bio-protocol

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Low-viscosity Matrix Suspension Culture for Human Colorectal Epithelial Organoids and Tumoroids

Tao Tan[1, 2], Yumiko Hirokawa[1], Jordan Clarke[1], Anuratha Sakthianandeswaren[1, 2] and Oliver M. Sieber[2, 3, 4, *]

[1]Personalised Oncology Division, The Walter and Eliza Hall Institute of Medial Research, Parkville, Victoria, Australia
[2]Department of Medical Biology, The University of Melbourne, Parkville, Victoria, Australia
[3]Department of Surgery, The University of Melbourne, Parkville, Victoria, Australia
[4]Department of Biochemistry and Molecular Biology, Monash University, Clayton, Victoria, Australia
*For correspondence: sieber.o@wehi.edu.au

Abstract

Three-dimensional culture of human normal colorectal epithelium and cancer tissue as organoids and tumoroids has transformed the study of diseases of the large intestine. A widely used strategy for generating patient-derived colorectal organoids and tumoroids involves embedding cells in domes of extracellular matrix (ECM). Despite its success, dome culture is not ideal for scalable expansion, experimentation, and high-throughput screening applications. Our group has developed a protocol for growing patient-derived colorectal organoids and tumoroids in low-viscosity matrix (LVM) suspension culture. Instead of embedding colonic crypts or tumor fragments in solid ECM, these are grown suspended in medium containing only a low percentage of ECM. Compared with dome cultures, LVM suspension culture reduces the labor and cost of establishing and passaging organoids and tumoroids, enables rapid expansion, and is readily adaptable for high-throughput screening.

Keywords: Patient-derived organoids, Tumoroids, Colorectum, Low-viscosity matrix, Suspension culture

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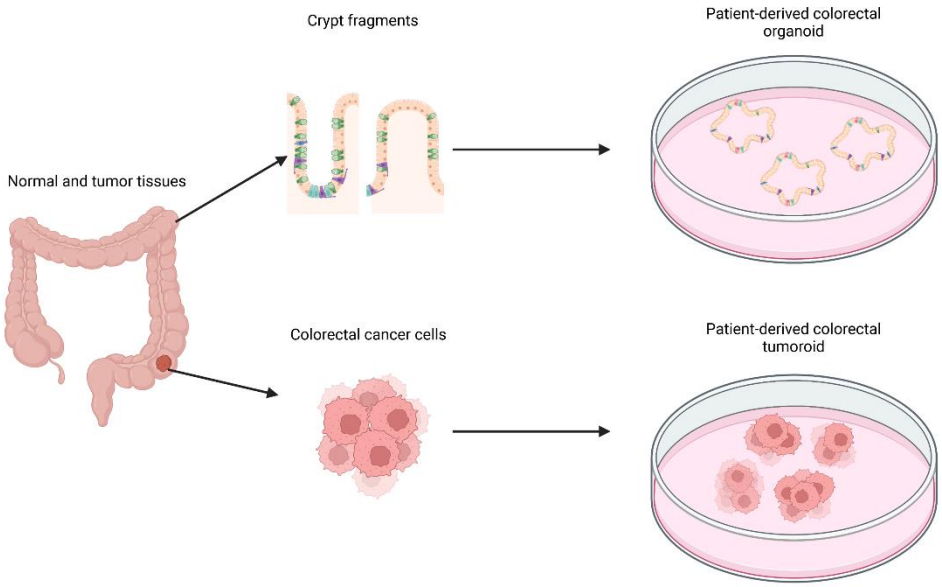
Graphic abstract:



Generation of organoids and tumoroids from human large intestine using LVM suspension culture (Created with BioRender.com).

Background

Patient-derived three-dimensional *in vitro* tissue models of normal and cancer tissues, referred to as organoids and tumoroids, have become an invaluable tool for the study of disease biology, and the development of precision medicine approaches. Organoids and tumoroids can be established from colorectal crypts and tumor fragments embedded in extracellular matrix (ECM), with medium containing growth factors and inhibitors supporting the growth of intestinal stem cells (Pinto *et al.*, 2003; Kuhnert *et al.*, 2004; Jung *et al.*, 2011; Sato *et al.*, 2011; Weeber *et al.*, 2015). While embedment into solid ECM is considered essential for intestinal epithelial organoid and tumoroid culture (Sato *et al.*, 2011), this is associated with technical challenges that hamper culture expansion and experimentation. Organoid and tumoroid growth in solid matrices is limited by the accumulation of solid stress, as well as the delivery of oxygen and nutrients (Roose *et al.*, 2003), requiring frequent passaging to enable culture maintenance and expansion. In addition, solid matrices must be removed mechanically or using enzymes to isolate organoids and tumoroids for propagation or downstream experimental applications (Sato *et al.*, 2011).

To address the limitations of organoid and tumoroid culture in solid ECM, we have developed a low-viscosity matrix (LVM) suspension culture method. Instead of ECM embedment, organoids and tumoroids are grown suspended in culture medium containing only a low percentage of ECM, reducing labor and cost. We have demonstrated the utility of our LVM suspension culture approach for organoid and tumoroid establishment, propagation, scalable expansion, and biobanking, as well as tumoroid high-throughput drug testing, as detailed in our related research article (Hirokawa *et al.*, 2021).

Materials and Reagents

1. Corning Tissue-culture treated culture Petri dishes 100 × 20 mm (Merck, catalog number: CLS430167)
2. Pipettes

3. Axygen filter tips 10 µL, 20 µL, 200 µL, 1,000 µL (Corning, catalog numbers: TF-1000-L-R-S, TF-200-L-R-S, TF-20-L-R-S, TF-10-L-R-S)
4. Greiner Bio-One 6 Well Suspension Culture Plates (Interpath, catalog number: 657185)
5. Corning™ Mini Bioreactor Centrifuge Tubes (Corning, catalog number: 431720)
6. Nunc 50 mL Conical Sterile Centrifuge Tubes (Thermo Fisher Scientific , catalog number: 339652)
7. Centrifuge tube 10 mL 100 × 16 mm (Sarstedt, catalog number: 62.9924.284)
8. Tube Micro 1.5 mL Centrifuge Polypropylene Clear Autoclaved (Eppendorf, catalog number: 0030121872)
9. Nalgene™ Rapid-Flow™ Sterile Single Use Vacuum Filter Units (Thermo Fisher Scientific, catalog number: 564-0020)
10. Human IntestiCult Organoids Growth Medium (Stemcell Technologies, catalog number: 6010)
11. Primocin (Invivogen, catalog number: ant-pm-1)
12. Penicillin-Streptomycin (10,000 U/mL) (Life Technologies, catalog number: 15140122)
13. Gibco™ Gentamicin (50 mg/mL) (Thermo Fisher Scientific, catalog number: 15750078)
14. Gibco Advanced DMEM/F-12 (Thermo Fisher Scientific, catalog number: 12634028)
15. Gibco GlutaMAX™ Supplement (Thermo Fisher Scientific, catalog number: 35050061)
16. HEPES (Sigma-Aldrich, catalog number: H3375-1KG)
17. Nicotinamide (Sigma-Aldrich, catalog number: 72340-1KG)
18. N-Acetyl-l-Cysteine (Sigma-Aldrich, catalog number: A7250-1KG)
19. B-27 Supplement (50×) (Life Technologies, catalog number: 17504001)
20. N-2 Supplement (100×) (Life Technologies, catalog number: 17502001)
21. Normocin (Invivogen, catalog number: ant-nr-2)
22. Human recombinant basic FGF (FGF2) (Life Technologies, catalog number: PHG0263)
23. Human recombinant EGF (Life Technologies, catalog number: PHG0313)
24. Y-27632 (Stemcell Technologies, catalog number: 72308)
25. Corning® Matrigel® Basement Membrane Matrix, *LDEV-Free, 10 mL (Corning, catalog number: 356234)
26. Dispase II (protease) (Merck, catalog number: D4693-1G)
27. Gibco Collagenase IV (Life Technologies, catalog number: 17104019)
28. TrypLE Express Enzyme (1×) (Thermo Fisher Scientific, catalog number: 12604021)
29. CryoStor® CS10 (Stemcell Technologies, catalog number: 07930)
30. Dulbecco's Phosphate Buffered Saline (Life Technologies, catalog number: 14040182)
31. Gibco DMEM/F12 (Life Technologies, catalog number: 11320082)
32. Gibco DMEM/F12 + GLUTAMAX (Life Technologies, catalog number: 10565042)
33. Ethylenediaminetetraacetic acid (EDTA) (Merck, catalog number: E9884)
34. Dithiothreitol (DTT) (Merck, catalog number: 11583786001)
35. A83-01 (Tocris Bioscience, catalog number: 2939)
36. SB202190 (Sigma-Aldrich, catalog number: S7067)
37. Bovine serum albumin (BSA) (Merck, catalog number: A9418)
38. Anti-Adherence Rinsing Solution (Stemcell Technologies, catalog number: 07010)
39. Tissue medium (see Recipes)
40. EDTA chelation buffer (see Recipes)
41. Colorectal tumoroid primary culture medium (see Recipes)
42. Colorectal tumoroid maintenance medium (see Recipes)

Equipment

1. Biosafety cabinet (Tissue culture hood)
2. Dissection Forceps and Scissors
3. Corning CoolCell LX Cell Freezing Container (Biocision, model: BCS-405)
4. Benchtop Centrifuge 5424 (Eppendorf, model: 5424)
5. Tabletop Centrifuge 5810R (Eppendorf, model: 5810R)

6. Bright-field inverted Tissue Culture Microscope
7. HERAcell VIOS 160i CO_2 incubator (Thermo Fisher Scientific, catalog number: HEA51030287)
8. Ultra low freezer (-80°C)

Procedure

A. Processing of human tissue samples

1. Tissues are kept in 4 mL of tissue medium (see Recipes) once collected after surgical resection.

   Note: Processing of the tissue within 24 h is recommended, although tissues can be stored in tissue medium at 4°C for up to 72 h.

2. Wash normal and tumour tissues in 10 mL of PBS containing 100 µg/mL Primocin and 100 µg/mL Gentamicin at room temperature for 15 min.

   Note: If tissue medium looks turbid when the tissue arrives, treat the tissue with 10 mL of 0.1% sodium hypochlorite diluted in PBS at room temperature for 5–10 min, as step A2.

3. Centrifuge in a tabletop centrifuge at 200 × *g* and 4°C for 5 min, and discard the supernatant.
4. Wash tissues another five times, with 10 mL of ice-cold PBS containing 100 U/mL Penicillin, 100 µg/mL Streptomycin, and 50 µg/mL Gentamicin, centrifuging at 200 × *g* and 4°C for 5 min.

B. Primary culture of organoids from normal colon tissues

1. Incubate tissue samples in 5 mL of EDTA chelation buffer (see Recipes) at room temperature for 30–60 min.
2. Transfer the samples from EDTA chelation buffer to 20 mL of ice-cold PBS.
3. Hold a sample with tweezers and shake it vigorously to release the colon crypts into PBS (Video 1 and Figure 1).

Video 1. Process of shaking off crypts from resected colon tissue.

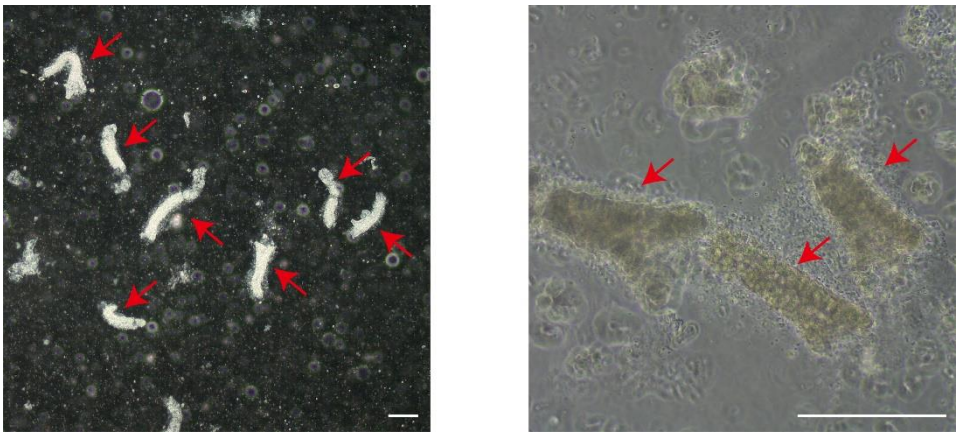


Figure 1. Representative images of isolated colorectal crypt fragments. Scale bar, 200 μm.

4. Remove the remaining tissue, centrifuge the crypt suspension in a benchtop centrifuge at 500 × *g* and 4°C for 3 min, and discard the supernatant.
5. Resuspend the isolated colonic crypts in 2 mL of freshly prepared DMEM/F12 containing 0.1 mg/mL Dispase II and 10 μM Y-27632.
6. Incubate at 37°C for 5–10 min, until the crypts are digested into fragments.

Note: Monitor crypt digestion using an inverted microscope at four times magnification.

7. Pipette the suspension 10–30 times with a 1,000 μL-tip, to help generate crypt fragments.
8. Centrifuge the suspension in a tabletop centrifuge at 500 × *g* and 4°C for 3 min.
9. Discard the supernatant, and resuspend the pellet in 3 mL of DMEM/F12 containing 10 μM Y-27632.
10. Centrifuge the suspension in a tabletop centrifuge at 500 × *g* and 4°C for 3 min.
11. Discard the supernatant and resuspend the pellet in 0.3–5 mL of DMEM/F12 containing 10 μM Y-27632.
12. Count the number of crypt fragments (~50 μm in size) using a haemocytometer (Figure 2), and calculate the required culture media volume as follows:
    a. 12,000–24,000 fragments in 3.5 mL/well for a 6-well suspension plate.
    b. 3,000–6,000 fragments in 1.5 mL/well for a 24-well suspension plate.
    c. 24,000–50,000 fragments in 5 mL/bioreactor tube, as shown in Figure 3.

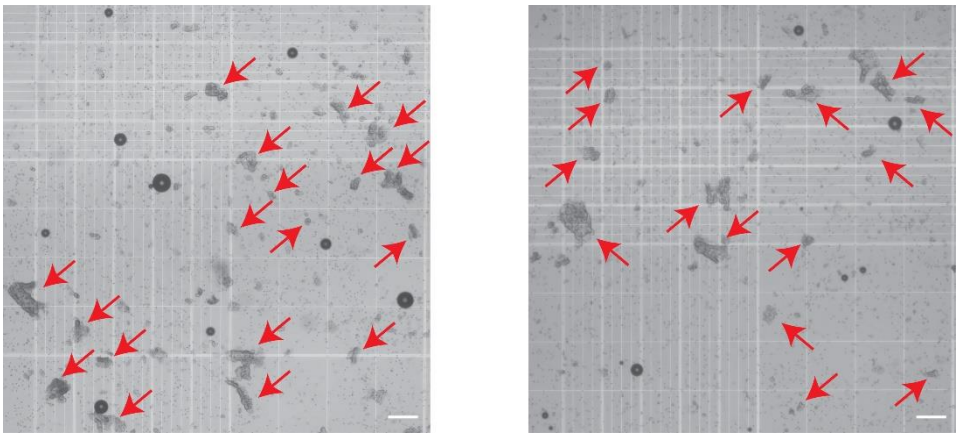


Figure 2. Counting of crypt fragments with a hemocytometer before plating. Red arrows indicate crypt

fragments. Scale bar, 200 μm.

Figure 3. Organoid/tumoroid cultures in bioreactor tubes in the tissue culture incubator.

13. Suspend crypt fragments in human IntestiCult organoids growth medium containing 5% Matrigel and 10 μM Y-27632, and transfer to plate wells.

    Note: Mix fragments and medium containing Matrigel by pipetting approximately 10 times with a 1,000-μL tip, without touching the bottom of the well. If using a bioreactor tube for culture, rinse the bioreactor tube with 10 mL of anti-adherence rinsing solution once and wash with 10 mL of DMEM/F12 three times before adding the crypt fragments.

14. Incubate in a 5% CO_2 incubator at 37°C.
15. Replace media with fresh human IntestiCult organoids growth medium containing 10 μM Y-27632 every two days. To change medium, allow the culture plates to settle in a tissue culture hood for 5 min, before removing supernatant. Remove half of the medium (2–3 mL) without disturbing the organoids. Tilt the plate to facilitate removal of medium if necessary. Add 2–3 mL of fresh medium, and mix by pipetting approximately 10 times with a 1,000-μL tip without touching the bottom of the well, to scramble up organoids. When using bioreactor tubes for culturing, tubes sit on the rack in the incubator. Shake tubes vigorously every 24 h to mix well. Agitating bioreactors with a shaker in the incubator can improve culture conditions with adequate air exchange.

Notes:

a. Y-27632 can be omitted once organoids are established.

b. Always use filter tips that are pre-rinsed with DMEM/F12 + 1% BSA, to avoid loss of organoids from pipetting. Observe cultures every day and scramble cells/organoids with pipettes, if these adhere to the bottom.

c. Medium change can also be performed by transferring cultures into centrifuge tubes, centrifuging in a tabletop centrifuge at 500 × g and 4°C for 5 min, replacing supernatant with fresh medium, and plating back into culture plates. When using bioreactor tubes, centrifuge in a tabletop centrifuge at 500 × g and 4°C for 10 min, so that the organoids settle down at the bottom before removing the supernatant.

d. To minimize the loss of organoids during medium change, centrifuge the collected supernatant in a tabletop centrifuge at 500 × g and 4°C for 5 min, or in a benchtop centrifuge at 6,000 rpm and room temperature for 1 min. Discard the supernatant, resuspend the pellet with 500 µL of fresh medium, and plate them back into the original well.

C. Primary culture of tumoroids from colorectal cancer (CRC) tissues

1. Mince CRC tissue into small pieces in a Petri dish on ice using forceps and scissors.

   Note: If the tissue is less than 30 mg, mince the tissue, resuspend in 3 mL of ice-cold PBS, and go to step C4.

2. Resuspend the minced tissues in 2 mL of freshly prepared DMEM/F12 containing 0.1 mg/mL Dispase II, 200 U/mL Collagenase IV and 10 µM Y-27632. Digest at 37°C for 30–60 min, and pipette the digestion mix and tissue pieces with 1,000-µL tips every 10 min, until the tissue pieces can pass through the tips easily.
3. Centrifuge the suspension in a tabletop centrifuge at 500 × *g* and 4°C for 5 min.

   Note: Check the supernatant for remaining cells under microscope before discarding. If there are still many cells in the supernatant, centrifuge the supernatant again at 500 × g and 4°C for 5 min.

4. Wash pellet with 10 mL of DMEM/F12 containing 10 µM Y-27632 at 500 × *g* and 4°C for 5 min.
5. Resuspend the pellets in 0.5 mL of colorectal tumoroid primary culture medium (see Recipes).
6. Count the number of fragments using a haemocytometer, and calculate the media volume as follows:
   a. 12,000–24,000 fragments in 3.5 mL/well for a 6-well suspension plate.
   b. 3,000–6,000 fragments in 1.5 mL/well for a 24-well suspension plate.
   c. 24,000–50,000 fragments in 5 mL/bioreactor tube.
7. Suspend crypt fragments in colorectal tumoroid primary culture medium (see Recipes) containing 5% Matrigel and 10 µM Y-27632. Transfer to plate wells.

   Note: Mix fragments and medium containing Matrigel by pipetting approximately 10 times with a 1,000-µL tip, without touching the bottom of the well. If using a bioreactor tube for culture, rinse the bioreactor tube with 10 mL of anti-adherence rinsing solution once, and wash with 10 mL of DMEM/F12 three times, before adding crypt fragments.

8. Incubate in a 5 % CO_2 incubator at 37°C.
9. Replace media with fresh colorectal tumoroid primary culture medium containing 10 µM Y-27632 every two days. To change medium, allow the culture plates to settle in a tissue culture hood for 5 min, before

removing supernatant. Remove half of the medium (2–3 mL) without disturbing the organoids. Tilt the plate to facilitate removal of medium if necessary. Add 2–3 mL of fresh medium, and mix by pipetting approximately 10 times with a 1,000-µL tip without touching the bottom of the well, to scramble up organoids. When using bioreactor tubes for culturing, tubes sit on the rack in the incubator. Shake tubes vigorously every 24 h to mix well. Agitating bioreactors with a shaker in the incubator can improve culture condition with adequate air exchange.

Notes:

a. *Y-27632 can be omitted once organoids are established.*
b. *Always use filter tips that are pre-rinsed with DMEM/F12 + 1% BSA, to avoid loss of organoids from pipetting. Observe cultures every day, and scramble cells/organoids with pipettes if these adhere to the bottom.*
c. *Medium change can also be performed by transferring cultures into centrifuge tubes, centrifuging in a tabletop centrifuge at 500 × g and 4°C for 5 min, replacing supernatant with fresh medium, and plating back into culture plates. When using bioreactor tubes, centrifuge in a tabletop centrifuge at 500 × g and 4°C for 10 min, so that the organoids settle down at the bottom before removing the supernatant.*
d. *To minimize the loss of organoids during medium change, centrifuge the collected supernatant in a tabletop centrifuge at 500 × g and 4°C for 5 min, or in a benchtop centrifuge at 6,000 rpm and room temperature for 1 min. Discard the supernatant, resuspend the pellet with 500 µL of fresh medium, and plate them back to the original well.*

D. Passaging

1. Colorectal organoids initially grow in round shapes (1–2 weeks); with longer term culture colorectal organoids to form crypt buds, as detailed in our previous publication (Hirokawa *et al.*, 2021). Organoids/tumoroids with dark cores are ready for passaging (Figure 4). If not passaged, dead cells will start to emerge from the organoids/tumoroids.

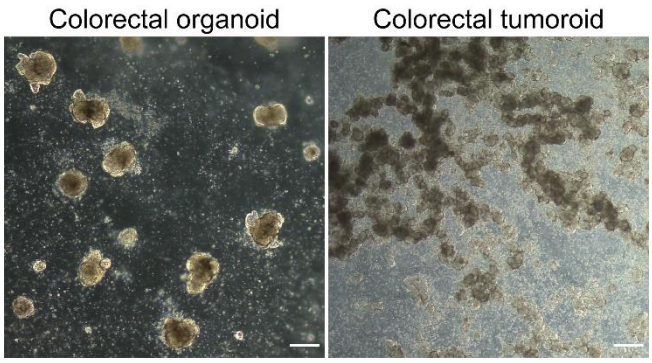


Figure 4. Bright-field images of colorectal organoids and tumoroids ready for passaging after 2 weeks in culture. Scale bars, 200 µm.

2. Centrifuge in a tabletop centrifuge at 500 × *g* and 4°C for 5 min.
3. Discard the supernatant, and resuspend the pellet in 1 mL of PBS.
4. Centrifuge in a benchtop centrifuge at 8,000 rpm and room temperature for 1 min.
5. Resuspend the pellet in 1 mL of TrypLE Express Enzyme, and incubate at 37°C for 10–15 min (depending on the size of the organoids).
6. Centrifuge in a benchtop centrifuge at 6,000 rpm and room temperature for 1 min.
7. Resuspend the pellet in 1 mL of 1 % (m/v) BSA in DMEM/F12.

8. Centrifuge in a benchtop centrifuge at 6,000 rpm and room temperature for 1 min.
9. Resuspend the pellet in 0.5–1 mL of human IntestiCult organoids growth medium or colorectal tumoroid maintenance medium (see Recipes) containing 10 µM Y-27632, pipetting with a 200-µL tip pre-rinsed with 1% BSA in DMEM/F12, to dissociate organoids to single cells or small fragments.
10. Count the number of fragments with a haemocytometer, and calculate the required media volume as follows:
    a. 100,000–300,000 cells, or 1,000–5,000 fragments in 3.5 mL/well for a 6-well suspension plate.
    b. 500,000 cells or 10,000 fragments in 17.5 mL in a bioreactor tube.
11. Resuspend single cells and/or fragments in human IntestiCult organoids growth medium or colorectal tumoroid maintenance medium (see Recipes) containing 5% Matrigel and 10 µM Y-27632. Transfer to wells or bioreactor tubes.
12. Incubate in 5% CO_2 incubator at 37°C.
13. Replace media with fresh human in human IntestiCult organoids growth medium or colorectal tumoroid maintenance medium containing 10 µM Y-27632 every two days. To change medium, allow the culture plates to settle in a tissue culture hood for 5 min, before removing supernatant. Remove half of the medium (2–3 mL) without disturbing the organoids. Tilt the plate to facilitate removal of medium if necessary. Add 2–3 mL of fresh medium, and mix by pipetting approximately 10 times with a 1,000-µL tip without touching the bottom of the well, to scramble up organoids. When using bioreactor tubes for culturing, tubes sit on the rack in the incubator. Shake tubes vigorously every 24 h to mix well. Agitating bioreactors with a shaker in the incubator can improve culture condition with adequate air exchange.

Notes:

a. Y-27632 can be omitted once organoids are established.

b. Always use filter tips that are pre-rinsed with DMEM/F12 + 1% BSA, to avoid loss of organoids from pipetting. Observe cultures every day, and scramble cells/organoids with pipettes if these adhere to the bottom.

c. Medium change can also be performed by transferring cultures into centrifuge tubes, centrifuging in a tabletop centrifuge at 500 × g and 4°C for 5 min, replacing supernatant with fresh medium, and plating back into culture plates. When using bioreactor tubes, centrifuge at in a tabletop centrifuge at 500 × g and 4°C for 10 min, so that the organoids settle down at the bottom before removing the supernatant.

d. To minimize the loss of organoids during medium change, centrifuge the collected supernatant in a tabletop centrifuge at 500 × g and 4°C for 5 min, or in a benchtop centrifuge at 6,000 rpm and room temperature for 1 min. Discard the supernatant, resuspend the pellet with 500 µL of fresh medium, and plate them back to the original well.

E. Cryopreservation

1. Collect organoids/tumoroids into centrifuge tubes.
2. Centrifuge in a tabletop centrifuge at 500 × *g* and 4°C for 5 min.
3. Discard the supernatant, and re-suspend the pellet with 5 mL of PBS.
4. Centrifuge in a tabletop centrifuge at 500 × *g* and 4°C for 5 min.
5. Discard the supernatant.

Notes:

a. *A turbid layer of ECM may be present after centrifugation. As long as the ECM does not contain organoids, it should be aspirated with pipettes and discarded.*
b. *For early organoids/tumoroids collected 3–5 days after last passaging, go to step E6 directly. For mature organoids/tumoroids that were cultured for 1–2 weeks, digest with 1 mL of TrypLE Express Enzyme at 37°C for 10–15 min as needed, followed by washing with 1 mL of 1% BSA in DMEM/F12 to terminate the digestion.*

6. Suspend pellets in 1 mL of CryoStor® CS10 freezing media per cryotube, to ensure a homogenous suspension.

Note: Organoids/tumoroids should be cryopreserved at the density of 10,000 organoids or 2 million viable cells per vial.

7. Cryopreserve organoids/tumoroids at a rate of 1°C/min, using a CoolCell LX Cell Freezing Container. Keep the freezing container in a -80°C freezer for more than 8 h before transferring the cryotubes to liquid nitrogen vapor phase, for long-term storage.

F. Thawing

1. Thaw the frozen organoids/tumoroids in a 37°C water bath.
2. Gently transfer organoid suspension to a centrifuge tube, using a 1,000-µL pipette.
3. Add 9 mL of DMEM/F12 containing 10 µM Y-27632 media.
4. Centrifuge in a tabletop centrifuge at 300–500 × *g* and 4°C for 3 min.
5. Discard supernatant and re-suspend pellets in 3.5 mL of fresh human IntestiCult organoids growth medium, or colorectal tumoroid maintenance medium, containing 5% Marigel and 10 µM Y-27632. Transfer to 6-well suspension culture plates.
6. Incubate in 5% CO_2 humidified incubator at 37°C.

Recipes

1. Tissue medium (once prepared, keep at 4°C)

Reagent	Final concentration	Amount
Y-27632 (10 mM)	10 µM	500 µL
Primocin (50 mg/mL)	100 µg/mL	1 mL
Gentamicin (50 mg/mL)	50 µg/mL	500 µL
DMEM/F12+GLUTAMAX	n/a	500 mL
Total	n/a	500 mL

2. EDTA chelation buffer (add DTT before use)

Reagent	Final concentration	Amount
EDTA	3 mM	876.72 mg
Dithiothreitol (DTT)	50 µM	7.71 mg
PBS	n/a	1,000 mL
Total	n/a	1,000 mL

3. Colorectal tumoroid primary culture medium

(Once prepared, keep at 4°C for up to 2 weeks. Freshly prepared medium is highly recommended. Add fresh human recombinant EGF and FGF2 before use.)

Reagent	Final concentration	Amount
Y-27632 (10 mM) **	10 µM	500 µL
Primocin (50 mg/mL)	100 µg/mL	1 mL
Penicillin-Streptomycin (10,000 U/mL)	100 U/mL and 100 µg/mL	5 mL
Gentamicin (50 mg/mL)	50 µg/mL	500 µL
Human recombinant Epidermal Growth Factor (EGF) (100 µg/mL)	50 ng/mL	250 µL
Human recombinant basic Fibroblast Growth Factor (FGF2) (20 µg/mL)	20 ng/mL	500 µL
Nicotinamide (1 M)	10 mM	5 mL
N-Acetyl-l-Cysteine (500 mM)	1 mM	1 mL
HEPES (1 M, pH 7.4)	10 mM	5 mL
Gibco GlutaMAX™ Supplement (100×)	100×	5 mL
B-27 Supplement (50×)	50×	10 mL
N-2 Supplement (100×)	100×	5 mL
Gibco Advanced DMEM/F-12	n/a	461.25 mL
Total	n/a	500 mL

**, Y-27632 is not needed after organoids or tumoroids are established.

4. Colorectal tumoroid maintenance medium

(Once prepared, keep at 4°C for up to 2 weeks. Freshly prepared medium is highly recommended. Human recombinant EGF and FGF2 can be added before use.)

Reagent	Final concentration	Amount
Y-27632 (10 mM) **	10 µM	500 µL
Normocin (50 mg/mL)	100 µg/mL	1 mL
Penicillin-Streptomycin (10,000 U/mL)	100 U/mL and 100 µg/mL	5 mL
Human recombinant Epidermal Growth Factor (EGF) (100 µg/mL)	50 ng/mL	250 µL
Human recombinant basic Fibroblast Growth Factor (FGF2) (20 µg/mL)	20 ng/mL	500 µL
Nicotinamide (1 M)	10 mM	5 mL
N-Acetyl-l-Cysteine (500 mM)	1 mM	1 mL
HEPES (1 M, pH 7.4)	10 mM	5 mL
Gibco GlutaMAX™ Supplement (100×)	100×	5 mL
B-27 Supplement (50×)	50×	10 mL
N-2 Supplement (100×)	100×	5 mL
Gibco Advanced DMEM/F-12	n/a	461.75 mL
Total	n/a	500 mL

**, Y-27632 is only needed when organoids or tumoroids were broken into single cells for passaging.

Acknowledgments

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Competing interests

The authors declare no competing interests.

Ethics

This study was conducted in accordance with the Declaration of Helsinki, the NHMRC Statement on Ethical Conduct in Human Research, and Institutional Human Research Ethics approval (HREC 2016.249, Walter and Eliza Hall Institute of Medical Research). All patients gave informed consent.

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