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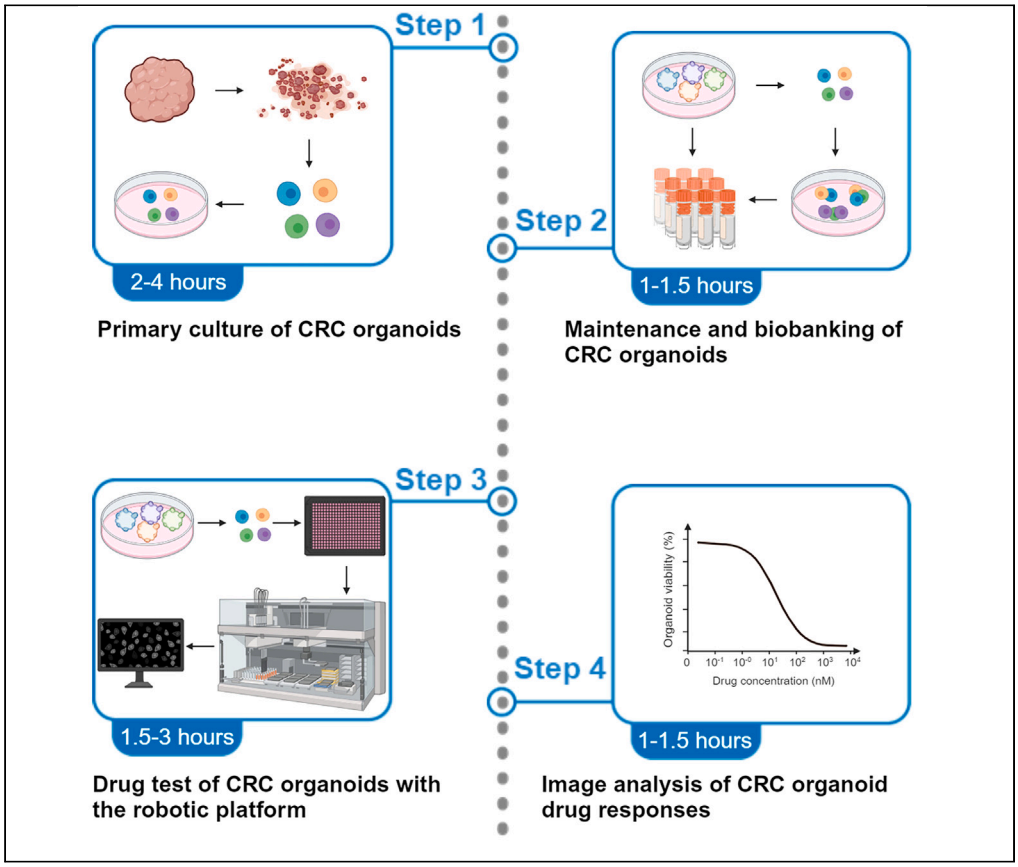
Protocol for generation of and high-throughput drug testing with patient-derived colorectal cancer organoids



Drug sensitivity testing of patient-derived tumor organoids (PDTOs) is a promising tool for personalizing cancer treatment. Here, we present a protocol for generation of and high-throughput drug testing with PDTOs. We describe detailed steps for PDTO establishment from colorectal cancer tissues, preparation of PDTOs for high-throughput drug testing, and quantification of drug testing results using image analysis. This protocol provides a standardized workflow for PDTO testing of standard-of-care therapies, along with exploring the activity of new agents, for translational research.

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

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Highlights

Suspension culture protocol to establish colorectal cancer patient-derived organoids

Steps to expand, cryopreserve, and thaw colorectal cancer organoids

Procedures to perform high-throughput drug testing with colorectal cancer organoids

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Protocol for generation of and high-throughput drug testing with patient-derived colorectal cancer organoids

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SUMMARY

Drug sensitivity testing of patient-derived tumor organoids (PDTOs) is a promising tool for personalizing cancer treatment. Here, we present a protocol for generation of and high-throughput drug testing with PDTOs. We describe detailed steps for PDTO establishment from colorectal cancer tissues, preparation of PDTOs for high-throughput drug testing, and quantification of drug testing results using image analysis. This protocol provides a standardized workflow for PDTO testing of standard-of-care therapies, along with exploring the activity of new agents, for translational research.

For complete details on the use and execution of this protocol, please refer to Tan et al.[1]

BEFORE YOU BEGIN

Approximately 90% of colorectal cancers (CRCs) exhibit aberrant activation of the WNT signaling pathway.[2–5] To select for outgrowth of CRC cells from tumor tissue specimens, WNT ligands are thus omitted from tumor organoid culture medium. The protocol below describes the specific steps for generating and drug sensitivity testing of CRC organoids in suspension culture with 3%–5% of Matrigel. Notably, the tumor organoid culture medium used in this protocol is not suitable for tumors that are dependent on WNT ligand-mediated signaling, such as DNA mismatch repair deficient tumors with loss-of-function mutations in RNF43.[6]

Institutional permissions

All patients provided written informed consent, and the study was approved by medical ethics committees at all sites (HREC 2016.249, WEHI).

Studies conducted using this protocol must obtain permission(s) from relevant institutions.

Preparation of components for colorectal cancer organoid culture medium

Timing: 90 min

1. Prepare B27 supplement aliquots (store at −20°C for up to 6 months).
   a. Thaw the bottle of B27 supplement at 4°C 12 h before use.
   b. Divide into 10-mL aliquots.
   c. Thaw the required number of 10 mL B27 aliquots at 4°C 12 h before use before use.
2. Prepare N2 supplement aliquots (store at −20°C for up to 6 months).
   a. Thaw the bottle of N2 supplement at 4°C 12 h before use .
   b. Divide into 5 mL aliquots.
   c. Thaw the required number of 5 mL N2 aliquots at 4°C 12 h before use.
3. Prepare nicotinamide (Stock concentration: 1 M, store at −20°C for up to 6 months).
   a. Weigh 61.06 *g* nicotinamide in a sterile environment.
   b. Dissolve nicotinamide in 500 mL sterile DPBS prefiltered with a 0.1 μm filter by inverting the bottle multiple times until all powder is dissolved and the solution becomes clear.
   c. Divide into 5 mL aliquots.
   d. Thaw required aliquots at 20°C–22°C before use.
4. Prepare A-83-01 (Stock concentration: 10 mM, store at −20°C for up to 6 months).
   a. Dissolve 5 mg A-83-01 in 1.19 mL sterile DMSO by vortexing.
   b. Divide into 25 μL aliquots.
   c. Thaw required aliquots at 20°C–22°C before use.
5. Prepare N-acetyl-L-cysteine (Stock concentration: 500 mM, store at −20°C for up to 6 months).
   a. Weigh 4.08 *g* N-acetyl-L-cysteine (NAC) in a 50 mL tube in a sterile environment.
   b. Add 50 mL Milli-Q water prefiltered with a 0.1 μm filter.
   c. Mix by inverting and incubate at 37°C until dissolved.
   d. Divide into 1 mL aliquots.
   e. Thaw required aliquots at 20°C–22°C before use.
6. Prepare human recombinant epidermal growth factor (EGF) (Stock concentration: 100 μg/mL, store at −80°C for up to 6 months).
   a. Dissolve 1 mg EGF in 10 mL sterile DPBS prefiltered with a 0.1 μm filter containing 0.1% BSA by inverting.
   b. Divide into 250-μL aliquots.
   c. Thaw required aliquots at 20°C–22°C before use.
7. Prepare human recombinant basic fibroblast growth factor (FGF2) (Stock concentration: 100 μg/mL, store at −80°C for up to 6 months).
   a. Dissolve 1 mg FGF2 in 10 mL sterile DPBS prefiltered with a 0.1 μm filter containing 0.1% BSA by inverting.
   b. Divide into 100-μL aliquots.
   c. Thaw required aliquots at 20°C–22°C before use.
8. Prepare HEPES (Stock concentration: 1 M, pH 7.4, store at 20°C–22°C for up to two years).
   a. Dissolve 119.15 *g* in 500 mL Milli-Q water by inverting.
   b. Adjust pH to 7.4 with NaOH.
   c. Filter through 0.1 μm filter.
9. Prepare Y-27632 (ROCK inhibitor) (Stock concentration: 10 mM, store at −80°C for up to 12 months).
   a. Dissolve 50 mg in 20.22 mL sterile DMSO by vortexing.
   b. Divide into 500-μL aliquots.
   c. Thaw required aliquots at 20°C–22°C before use.

Preparation of tissue collection medium

Timing: 30 min

10. Add 500 μL 50 mg/mL gentamicin into 500 mL DMEM/F12 containing GlutaMAX medium.
11. Divide tissue collection medium into 4 mL aliquots in 5 mL tubes.
12. Store at 4°C for up to 6 months.

Preparation of tissue digestion medium

Timing: 30 min

13. Weigh 500 mg collagenase IV in a sterile environment.
14. Add 500 mL DMEM/F12 containing GlutaMAX medium to dissolve collagenase. The final concentration of collagenase solution is 1 mg/mL, which equals to 200 U/mL.
15. Add 500 μL 10 mM Y-27632 to reach a final concentration of 10 μM.
16. Divide tissue collection medium into 1 mL aliquots in 1.5 mL Eppendorf tubes (store at −20°C for up to 12 months).
17. Thaw required aliquots at 20°C–22°C before use.

Preparation of colorectal cancer organoid maintenance and primary culture medium

Timing: 30 min

18. Remove 37.9 mL medium from the 500 mL bottle of Advanced DMEM/F-12.
19. Add 5 mL 100x N2 supplement (final concentration 1x).
20. Add 10 mL 50× B27 (final concentration 1x).
21. Add 1 mL 500 mM NAC (final concentration 1 mM).
22. Add 5 mL 1 M nicotinamide (final concentration 10 mM).
23. Add 100 μL 100 μg/mL human recombinant FGF2 (final concentration 20 ng/mL).
24. Add 250 μL 100 μg/mL human recombinant EGF (final concentration 50 ng/mL).
25. Add 5 mL 1 M HEPES (final concentration 10 mM).
26. Add 5 mL Gibco GlutaMAX supplement (100X) (final concentration 1x).
27. Add 5 mL penicillin-streptomycin (containing 10,000 units/mL of penicillin and 10,000 μg/mL of streptomycin.) (final concentration 100 U/mL and 100 μg/mL).
28. Add 1 mL 50 mg/mL normocin (final concentration 100 μg/mL).
29. Add 25 μL 10 mM A-83-01 (final concentration 500 nM).
30. Add 500 μL 10 mM Y-27632 (final concentration 10 μM).
31. Filter through 0.22 μm filter. Store made-up medium at 4°C for up to 2 weeks or aliquot into 45-mL aliquots in 50-mL Falcon centrifuge tubes and store the medium at −20°C for up to 3 months.

⚠ CRITICAL: For primary culture, change step 18 to "remove 32.9 mL medium" and replace steps 27 and 28 by "adding 1 mL 50 mg/mL primocin (final concentration 100 μg/mL)".

Preparation of equipment for processing colorectal cancer tissues

Timing: 50 min

32. Scissors and Tweezers need to be soaked in 75% Ethanol for 10 min at 20°C–22°C.
33. Wipe scissors and tweezers clean with paper towel pre-wet with 80% ethanol.
34. Put the clean scissors and tweezers on racks in a biosafety cabinet to undergo 30-min UV.

Optional: Label two petri dish with allocated patient/organoid IDs and prepare motifs for OCT embedment.

Optional: Prepare cryopreservation tubes filled with 800 μL CryoStor CS10 medium.

Preparation of washing PBS

Timing: 3 min

35. Add 40 μL 50 mg/mL gentamycin into 40 mL PBS (40 mL is sufficient for processing each tumor tissue).

⚠ CRITICAL: Good aseptic technique is required when preparing all reagents.

Preparation of washing medium

Timing: 3 min

36. Add 500 μL 10 mM Y-27632 into 500 mL DMEM/F12 containing GlutaMAX medium (100 mL is sufficient for processing each tumor tissue; remaining washing medium can be stored at 4°C for 1 month).

⚠ CRITICAL: Good aseptic technique is required when preparing all reagents.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological samples		
Human colorectal cancer tissues	This paper	N/A
Human normal colon tissues	This paper	N/A
Buffy coat from patient blood samples	This paper	N/A
Chemicals, peptides, and recombinant proteins		
Gibco Advanced DMEM/F-12	Thermo Fisher Scientific	Cat#12634028
Gibco DMEM/F12 (containing GlutaMAX)	Life Technologies	Cat#10565042
GlutaMAX supplement	Gibco	Cat#35050061
HEPES	Sigma-Aldrich	Cat#H3375-1KG
B-27 supplement	Life Technologies	Cat#17504001
N2 supplement	Life Technologies	Cat#17502001
Nicotinamide	Sigma-Aldrich	Cat#72340-1KG
N-acetyl-L-cysteine	Sigma-Aldrich	Cat#A7250-1KG
Recombinant human basic fibroblast growth factor (FGF2)	Life Technologies	Cat#PHG0263
Recombinant human epidermal growth factor (EGF)	Life Technologies	Cat#PHG0313
A83-01	Tocris Bioscience	Cat#2939
Primocin	InvivoGen	Cat#ant-pm-2
Y27632	STEMCELL Technologies	Cat#72308
Matrigel	Corning	Cat#356234
Penicillin-streptomycin	Life Technologies	Cat#15140122
Normocin	InvivoGen	Cat#ant-nr-2
Gentamicin	Thermo Fisher Scientific	Cat#15750078
Collagenase IV	Life Technologies	Cat#17104019
TrypLE Express enzyme	Thermo Fisher Scientific	Cat#12604021
Tissue-Tek O.C.T Compound	Emgrid	Cat#4583
Bovine serum albumin (BSA)	Merck	Cat#A9418
Dulbecco's phosphate-buffered saline (DPBS) 500 mL	Thermo Fisher Scientific	Cat#14190136
DMSO	Sigma-Aldrich	Cat#D4540
CryoStor CS10 medium	STEMCELL Technologies	Cat# 07930
Trypan blue	Thermo Fisher Scientific	Cat#15250061
3D CellTiter-Glo	Promega	Cat#G9683
5FU	Selleckchem	Cat#S1209
SN-38	Selleckchem	Cat#S4908
Oxaliplatin	Selleckchem	Cat#S1224
Regorafenib	Selleckchem	Cat#S5077
TAS-102	Selleckchem	Cat#S8539

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Gemcitabine	Selleckchem	Cat#S1149
Pemetrexed	Selleckchem	Cat#S5971
Temozolomide	Selleckchem	Cat#S1237
Erlotinib	Selleckchem	Cat#S7786
Critical commercial assays		
LookOut Mycoplasma PCR Detection Kit	Sigma-Aldrich	MP0035-1KT
Experimental models: Organisms/strains		
Patient-derived colorectal cancer organoids	This paper	N/A
Software and algorithms		
ImageJ software (NIH Image)	Schindelin et al.[7]	https://imagej.nih.gov/ij/
R (version 4.2.0)	R Development Core Team[8]	https://www.R-project.org/
NIS-Elements AR software (version 5.21.03, 64-bit)	Nikon	N/A
Other		
MANTIS Microfluidic liquid dispenser	Formulatrix	Cat#0695
JANUS G3 Liquid Handler Workstation	PerkinElmer	G3
384 PinTool addition accessory	PerkinElmer	N/A
Nikon Eclipse Ti2 inverted microscope system	Nikon	Eclipse Ti2
Drug stock plate, microplate 384-well, (U)-bottom, polypropylene, low volume, 35 µL, case of 50	PerkinElmer	Cat#6008890
MicroClime Environmental lid, COC, SBS fit, sterile,	Labcyte	Cat#LLS-0310-IP
6-well multiwell plate for suspension cultures	Greiner Bio-One	Cat#657185
PDTO assay plate, 384-well black/clear bottom plate, non-treated surface	Thermo Fisher Scientific	Cat#NUN242764
Nunc microplate lids, 384 plates, sterile	Thermo Fisher Scientific	Cat#264611
70-µm cell strainers	Bio-Strategy	Cat#BDAA352340
Needle 26G × 13 mm single use sterile	Terumo Australia	Cat#AN2613R1
Syringe hypodermic 1 mL tuberculin single use individually wrapped sterile	Science Supply Australia	Cat#10002122
Filter unit 500 mL 0.2 µm PES 75 mm diameter membrane sterile 12/box	Merck	Cat#10016724
Filter unit 250 mL 0.2 µm PES 50 mm diameter membrane sterile 12/box	Merck	Cat#10016723
Filter, PES, 1,000 mL,90 mm, 0.1 µm case of 12 (Nalgene Rapid-Flow sterile disposable filter units with PES membrane, 1,000 mL, 0.1 µm pore, 90 mm membrane)	Thermo Fisher Scientific	Cat#567-0010
Hemocytometer	STEMCELL Technologies	Cat#100-1181
Cool cell LX cell freezing container	STEMCELL Technologies	Cat#200-0644
Tabletop centrifuge	Eppendorf	Cat#5425R
Benchtop centrifuge	Eppendorf	Cat#5810R
Nikon eclipse TS100 microscopy	Nikon	Eclipse TS100

MATERIALS AND EQUIPMENT

Recipes

Tissue collection medium

Reagent	Final concentration	Amount
Gentamicin	50 µg/mL	500 µL
DMEM/F12+GLUTAMAX	N/A	500 mL
Total	N/A	500 mL

[Once prepared, keep at 4°C for up to 6 months]

Colorectal cancer organoid primary culture medium

Reagent	Final concentration	Amount
Y-27632 (10 mM)	10 µM	500 µL
Primocin (50 mg/mL)	100 µg/mL	1 mL

(*Continued on next page*)

Continued		
Reagent	Final concentration	Amount
Human recombinant epidermal growth factor (EGF) (100 μg/mL)	50 ng/mL	250 μL
Human recombinant basic fibroblast growth factor (FGF2) (100 μg/mL)	20 ng/mL	100 μL
A83-01 (10 mM)	500 nM	25 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 (100X)	100X	5 mL
B-27 supplement (50X)	50X	10 mL
N-2 supplement (100X)	100X	5 mL
Gibco Advanced DMEM/F-12	N/A	461.6 mL
Total	N/A	500 mL
[Once prepared, keep at 4°C for up to 2 weeks or aliquot and freeze at −20°C for up to 3 months]		

Colorectal cancer organoid maintenance medium		
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) (100 μg/mL)	20 ng/mL	100 μL
A83-01 (10 mM)	500 nM	25 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 (100X)	100X	5 mL
B-27 supplement (50X)	50X	10 mL
N-2 supplement (100X)	100X	5 mL
Gibco Advanced DMEM/F-12	N/A	462.1 mL
Total	N/A	500 mL
[Once prepared, keep at 4°C for up to 2 weeks or aliquot and freeze at −20°C for up to 3 months]		

STEP-BY-STEP METHOD DETAILS

Preparation of tumor tissues for histological analysis, genomic analysis, and tumor organoid generation

Timing: 30 min per culture

This section describes how to prepare colorectal cancer tissues for PDTO generation, histological characterization and DNA extraction.

1. Transfer each tissue sample from tissue collection medium into a 50 mL centrifuge tube containing 10 mL washing PBS with clean tweezers and allow the tissue to settle down by gravity for 10 min at 20°C–22°C.

⚠ CRITICAL: After each use, wash tweezers with 80% ethanol and then wipe tweezers clean with paper towel pre-wetted with 80% ethanol.

Note: Tissues are kept in tissue collection medium once collected after surgical resection or biopsy.

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

Pause point: If not processed immediately, add 4 μL 10 mM Y-27632 and 8 μL 50 mg/mL primocin to every 4 mL of tissue collection medium and stored tissues in tissue collection medium at 4°C for up to 72 hours.

⚠ CRITICAL: Perform good aseptic operations.

2. Remove the supernatant by vacuum suction, add another 10–20 mL washing PBS and let the tissue settle down by gravity for 10 min at 20°C–22°C.
3. Transfer tissues with clean tweezers into petri dishes.

⚠ CRITICAL: Perform good aseptic operations. After each use, wash tweezers with 80% ethanol and then wipe tweezers clean with paper towel pre-wetted with 80% ethanol.

4. Quickly weigh each tissue to avoid dryness and take photos of the tissue for record.

Note: For needle biopsies, go to step 5 directly.

5. Add 2 mL DPBS to cover the tissue and prevent it from drying.
6. Cut 1 mm^3 pieces to be embedded in OCT compound for later histology analysis and DNA/RNA extraction.
7. Cut a 3 mm × 3 mm × 1 mm piece for tumor organoid generation.
8. Cut any remaining tissue into 1 mm^3 pieces and place every three pieces into 1 mL CryoStor medium for cryopreservation.

Note: If available, a 1 mm^3 piece of adjacent normal colorectal tissue from surgery should be preserved in OCT compound for DNA extraction for annotation of gene mutations and identification of cross-contamination between tumor organoids from different patients. If a normal tissue is not available, buffy coat extracted from 15 mL peripheral blood sample of a patient can be used for DNA extraction.

9. Add 5 mL washing PBS into the dish and mince the 3 mm × 3 mm × 1 mm CRC tissue in the dish with scissors until the tissue suspension can pass through 1 mL tips.
10. Collect the tissue suspension into a 10 mL conical centrifuge tube.

Tissue digestion and generation of CRC PDTOs in suspension culture

Timing: 90–120 min per culture

This section describes how to digest CRC tissues into fragments and seed the fragments to generate PDTOs.

11. Centrifuge the tissue suspension at 200 × *g* for 5 min.
12. Remove the supernatant by vacuum suction.
13. Resuspend the minced tissues in 2 mL tissue digestion medium.
14. Digest at 37°C incubator for 30–60 min.

⚠ CRITICAL: pipette the digestion mix and tissue pieces with 1 mL tips every 10 min until the tissue pieces can pass through the tips easily. Example picture of digested tissue fragments is in Figure 1A.

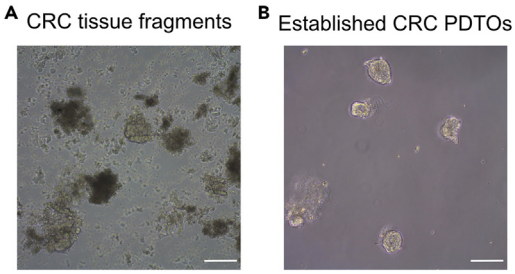

Figure 1. Representative pictures of digested CRC tissue fragments and established PDTOs

(A) Fragments from digested CRC tissues before the washing step.

(B) CRC PDTOs established 24 h after tissue processing. Images were all taken using the 10X objective of Nikon eclipse TS100 microscopy. Scale bar, 200 μm.

15. Centrifuge the suspension at 200 × *g* for 5 min.

Note: Check the supernatant for remaining cells under a microscope before aspirating the supernatant. If there are still many cells floating in the supernatant, centrifuge the supernatant again at 400 × *g* for 5 min.

16. Remove the supernatant by vacuum suction.
17. Add 10 mL washing medium to resuspend the digested tissue.
18. Centrifuge at 200 × *g* for 5 min.
19. Remove the supernatant by vacuum suction.
20. Repeat steps 13–14 four times.
21. Resuspend the pellets in 1 mL colorectal cancer organoid primary culture medium (See materials and equipment setup section for recipes).
22. Take 10 μL out of suspension, dilute 1:1 with 0.4% crystal violate and count viable fragments (those not stained blue) using hemocytometer.
23. Plate 12,000–24,000 fragments in 3 mL colorectal cancer organoid primary culture medium with 150 μL Matrigel for each well of a 6-well suspension plate.
24. Incubate at 37°C in 5% CO_2 incubator. CRC PDTOs form within 1–7 days. Example picture of established CRC PDTOs is in Figure 1B.

⚠ CRITICAL: Observe primary cultures carefully during the first 72 hours. Contamination by microorganisms mostly happens in the first 72 hours. If contamination happens, see Troubleshooting section, problem 1 and possible solution.

⚠ CRITICAL: Some tumor organoids can adhere to the bottom of plates or tubes due to gravity and the low percentage of Matrigel. Check cultures daily. Scramble up organoids to keep healthy organoid growth if they adhere to the bottom of plates.

Note: Always use filter tips that are pre-rinsed with DMEM/F12 containing GlutaMAX medium +1% BSA when resuspending organoids to avoid loss of organoids from pipetting.

⚠ CRITICAL: Matrigel might get digested by enzymes secreted by some primary tumor organoid cultures. For these primary cultures, collect these into 10 mL conical centrifuge tubes, centrifuge at 200 × *g* for 5 min, remove supernatant by vacuum suction, add fresh colorectal cancer organoid primary culture medium containing 5% Matrigel and plate cultures into culture plates.

25. Every two to three days, refresh colorectal cancer organoid culture medium.
    a. Leave culture plates in a tissue culture hood for 5 min to let organoids settle down with solidified Matrigel to the plate bottom by gravity.
    b. Replace half of the supernatant with fresh medium by pipetting without disturbing the layer of organoids and Matrigel at the plate bottom. See Figure 2 for illustration about how the medium is changed.

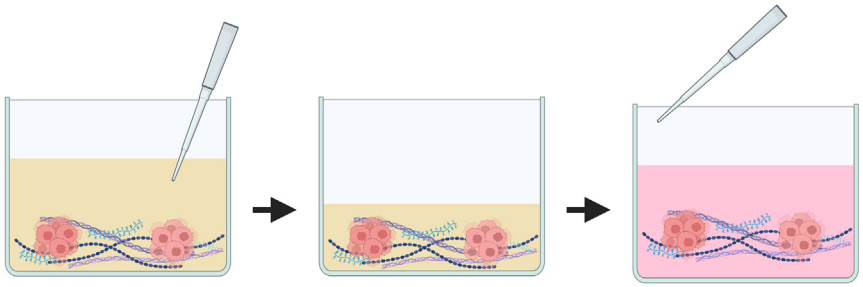

Figure 2. Illustration of changing medium for CRC PDTO cultures
Half of the medium was removed without disturbing PDTOs and Matrigel at the bottom of the well. Fresh organoid maintenance medium was then added against the wall of the well. Created with BioRender 2024.

c. Pipette the mixture of tumor organoids, Matrigel and medium approximately ten times with a 1 mL tip.

⚠ CRITICAL: Two weeks after the initiation of the culture, collect the supernatant of tumor organoid cultures to check for mycoplasma status using the LookOut Mycoplasma PCR Detection Kit.

Passaging of CRC PDTOs

Timing: 30 min per culture

This section describes maintenance of CRC PDTOs via passaging.

26. Collect all tumor organoids into 10 mL conical centrifuge tubes. Pipette as in Step 21c.

Note: Healthy tumor organoids have smooth edges. See Figure 3A for representative images of organoids growing well.

Note: Tumor organoids are ready for passaging when the cores of the tumor organoids are dark and the edges start to become rough. See Figure 4 for representative images of organoids ready for passaging.

Note: Most tumor organoids are ready for passaging 7–14 days from last passage. However, for tumor organoids growing slowly, culturing for up to 3 months is required, with medium change every 2–3 days and replenishing of Matrigel every three weeks. See Figures 3A and 3B for examples of organoids with different growth rates. Check Troubleshooting section, problem 2 and possible solutions.

27. Centrifuge at 200 × *g* for 5 min.
28. Remove the supernatant by vacuum suction.

Note: A hazy layer of liquid Matrigel may be present above white / yellow organoid pellets after centrifugation. This will be removed by PBS washing at the next step.

29. Resuspend the pellet in 1 mL PBS and transfer to 1.5 mL autoclaved Eppendorf tubes.
30. Centrifuge at 1500 × *g* (Tabletop centrifuge) for 2 min.
31. Remove the supernatant by vacuum suction.
32. Resuspend the pellet in 1 mL TrypLE Express Enzyme and incubate at a 37°C incubator for 10–15 min (Larger organoids takes longer for digestion).

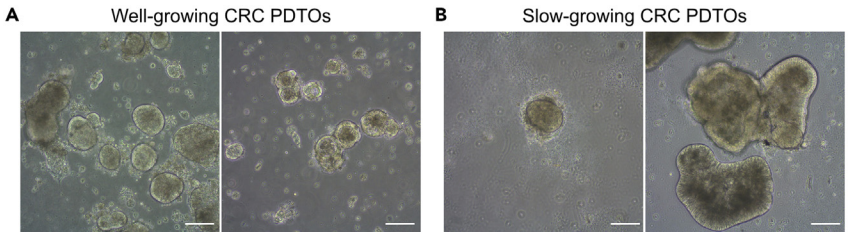

Figure 3. Representative images of CRC PDTOs at different growth rates

(A) Fast-growing healthy CRC PDTOs. Images were taken one week since last passage.

(B) Slow-growing CRC PDTOs. Images were taken after two months of culture. Images were all taken using the 10X objective of Nikon eclipse TS100 microscopy. Tumor organoid sizes could vary between cultures due to their different growth rates. Scale bar, 200 μm.

33. Centrifuge at 1500 × *g* (Tabletop centrifuge) for 2 min.
34. Remove the supernatant by vacuum suction.
35. Resuspend the pellet in 1 mL of 1% (m/v) sterile BSA in DMEM/F12 containing GlutaMAX medium.
36. Centrifuge at 1500 × *g* (Tabletop centrifuge) for 2 min.
37. Resuspend the pellet in 1 mL colorectal cancer organoid maintenance medium. See Figure 5 for example images of organoid fragments after digestion.
38. Dissociate organoids into small fragments by pipetting.
39. Take 10 μL out of suspension, dilute 1:1 with 0.4% crystal violate and count viable fragments (those not stained blue) using hemocytometer.
40. Resuspend 100,000–300,000 cells in 3 mL tumor organoid culture medium and 150 μL Matrigel and plate into 6-well suspension culture plates.
41. Incubate at 37°C in 5% CO_2 incubator.
42. Every two to three days, refresh tumor organoid culture medium (As Step 25).

Cryopreservation

Timing: 15 min per culture

This section describes cryopreservation of CRC PDTOs for long-term storage.

43. Collect tumor organoids into 10 mL conical centrifuge tubes.
44. Centrifuge at 200 × *g* (Benchtop centrifuge) for 5 min.
45. Remove the supernatant by vacuum suction.

Note: A hazy layer of liquid Matrigel may be present after centrifugation. This will be removed by PBS washing at the next step.

46. Resuspend the pellet with 3 mL PBS.
47. Centrifuge at 200 × *g* (Benchtop centrifuge) for 5 min.
48. Remove the supernatant by vacuum suction.

⚠ CRITICAL: For mature tumor organoids (those cultured for more than one week), go to step 45; For tumor organoids cultured for 3–5 days since last passage, go to step 51 directly.

49. Add 1 mL TryPLE to resuspend the pellet and incubate at a 37°C incubator for 10 min.
50. Centrifuge at 200 × *g* (Benchtop centrifuge) for 5 min.
51. Remove the supernatant by vacuum suction.

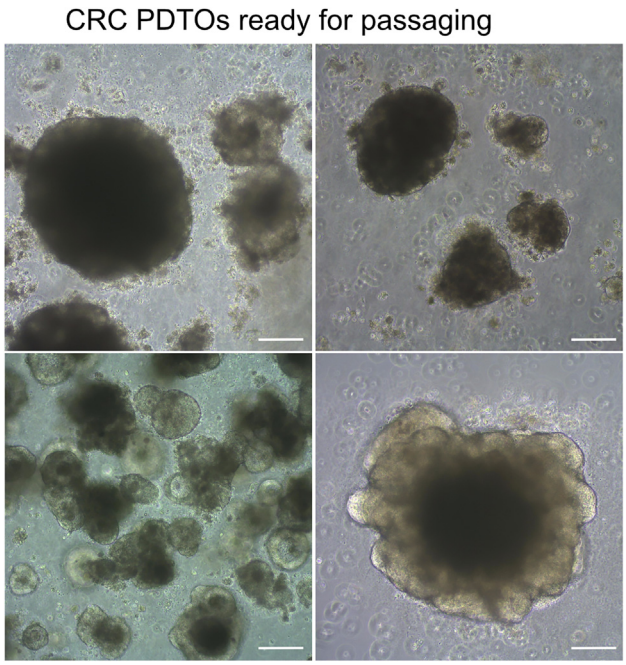

Figure 4. Representative images of CRC PDTOs ready for passaging
Images were all taken using the 10X objective of Nikon eclipse TS100 microscopy. Tumor organoid sizes could vary between cultures due to their different growth rates. Scale bar, 200 μm.

52. Add 1 mL of 1% BSA in DMEM/F12 containing GlutaMAX medium to stop digestion.
53. Centrifuge at 200 × *g* (Benchtop centrifuge) for 5 min.
54. Remove the supernatant by vacuum suction.
55. Resuspend pellets in 1 mL CryoStor and transfer to a cryotube.

Note: Tumor organoids should be cryopreserved at a density of 10,000 organoids or 2 million viable cells per vial.

56. Put cryotubes in CoolCell LX Cell Freezing Container. Keep the container in −80°C for more than 8 h before transferring frozen organoid stocks to liquid nitrogen facility.

⚠ CRITICAL: Store frozen organoid stocks in vapor phase liquid nitrogen for long-term storage and avoidance of cross-contamination.

Thawing

Timing: 15 min

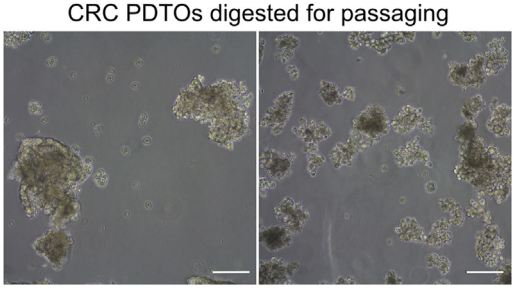

Figure 5. Representative images of fragments from digested CRC PDTOs
Images were all taken using the 10X objective of Nikon eclipse TS100 microscopy. Scale bar, 200 μm.

This section describes how to revive cryopreserved CRC PDTOs.

57. Add 4 mL colorectal cancer organoid maintenance medium into a 10 mL centrifuge tube.
58. Warm frozen tumor organoid stocks at 37°C water bath to thaw rapidly.
59. Gently transfer the thawed organoid stock to a 10 mL conical centrifuge tube.
60. Centrifuge at 200 × *g* for 5 min.
61. Remove supernatant by vacuum suction.
62. Resuspend pellets with 3 mL colorectal cancer organoid maintenance medium (See recipes) containing 5% Matrigel and plate them into 6 well suspension culture plates.
63. Incubate at 37°C in 5% CO_2 in a humidified incubator.

Preparation for drug screening

Timing: 60–90 min

This section describes how to prepare single-cell suspension of CRC PDTOs to be seeded into 384-well plates for drug screening.

64. Collect PDTOs ready for drug assay (2–4 weeks since last time CRC PDTOs were passaged) into 15 mL tubes.
65. Centrifuge at 200 × g for 5 min and then remove supernatant.
66. Resuspend PDTOs with 1 mL PBS.
67. Centrifuge at 200 × *g* for 5 min and then remove supernatant.
68. Resuspend PDTOs with 1 mL TrypLE Express enzyme and incubate at 37°C for 10–15 min.
69. Centrifuge at 200 × *g* for 5 min and then remove supernatant.
70. Resuspend PDTOs with 5 mL DMEM/F12 containing GlutaMAX medium with 1% bovine serum albumin (BSA).
71. Centrifuge at 200 × *g* for 5 min and then remove supernatant.
72. Resuspend PDTOs with 1 mL colorectal cancer maintenance medium.
73. Dissociate PDTOs into single cells with 26G needles and 1 mL syringes and filter through 70-μm cell strainers into a Falcon centrifuge tube to obtain single cell suspension.
74. Mix 10 μL of single cell suspension with 10 μL 0.4% trypan blue solution and count live cells (cells not stained blue) using hemocytometers.

Note: Cell viability should be more than 90%, which means that less than 10% of the cells in the hemocytometer should be stained blue.

75. Resuspend single cells in colorectal cancer organoid maintenance medium containing 3% Matrigel.

Note: For assay on one 384-well plate, suspend 750,000 cells in 15 mL colorectal cancer organoid maintenance medium containing 450 μL Matrigel. Multiply the cell number and medium volume by the number of assay plates if needed.

⚠ CRITICAL: Wells of the outside two columns and two rows are not used for drug screening and instead topped up with 100 μL PBS per well to minimize the impact of edge effects on assay results. Therefore, only the 12 × 20 = 240 wells in the center of a 384-well assay plate are plated with cells for drug screening, which requires at least 240 × 3000 = 720,000 cells in 240 × 60 = 14,400 μL colorectal cancer organoid maintenance medium containing 3% Matrigel. Prepare extra amount of cells and medium to account for dead volume during cell plating.

76. Seed cells into 384-well PDTO assay plates at the density of 3,000 cells per 60 μL per well using a MANTIS liquid handling robot.

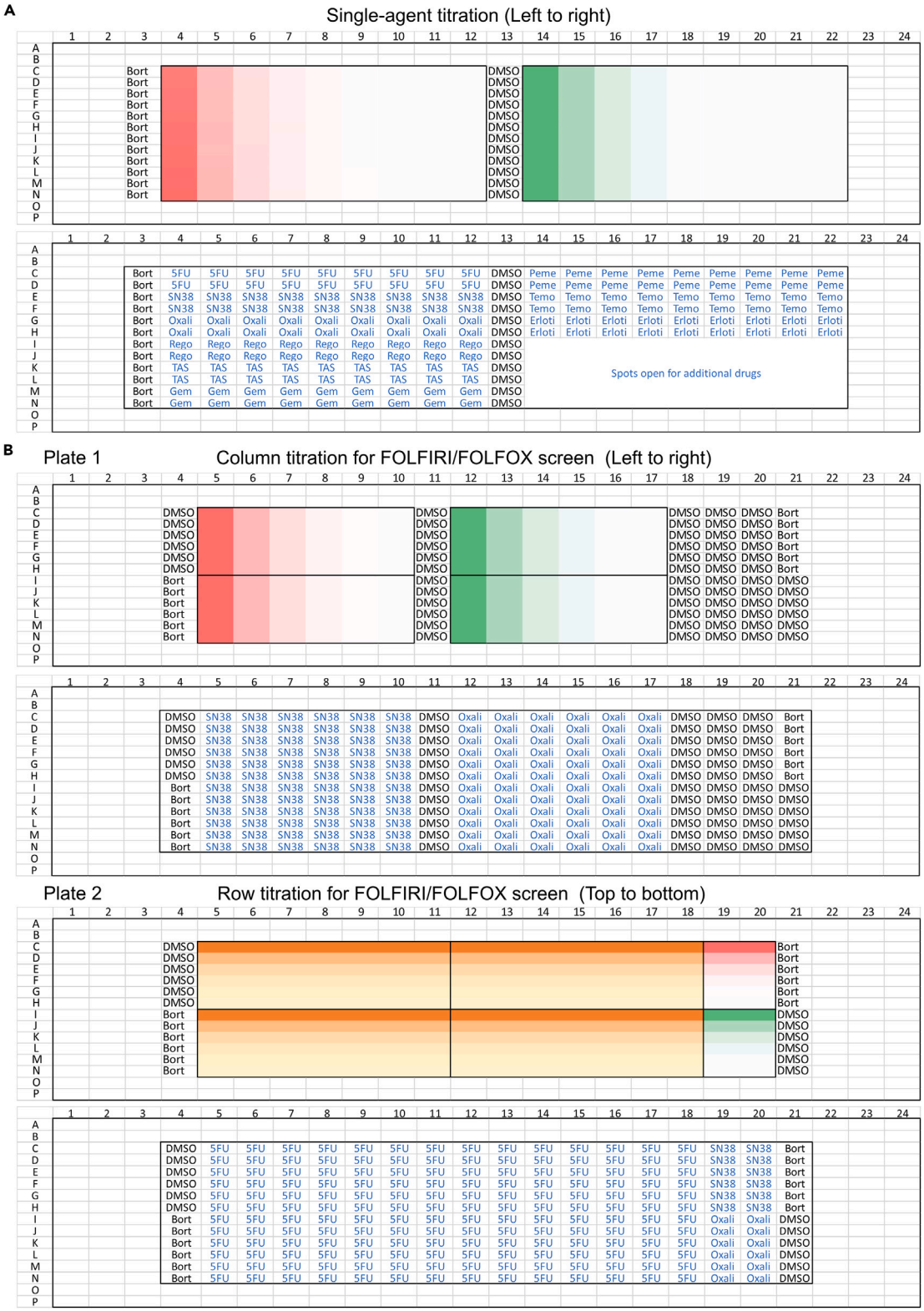
A
Single-agent titration (Left to right)
B
Plate 1
Column titration for FOLFIRI/FOLFOX screen (Left to right)
Plate 2
Row titration for FOLFIRI/FOLFOX screen (Top to bottom)
Spots open for additional drugs

Figure 6. Illustration of compound plate design

(A) Design of the compound plate for single-agent screen.

(B) Design of the compound plates for FOLFIRI/FOLFOX screen. Drug titration directions are shown by fading colors. The darkest colors indicate the locations of drugs with the highest concentration. Bort, positive control (Bortezomib); DMSO, negative control; 5FU, 5-fluorouracil; SN38, SN-38; Oxali, oxaliplatin; Rego, regorafenib; TAS, TAS-102; Gem, gemcitabine; Peme, pemetrexed; Temo, temozolomide; Erloti, Erlotinib.

Note: If fast-growing PDTOs become too confluent during organoid drug assay, repeat organoid assay with 2,000 cells per well.

77. Use Microclime lids to minimize edge effect.
78. Culture plated cells for 3 days at 37°C in 5% CO_2 in a humidified cell culture incubator.

Drug stock plate preparation and drug addition

Timing: 60–90 min

This section describes preparation of drug stock plates for adding drug treatments to CRC PDTO drug testing plates.

79. To prepare drug stock plates at 200X (for single-agent screen) or 600X (for FOLFOX or FOLFIRI combination matrix screen) stock concentrations, add 15 μL DMSO (negative control) to corresponding positions on drug stock plates. Drug stock plate designs are shown in Figure 6.

Note: Use one drug stock plate for single-agent screen but use two drug stock plates for combination matrix screen due to the two-drug matrix design.

80. Add 15 μL Bortezomib (positive control, 300 μM for single-agent screen and 200 μM on stock plates for combination matrix screen) to corresponding positions on drug stock plates. Drug stock plate designs are shown in Figure 6.
81. Add 15 μL drugs at the highest concentrations of titration series to corresponding positions on drug stock plates. Drug stock plate designs are shown in Figure 6.

Note: For single-agent screen, the highest drug concentrations on stock plates are 10 mM for 5-fluorouracil, 100 μM for SN-38, 15 mM for oxaliplatin, 10 mM for regorafenib, 10 mM for TAS-102, 3 mM for gemcitabine, 2 mM for pemetrexed, 150 mM for temozolomide and 10 mM for erlotinib.

Note: For FOLFOX or FOLFIRI combination matrix screen, the highest drug concentrations on stock plates are 30 mM for 5-fluorouracil, 300 μM for SN-38, 45 mM for oxaliplatin.

82. Use JANUS liquid handling robot to titrate drugs from highest to lowest concentrations (9-point, 4-fold dilution series on stock plates for single-agent screen, 6-point 4-fold dilution series on stock plates for FOLFOX/FOLFIRI matrix screen) according to the drug stock plate design (Figure 6).

Note: For 4-fold dilution series, add 3 μL drugs into 9 μL DMSO and mix well for each dilution.

83. Add drugs into 384-well assay plates from stock plates using a JANUS liquid handling robot with PinTool addition accessory.

Note: PinTool adds 100 nL drugs per addition. Thus, for single-agent screen, perform 3 PinTool addition from single-agent drug stock plates into assay plates to add 300 nL of the 200X stock drugs into 60 μL medium in each well. For FOLFOX or FOLFIRI combination matrix

screen, perform 1 PinTool addition from each combination matrix screen stock plate into assay plates to add 100 nL of the 600X stock drugs into 60 μL medium in each well.

84. Switch the Microclime plate lids to transparent assay lids for Nunc 384-well plates.
85. Image each well of PDTO assay plates with an automated Nikon Eclipse Ti2 Inverted Microscope System (NIS-Elements AR software version 5.21.03, 64-bit) with a 4X bright-field objective in a 25 μm Z-stack over a 500-μm range every 24 h for 7 consecutive days.

Organoid drug sensitivity analysis

Timing: 60–90 min

This section describes image analysis of organoid viability changes under drug treatment to generate drug dose-response curves.

86. Measure size changes of PDTOs treated with different drug doses/combinations via ImageJ.[7]
    a. Open Nikon ND2 file.

```
>run("Bio-Formats Macro Extensions")
>idnd2 = "FileName.nd2" //path to ND2 file
>EDOFFile = "Output.tif" //path to tiff output
>s = 1 //Select well within the nd2 file
```

Note: Need to install ImageJ plugins: Bio-Formats and Extended Depth of Field (http://bigwww.epfl.ch/demo/edf/).

    b. Extract z-stack and flatten image.

```
>run("Bio-Formats Importer", "open=["+idnd2+"] color_mode=Composite split_channels
view=Hyperstack stack_order=XYCZT series_"+s)
>run("EDF Easy mode","quality='1' topology='1' show-topology='off' show-view='off'")
>run("8-bit")
>saveAs("tiff", EDOFFile)
```

    c. Generating files including organoid area text file and a PNG image overlaying identified organoids over original tiff image.

```
>tifFile = "Output.tif" //path to tiff input from Step 1
>outPNG = "Output_overlay.png" //path overlayed output image
>outMeas = "Output_measurements.csv" //path organoid measurement
```

    d. Open tiff image.

```
>open(tifFile)
>name = getTitle
>mainTitle=getTitle()
>run("Duplicate...", "title=originalimage")
>selectWindow(mainTitle)
```

e. Subtract background and enhance contrast.

```
>run("Subtract Background...", "rolling=10 light sliding")

>run("Enhance Local Contrast (CLAHE)", "blocksize=127 histogram=256 maximum=3 mask=*None*
fast_(less_accurate)")

>setAutoThreshold("MaxEntropy")

>setOption("BlackBackground", true)
```

f. Identify organoids.

```
>run("Convert to Mask")

>run("Analyze Particles...", "size=200-Infinity show=Masks")

>run("Invert LUT")

>run("Fill Holes")

>run("Watershed")

>run("Set Measurements...", "area mean standard modal min centroid center perimeter bounding
fit shape feret's integrated median skewness kurtosis area_fraction display redirect=origi-
nalimage decimal=3")

>run("Analyze Particles...", "size=200-12000 circularity=0.20-1.00 show=Outlines display
exclude clear summarize")
```

g. Output measurements and PNG files

```
>rename("mask_of_organoids")

>run("Convert to Mask")

>run("Merge Channels...", "c1 = [mask_of_organoids] c4 = originalimage create")

>saveAs("PNG", outPNG)

>saveAs("Measurements", outMeas)
```

87. For single-agent screen, fit drug curves using a four-parameter log-logistic function of the drc R library for full models with normalization to negative controls (DMSO) and positive controls (Bortezomib) to determine effective concentration metrics (EC). Quantify drug response as area under the dose response curve (AUC).
88. For FOLFOX/FOLFIRI matrix screen, calculate pan-matrix relative activity score by averaging the inhibition of PDTO viability over the matrix.
89. Calculate the robust Z′ score and CV% of negative control to confirm the assay quality.

Robust Z′ factor: RZ′ = 1–3 X (m.a.d.(Cpos)+m.a.d.(Cneg))/abs(median(Cpos)-mediam(Cneg)).

Coefficient of variation: CV = s.d. / mean X 100%.

⚠ CRITICAL: Tumor organoids treated with vehicle and positive controls should exhibit distinct differences in sizes, which reflect changes in viability.[9] Robust Z′ factor is a measure that is sensitive to any change in assay conditions and an indicator of assay quality in a high-throughput screen. Plates run through the PDTO platform should have CVs of DMSO control wells lower than 20%, and robust Z′ factors higher than 0.5.

EXPECTED OUTCOMES

This protocol allowed establishment of colorectal cancer organoids at an 82% success rate from tumor resections and 63% from biopsies.[1] Organoids can be expanded, biobanked and drug tested in a high-throughput manner. Notably, a wide range of growth rates are observed among colorectal cancer organoids. After initial seeding, colorectal cancer organoids can first be seen as small round or folded structures at 24–72 h. It takes 1–4 weeks after initial seeding for the culture to be able to be passaged for the first time. It takes 1–3 weeks after the first passage before organoids are ready for the second passage. After the second passage, tumor organoids can be considered as successfully expanded cultures and can be passaged every two weeks. There are generally no limitations on the expansion span of successfully expanded tumor organoids, although limiting passages is recommended to avoid genomic and phenotypic drift in culture.

Visible changes in tumor organoids treated with drugs of interest compared to vehicle control can be detected after a 72 h incubation. Drug screening assays with $Z' > 0.5$ and CV (DMSO) < 20% are considered highly reproducible assays. Factors affecting time from organoid generation to completion of drug screening have been discussed in detail in Tan et al.[1] Measured tumor organoid sizes can be used to generate dose response curve and calculate parameters such as EC_{50} and AUC.

LIMITATIONS

Success rate of tumor organoid establishment

The success rate of generating colorectal cancer organoids from biopsies with this protocol is currently 63% and needs to be improved. The outgrowth efficiency of CRC PDTOs from biopsies could be affected by several factors. First, sufficient number of viable tumor epithelial cells within each biopsy could be key in effective generation of CRC PDTOs.[10,11] Second, measures to reduce delays before PDTO generation might lead to higher success rates, including addition of Y-27632 to tissue collection medium, minimized time of sample shipment, storage at 4°C during shipment and processing upon delivery. Third, optimal organoid culture condition varies among CRC PDTOs due to the different molecular alterations that they harbor.[6,12] Different organoid culture conditions (for example, with or without WNT ligands, hypoxia vs. normoxia) should thus be tested to enhance the outgrowth of tumor organoids.

Quality control when establishing patient-derived organoid cultures

Cross-contamination by normal colon cells or other CRC PDTO cultures derived from different donors could pose a threat to the reliability of data obtained. Tumor resections and biopsies should be examined by professional pathologists to confirm the percentage of tumor cells. Caution should also be taken when handling multiple CRC PDTO cultures. Measures such as DNA sequencing of organoid cultures, SNP fingerprinting and marker expression by immunohistochemistry should be used for authentication of CRC PDTOs and to evaluate to what extent the organoids are representative of the original tumor.

Highly variable time to assay

Our experience is that tumor organoids with daily growth rates lower than 10% are less likely to produce reliable assay results. To drug test these organoids, the number of cells seeded in each assay well should be increased to 4,000–5,000 cells, if possible. Alternatively, end-point 3D Celltiter-glo (CTG) assays may be used. However, our experience is that slow-growing PDTOs not suitable for image analysis are neither able to yield high luminescence signals or reliable 3D CTG data possibly due to the fewer number of proliferating cells as compared to fast-growing CRC PDTOs. In addition, the use of 3D CTG assays requires establishment of accompanying 3D CTG database and increased number of replicates.

Long assay time

Another limitation of performing high-throughput CRC PDTO drug testing is the time it takes to image each 384-well assay plate in Z-stacks. A full 384-well plate can take an hour to image at a time

using our PDTO drug testing platform. This limits the number of assay plates in one PDTO assay run where there is limited microscope access, the number of wells per experiment, the number of fluorescent channels and Z-steps, etc.

TROUBLESHOOTING

Problem 1

The organoid culture is contaminated by bacteria or fungi, due to microorganisms resistant to the antibiotics added to the culture. (Step 24 at the "step-by-step method details" section).

Potential solution

Discard the culture and restart the culture by using a cryopreserved piece of tumor tissue. This time, wash tissue pieces 10 times after mechanical dissociation with scissors and tissue digestion.

It is strongly advised not to continue to use the culture plate that had contaminated organoid cultures. If have to use the other clean wells on the same culture plate, the contaminated wells can be disinfected. Carefully remove the lid of the culture plate without letting medium touch the lid and remove the contents of the wells (<4 mL) by vacuum suction. Add 6 mL 0.1% bleach against the wall into the wells. Put the lid back and incubate in the biosafety cabinet for 15 min. Remove the 0.1% bleach by vacuum suction. Add 7 mL 70% ethanol solution and then remove by vacuum suction.

Problem 2

Formed tumor organoids do not grow or grow very slowly (No obvious increase in size in 2 weeks). (Step 26 at the "step-by-step method details" section).

Potential solution

Collect tumor organoid cultures into 10 mL conical centrifuge tubes. Centrifuge at 200 × *g* for 5 min. Remove the supernatant by vacuum suction. Resuspend the organoid pellet with fresh organoid culture medium and 5% Matrigel. Split the culture into two suspension culture plates. Incubate one plate at 37°C in 5% CO_2 in normoxia and the other one at 37°C in hypoxia (3% O_2).

Do not passage tumor organoids or proceed to drug screening if organoids are growing slowly, this would lead to loss of the culture or failure of drug assays.

Problem 3

Tumor organoids were passaged late. (Step 26 at the "step-by-step method details" section).

Potential solution

Collect everything in the culture medium into 10 mL conical centrifuge tubes. Centrifuge at 200 × *g* for 5 min. Remove the supernatant by vacuum suction. Add 5 mL DPBS to resuspend the pellet, centrifuge at 200 × *g* for 5 min and remove the supernatant by vacuum suction. Resuspend the pellet with 2 mL fresh organoid culture medium and transfer them into a 6-well plate. Observe the culture under the microscope at both 4X and 10X magnification.

If there are still bright organoids with smooth edges, add 5% Matrigel into the medium, mix well and incubate at 37°C in 5% CO_2 for 2 days before proceeding to normal CRC organoid passaging as "Passaging of CRC PDTOs" in the "step-by-step method details" section.

If there are no bright organoids and all organoids have fallen apart, restart the culture by thawing a cryopreserved stock or a cryopreserved CRC tissue.

Problem 4

Tumor organoids were passaged early. (Step 26 at the "step-by-step method details" section).

Potential solution

Maintain the culture normally as described in Step 25 in the "step-by-step method details" section by changing medium every 2–3 days. Organoids can sometimes recover. If there is still little growth after 4 weeks, restart the culture by thawing a cryopreserved stock or a cryopreserved CRC tissue.

Problem 5

Drug assays did not work due to the low proliferation rate of some CRC PDTOs. (Step 85 at the "step-by-step method details" section).

Potential solution

Re-expand the CRC PDTOs. Perform drug assays only with cultures in optimal growth. Increase the number of seeded cells when re-setting up the assays. If the drug assay is still not working, the CRC PDTO culture might not be suitable for the screening.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Oliver Sieber (sieber.o@wehi.edu.au).

Technical contact

Further request for technical details can be sent to and will be fulfilled by the technical contact, Tao Tan (tan.tao@wehi.edu.au).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This paper does not report the original code.

Any additional information is available from the lead contact upon request.

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AUTHOR CONTRIBUTIONS

Conceptualization, T.T., D.M., and O.M.S.; funding acquisition, P.G. and O.M.S.; investigation, T.T. and O.M.S.; methodology, T.T., D.M., and O.M.S.; supervision, P.G. and O.M.S.; visualization, T.T., D.M., and O.M.S.; writing – original draft, T.T., D.M., and O.M.S.; writing – review and editing, T.T., P.G., D.M., and O.M.S.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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