUTIONS

N.A., R.A., and T.-L.d.P. performed sample collection and culturing and contributed to various techniques described in this protocol. N.A. wrote the initial draft, with additional contributions and edits from R.A. and T.-L.d.P. B.B. surgically collected samples from the individuals. M.K. conceptualized the study; acquired ethics clearance; helped with designing and troubleshooting, editing, and supervision; generated funds; and established collaborations.

DECLARATION OF INTERESTS

The authors declare no competing interests.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2025.103970.

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