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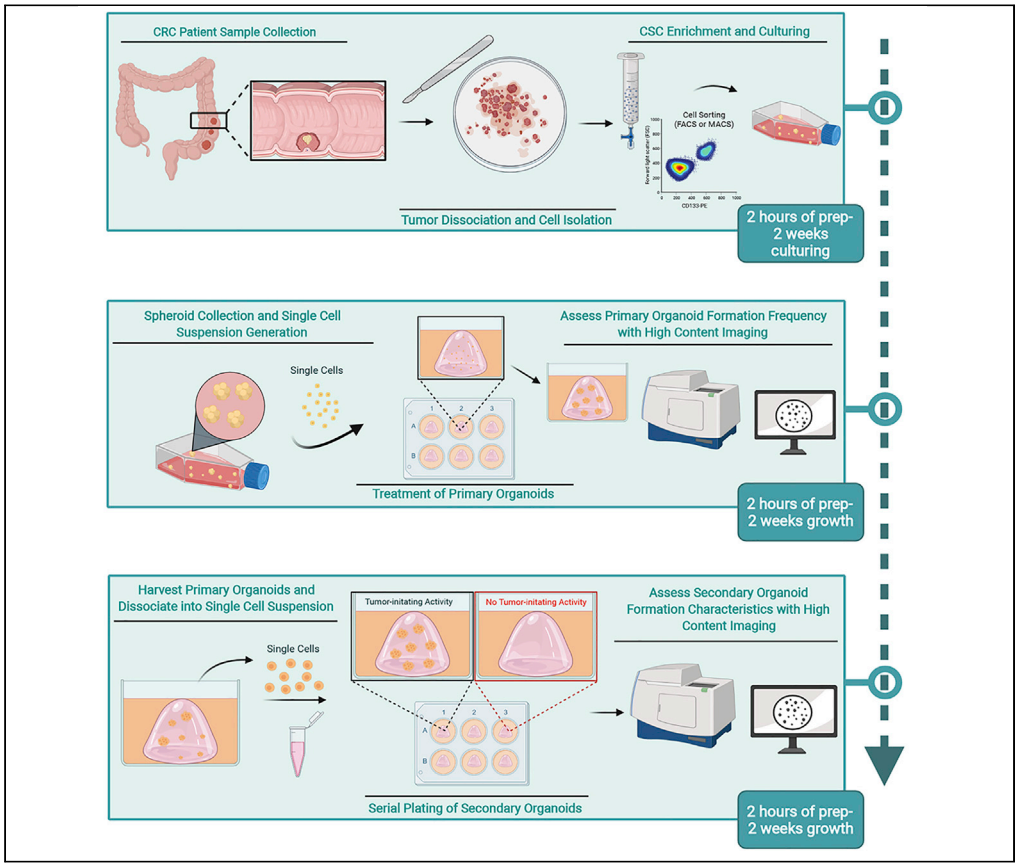
Protocol for serial organoid formation assay using primary colorectal cancer tissues to evaluate cancer stem cell activity



Organoids can enable the study of solid tumors initiated from a single cancer stem cell (CSC) *ex vivo*. We describe a serial tumor organoid plating protocol using primary colorectal cancer (CRC) tissues as a rapid and cost-efficient approach to evaluate the impact of therapeutic interventions on CSC functions. We detail the isolation of primary colorectal CSCs, organoid embedding, serial passaging, and CSC-related analytical techniques.

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Highlights

Rapid and cost-efficient assessment of tumor-initiating cell activity *ex vivo*

Preclinical tool evaluating the impact of drugs on cancer stem cell populations

Describes cancer stem cell enrichment and culture from primary colon tumor tissues

Compatible with complementary quantitative protocols to study tumor heterogeneity

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Protocol for serial organoid formation assay using primary colorectal cancer tissues to evaluate cancer stem cell activity

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SUMMARY

Organoids can enable the study of solid tumors initiated from a single cancer stem cell (CSC) *ex vivo*. We describe a serial tumor organoid plating protocol using primary colorectal cancer (CRC) tissues as a rapid and cost-efficient approach to evaluate the impact of therapeutic interventions on CSC functions. We detail the isolation of primary colorectal CSCs, organoid embedding, serial passaging, and CSC-related analytical techniques.

For complete details on the use and execution of this protocol, please refer to Masibag et al. (2021) and Bergin et al. (2021).

BEFORE YOU BEGIN

Cancer stem cells (CSC) represent a small population of tumor cells playing a pivotal role in cancer initiation, growth, and metastasis (de Sousa e Melo et al., 2017; Kreso and Dick, 2014; Visvader and Lindeman, 2012). Recognized as the root of cancer, CSCs present distinct functional hallmarks compared to bulk tumor cells, such as self-renewal and tumor-initiation activity (Bergin et al., 2021; Boyd et al., 2018; Kreso et al., 2014; O'Brien et al., 2007). The development of novel therapeutics targeting CSCs represents one of the most important challenges in current cancer drug discovery. Colorectal tumors are organized according to a cellular hierarchy, governed by populations of CSCs (Bergin et al., 2021; de Sousa e Melo *et al.*, 2017; O'Brien *et al.*, 2007). While specific surface markers were proven useful to enrich for CSCs in tumor cell fractions (Du et al., 2008; Hatano et al., 2017; O'Brien *et al.*, 2007), no absolute CSC markers were yet identified. Thus, monitoring bona fide CSC activity relies on functional assays such as complex *in vivo* serial tumor transplantation experiments (Benoit et al., 2017; Lima-Fernandes et al., 2019; Masibag et al., 2021). We established a serial tumor organoid plating protocol, using primary CRC tissues or tumorigenic cell lines to test the impact of small molecules, RNAi-based silencing, or gene editing tools on CSC functions. This assay represents a rapid and cost-efficient method to evaluate the response of a broad range of patient-specific tissues (male vs. female, clinical subtypes, mutational status, etc.) to different treatments, and a valuable pre-clinical *ex vivo* approach to predict the outcome of pharmacological interventions on CSC populations *in vivo*. The protocol below describes the key steps of CSC isolation from freshly resected patient tissues, CSC enrichment in spheroid cultures, 3D embedding of single-cell preparations in basement membrane extracts (BME), serial organoid passaging, and relevant analytical procedures to measure the impact of molecular interventions on colorectal CSC activity. Successful execution of this protocol requires access to freshly resected colorectal tumor tissues or established CSC cultures. Collection of such samples require informed consent and institutional research ethic board approval. Alternatively, this serial organoid plating assay can also be conducted using common CRC cell lines with demonstrated tumor-initiating activity. All the following procedures must be performed under a Class II Biological Safety Cabinet to

ensure sterility of experimental samples and to protect investigators from risks relating to biological materials. A tissue culture incubator adjusted at 37°C, and 5% CO_2 is also necessary.

Institutional permissions

Primary colorectal tumor tissues used to generate the data presented in this protocol were obtained with patient consent, as approved by the Ottawa Health Science Network Research Ethics Board, the University of Ottawa Office of Research Ethics and Integrity, and the University Health Network Research Ethics Board.

Basement membrane extract (BME) preparation

Timing: 12–18 h

This initial step is critical since inappropriately processed basement membrane stocks from commercial sources may lead to the failure of tumorigenic cell embedding and subsequent organoid growth.

1. A fresh bottle of 10 mL stock BME - e.g., Matrigel® or equivalent, ≥8–12 mg/mL of protein concentration - should be removed from −80°C storage and placed at 4°C in a box filled with ice for a 12–16 h incubation to ensure the BME has gradually thawed.
   a. Pre-cool serological pipettes and 15 mL conical tubes at −20°C for at least 30 min prior starting BME preparation.
2. Once BME is 90% thawed, put the stock container on ice into a sterile biological safety cabinet along with a bottle of Knockout (KO) DMEM.
3. Using a chilled serological pipette, transfer 10 mL of KO DMEM into the BME stock bottle, and slowly pipette its content by doing "up and downs" to prepare a 50% BME / 50% media solution.
   a. Repeat up and downs until the remainder of BME has thawed.
4. Replace the chilled serological pipette every 5 min to prevent BME polymerization.
5. Once the BME suspension is thawed and homogenous, prepare 0.5 mL–1 mL aliquots in sterile conical tubes (15 mL) by consistently keeping both, conical tubes and the BME container on ice.
6. Aliquots can be stored at −80°C for up to 24 months.

Note: BME aliquots must be thawed only once, a few minutes before its use during the experiment. We do not recommend re-freezing or reusing aliquots once they have reached a temperature above 5°C–6°C.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Mouse Anti-Ki-67 Monoclonal Antibody; Dilution 1:500	BD Biosciences	Cat# 556003; RRID: AB_396287
BMI-1 Antibody (JJ093-3); Dilution 1:250	Novus Biologicals	Cat# NBP2-67916; RRID: AB_2809763
E-Cadherin (24E10) Rabbit mAb Antibody; Dilution 1:500	Cell Signaling Technology	Cat# 3195; RRID: AB_2291471
Anti-Human CD133 Mouse Monoclonal Antibody, PE Conjugated; Dilution 1:500	BD Biosciences	Cat# 566593 RRID: AB_2744281
Biological samples		
Human: CRC primary sample, $APC^{Mut}/BRAF^{Mut}/p53^{Mut}$, Stage IV, Female, 45 years old	(Bergin *et al.*, 2021)	#92
Human: CRC primary sample, $KRAS^{Mut}/BRAF^{Mut}/p53^{Mut}$, Stage III, Male, 65 years old	(Bergin *et al.*, 2021)	#146
Human: CRC primary sample, $APC^{Mut}/KRAS^{Mut}/BRAF^{Mut}/p53^{Mut}$, Stage III, Female, 63 years old	(Bergin *et al.*, 2021)	#162

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Human: Colon Cancer Stem Cell - Frozen Vial	Celprogen, Inc.	Cat# 36112-39
Human: Colorectal Cancer Cell line, HCT116, Stable pLenti-GFP reporter, Male	(Masibag *et al.*, 2021)	n/a
Chemicals, peptides, and recombinant proteins		
BIX01294	AdipoGen Life Sciences	Cat# AG-CR1-0150-M001
UNC0642	Cayman Chemical	Cat# 14604-1
YB-0158	MedChemExpress	Cat# HY-136541
Hoechst 33342	Thermo Fisher Scientific	Cat# H3570
DMSO, sterile filtered	R&D Systems	Cat# 3176
Calcein AM	Tocris Bioscience	Cat# 5119
Sterile PBS (phosphate buffered saline), pH 7.4	Thermo Fisher Scientific	Cat# 10010023
Solution Formaldehyde 37%	Millipore Sigma	Cat# 47608
EDTA 0.5 M Solution, pH 8.0	Thermo Fisher Scientific	Cat# BP2482500
Corning™ Matrigel™ Membrane Matrix	Fisher Scientific	Cat# CB-40234
DMEM/F12 (1:1 Ratio)	Thermo Fisher Scientific	Cat# 11320033
Knockout DMEM	Thermo Fisher Scientific	Cat# 10829018
L-Glutamine	Thermo Fisher Scientific	Cat# 25030081
Non-Essential Amino Acids	Thermo Fisher Scientific	Cat# 11140050
Sodium Pyruvate	Thermo Fisher Scientific	Cat# 11360070
HEPES	Thermo Fisher Scientific	Cat# 15630080
Heparin	STEMCELL Technologies	Cat# 07980
Lipid Mixture 1	Millipore Sigma	Cat# L0288
Penicillin Streptomycin	Cytiva Hyclone	Cat# SV30010
Amphotericin B	Thermo Fisher Scientific	Cat# 15290018
N2 Supplement	Thermo Fisher Scientific	Cat# 17502048
B27 Supplement	Thermo Fisher Scientific	Cat# 17504044
Gentle Cell Dissociation Reagent	STEMCELL Technologies	Cat# 07174
Collagenase Type IV	STEMCELL Technologies	Cat# 07909
RNase-Free DNAse Set	QIAGEN	Cat# 79254
Trypan Blue Stain	Thermo Fisher Scientific	Cat# T10282
Ammonium Chloride Solution	STEMCELL Technologies	Cat# 07850
TrypLE Express Enzyme (1×) Phenol Red	Thermo Fisher Scientific	Cat# 12605010
Human recombinant EGF	Wisent Bioproducts	Cat# 511-110-EU
Recombinant Human FGF-basic	PeproTech	Cat# 100-18B
Cytiva Ficoll-Paque™ PLUS Media	Thermo Fisher Scientific	Cat# 36-101-6383
KnockOut™ Serum Replacement	Thermo Fisher Scientific	Cat# 10828028
Critical commercial assays		
MiniMACS Starting Kit	Miltenyi Biotec	Cat# 130-090-312
CD133 MicroBead Kit – Tumor Tissue	Miltenyi Biotec	Cat# 130-100-857
CD326 (EpCAM) MicroBeads, human	Miltenyi Biotec	Cat# 130-061-101
Dead Cell Removal kit	Miltenyi Biotec	Cat# 130-090-101
Software and algorithms		
CellReporterXpress	Molecular Devices	https://www.moleculardevices.com/products/cellular-imaging-systems/acquisition-and-analysis-software/cellreporterxpress#gref
ZEN pro software	Zeiss	https://www.zeiss.com/microscopy/int/products/microscope-software/zen.html
Imaris 9.5	Oxford Instruments	https://imaris.oxinst.com/versions/9-5
ImageJ	U.S. National Institutes of Health	https://imagej.nih.gov/ij/
Other		
Falcon Cell strainers, 70um	Thermo Fisher Scientific	Cat# 08-771-2
Progene® Cell Culture Dishes, 100 mm	Ultident Scientific	Cat# 229621
CellTreat 6-well plates	CELLTREAT Scientific Products	Cat# 229106
Thermo Scientific™ Nunclon™ Sphera™ Flasks	Thermo Fisher Scientific	Cat# 12-566-439
15 mL Conical Tubes, Sterile/Graduated	Sarstedt, Inc.	Cat# 62-554-100
50 mL Conical Tubes, Sterile/Graduated	Sarstedt, Inc.	Cat# 62-547-100

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Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Countess™ Cell Counting Chamber Slides	Thermo Fisher Scientific	Cat# C10228
Countess™ 3 Automated Cell Counter	Thermo Fisher Scientific	https://www.thermofisher.com/ca/en/home/life-science/cell-analysis/cell-analysis-instruments/automated-cell-counters/models/countess-3.html
ImageXpress Pico Automated High Content Imaging System	Molecular Devices	https://www.moleculardevices.com/products/cellular-imaging-systems/high-content-imaging/imagexpress-pico#gref
Zeiss LSM800 Confocal Microscope	Zeiss	https://www.zeiss.ca/microscopy/en/dynamic-content/news/2015/news-lsm800.html

MATERIALS AND EQUIPMENT

Cancer Stem Cell Culture Media		
Reagent	Final concentration	Amount
DMEM/F12 (1:1 Ratio)	n/a	500 mL
L-Glutamine (200 mM)	2 mM	5 mL
Non-Essential Amino Acids (100×)	1×	5 mL
Sodium Pyruvate (100 mM)	1 mM	5 mL
HEPES (100×)	1×	5 mL
Heparin (2 mg/mL)	4 μg/mL	1 mL
Lipid mixture	n/a	1 mL
Penicillin/Streptomycin	1%	5 mL
EGF (100 μg/mL stock)	20 ng/mL	100 μL
bFGF (100 μg/mL stock)	10 ng/mL	50 μL
N2 Supplement (100×)	1×	5 mL
B27 Supplement (50×)	1×	10 mL
Total	**n/a**	**538 mL**
Stable at 4°C for 1 month. Can be aliquoted and stored at −80°C for long term storage (>1 month).		

⚠ CRITICAL: All reagents must be added to the media formulation in a sterile biological safety cabinet.

Optional: Amphotericin B can be added to the CSC culture media, at 1μg/mL, to further prevent fungi contaminations.

Tumor Dissociation Solution		
Reagent	Final concentration	Amount
Collagenase IV	1 mg/mL	10 mL
Penicillin Streptomycin (100×)	1%	100μL
RNase-Free DNase	30 Kunitz Units	variable
Total	**n/a**	**~10.2 mL**
Stable at 4°C for 1 month. Can be aliquoted and stored at −20°C for long term storage (< 9 months).		

STEP-BY-STEP METHOD DETAILS

Isolation of cancer cells from primary tumor tissue

Timing: 2 h

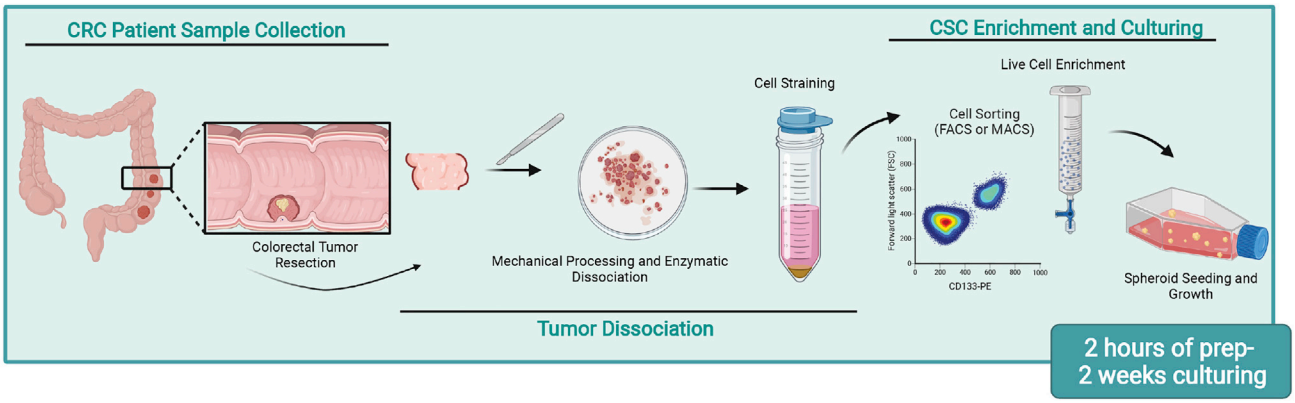

Figure 1. Schematic description of the method used to establish CSC-enriched cultures from human primary colorectal tumor tissues

Here we are describing the method employed to isolate and enrich primary CSCs from freshly resected human colorectal tumor tissues (Figure 1). Patient-derived samples are obtained with informed consent and are mechanically dissociated into smaller fragments, followed by enzymatic dissociation. The resulting cell suspension is strained to eliminate debris and aggregates. Upon lysis of red blood cells and exclusion of dead cells, a sorting procedure is applied to isolate the $CD133^{+}$ fraction. Cell sorting increases chances of successfully initiate spheroid formation over a subsequent culture period in non-adherent conditions. This section is an adaptation of a method described by Kreso et O'Brien. (Kreso and O'Brien, 2008).

Note: Fresh primary colorectal tumor tissues obtained from surgical procedures in the clinic must be preserved on ice, in a 15 mL conical tube (or equivalent) containing a physiological saline solution such as PBS and must not be frozen. Case-specific information may be provided be the clinical team, such as the age and the biological sex of the patient, as well as the tumor-node-metastasis (TNM) stage, the localization, the mutational status, and other pathological parameters of the neoplasm depending on Research Ethics Board approvals.

1. Obtain a freshly resected primary CRC sample in a 50 mL collection tube on ice, and immediately proceed to tissue dissociation in a biological safety cabinet.
2. Transfer the CRC tumor fragment to a 35 mm petri dish containing 5 mL of ice-cold phosphate-buffered saline (PBS) solution. Use the PBS to wash blood and/or debris.
3. Aspirate the PBS and perform a second wash of the tumor fragment using 3 mL of cold CSC media. Aspirate and replace the media with 3 mL of fresh CSC media.

Optional: If biobanking is advised, dissect the tissue into multiple fractions, for either protein/RNA/DNA extracts or histology embedding. Make sure to preserve a tissue section of $\geq 1 cm^3$ for CSC isolation.

4. Utilizing forceps to immobilize the tumor fragment, use a razor blade to mince the tumor fragment into ~2 mm pieces in CSC media.

⚠ CRITICAL: Mincing the tissue into smaller pieces maximizes the number of cells obtained through the isolation process.

5. Use scissors to cut the aspirating end of a p1000 tip to make it wider and use it to transfer the minced tumor fragment suspension to a 50 mL conical tube containing 5 mL of the Tumor Dissociation Solution.
6. Transfer tube containing the suspension to a 37°C incubator or water bath for 1 h.

a. Every 15 min, vigorously flick the tube 10–15 times to aid the enzymatic dissociation of the tumor sample. Alternatively, 10–15 up and down pipetting with a sterile 5 mL serological pipette can be performed.

7. Pass the digested tumor suspension through a sterile 70 μm cell strainer and collect the filtrate into a new sterile 50 mL conical tube. Wash the strainer with 1–2 mL of CSC media.
8. Centrifuge the filtrate at 450×*g* for 5 min at 4°C. Extend the centrifugation time if no substantial cell pellet can be observed. Aspirate and discard the supernatant.
9. Resuspend the cell pellet in 5 mL of a solution of 0.8% (w/v) Ammonium Chloride 0.8% + 0.1 mM EDTA to lyse red blood cells. Incubate on ice for 10 min to allow red blood cells lysis.

⚠ CRITICAL: If red blood cell lysis is incomplete and marked by residual red coloration, increase the volume of Ammonium Chloride solution, and let the cells to deposit for an extra 10 min.

10. Add an equal volume of CSC media to the cell suspension and centrifuge at 450×*g* for 5 min at 4°C. Aspirate and discard the supernatant.
11. Resuspend the pellet in 10 mL of fresh CSC media and remove dead cells from the tumor cell suspension using either Ficoll or a Dead Cell Removal kit following the manufacturer's guidelines.
12. Resuspend the resulting live cell fraction into desired volume of CSC media and perform cell viability counts using trypan blue and a hemocytometer, or an automated cell counter (e.g., Countess™ 3 Automated Cell Counter or equivalent). See troubleshooting 1

Note: If cell sorting methods are not available, it is possible to proceed to step-15 and initiate spheroid growth without enriching for $CD133^+$ cells. However, based on our experience, the success rate of spheroid formation is much lower without cell sorting.

13. Proceed to Magnetic-Activated Cell Sorting (MACS) of the primary CRC cell suspension to obtain a fraction enriched in $CD133^+$ tumor-initiating cells (O'Brien *et al.*, 2007). Use the Mini-MACS Starting Kit (or equivalent) with the CD133 MicroBead Kit according to the manufacturer's protocol.

Note: Fluorescence-Activated Cell Sorting (FACS) can be used instead of MACS to enrich $CD133^+$ colorectal tumor cells prior to establish spheroid cultures. However, we recommend the use of MACS for manipulators relying on core facility based FACS infrastructures with schedule restrictions. Since the access to primary tumor tissues can sometimes be difficult to plan, MACS represents a more flexible option for spontaneous tissue processing.

Optional: An initial MACS sorting step using EpCAM/CD326-conjugated MicroBeads, prior to $CD133^+$ cell enrichment can help excluding stromal, endothelial and hematopoietic cells from the CRC tumor cell mixture (Kreso and O'Brien, 2008).

14. Resuspend the $CD133^+$ tumor cell fraction in 10 mL of CSC media and perform a cell count using Trypan blue and a hemocytometer, or an automated cell counter. See troubleshooting 1
15. Transfer 5 × 10^4 cells/mL into non-adherent flasks and culture the cells at 37°C, 5% CO_2 for ~2 weeks or until visible multicellular aggregates emerge from the cultures (Figure 2A). See troubleshooting 2

Note: Since independent patient samples differ in terms of CSC frequency, the success rate for spheroid formation cannot be 100%. Moreover, some CRC primary samples will yield faster and more robust spheroid growth. Lastly, the spheroids shape often differ from a patient to another, forming either spherical or ruffled/irregular structures (Figures 2B and 2C).

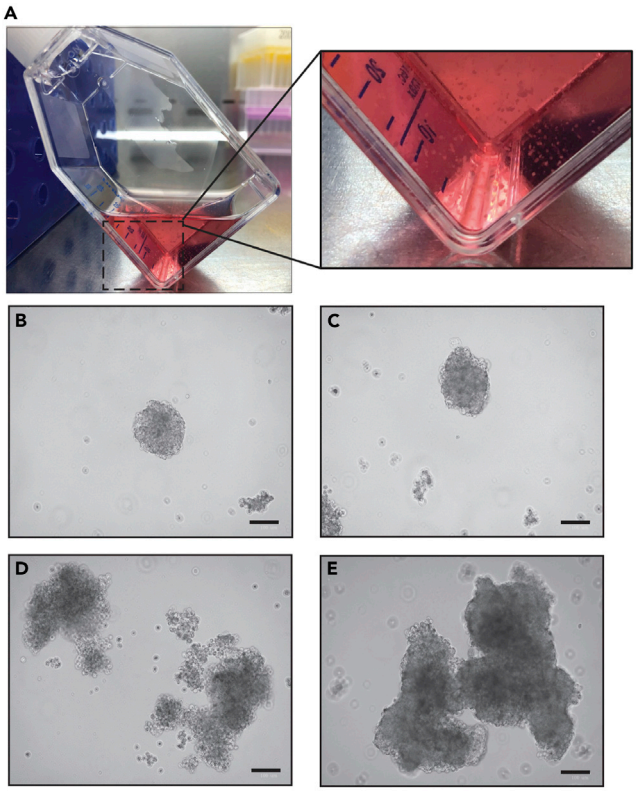

Figure 2. Macroscopic assessment of CSC-enriched spheroid growth and integrity

(A) Image of colorectal CSC-enriched spheroids growing in suspension.

(B–E) The high magnification inset is indicating how users should see spheroids by eye. Representative images of typical (B) spherical and (C) ruffled shaped spheroids suitable to use for organoid plating. Representative images of (D) disrupted and (E) overgrown spheroids, which should not be used for organoid plating. Scale bar = 100 μm.

Maintenance of colorectal CSC spheroids

Timing: 1 week

This step describes the timing and culture needs to maintain robust CSC-enriched spheroids in suspension. Our previous work demonstrated that 90%–95% of the cells constituting such spheroids are simultaneously expressing the surface markers CD44 and CD133, which are related to colorectal CSCs (Bergin *et al.*, 2021; Du *et al.*, 2008; O'Brien *et al.*, 2007). Spheroids can be passaged using either a mechanical or chemical/enzymatic dissociation method. It is noteworthy that CSC-enriched spheroid cultures can be established from patient-derived tumor tissues, as detailed in the previous section, or from commercial sources of colorectal CSCs (e.g., Celprogen Inc., Torrance, CA). Specific CRC cell lines such as HCT116 can also efficiently form spheroid suspensions when cultured in CSC media (serum-free) in non-adherent flasks. These provide additional options for users to execute this serial organoid plating protocol.

16. Once established, spheroid cultures must be monitored daily, and CSC media changed at least once every 3 days.
    a. To proceed to media change, place the flasks containing the spheroid cultures in a sterile biological safety cabinet and transfer the entire volume to a 15 mL conical tube.
    b. Allow spheres to deposit at the bottom of the tube or proceed to a brief, gentle centrifugation (450×*g*, 30 s).

c. Aspirate the culture media without disturbing the pellet and carefully resuspend spheroids in 8–10 mL of fresh CSC media.

⚠ CRITICAL: When changing the media of spheroid cultures, proceed gently and avoid harsh or repetitive pipetting to preserve the integrity of the structures.

17. CSC-enriched spheroid passaging must be performed once a week, or earlier if the user notices a fast growth rate. We recommend mechanical disruption of spheroids as a rapid and efficient method for passaging.

⚠ CRITICAL: Under-passaging CSC-enriched spheroids increases cell death and impact the integrity of CSCs for subsequent organoid formation.

Note: Fast spheroid growth can be monitored by the acidification rate of the CSC media (shift in DMEM/F12 pH indicator) and by microscopic observations revealing deteriorated and overgrown spheroids (Figures 2D and 2E).

18. When a spheroid culture is ready to be split, transfer the spheroids suspension to a 15 mL conical tube using a sterile 10 mL serological pipette.

Note: Larger cultures with high spheroid density should be separated into two tubes to ensure ease with disruption.

19. Mechanically disrupt the spheroids by executing vigorously up and down pipetting for 5 min using a sterile 5 mL serological pipette.
    a. Assess by eye the presence of residual spheroids and continue up and down pipetting for an additional 5 min if needed.
20. Once no spheroids are visible, filter the cell suspension through a 70 μm cell strainer to eliminate cell aggregates. Resuspend the cell suspension in the desired volume of CSC media. Perform cell counts and transfer 5×10^4 cells/mL into 8 mL of CSC media in a new series of non-adherent culture flasks.

⚠ CRITICAL: Since patient-derived spheroid are valuable samples, we recommend freezing stocks at early passages. Whole CSC-enriched spheroids are cryopreserved in a freezing media, which consists in a 10% DMSO, 30% KnockOut Serum Replacement, and 60% CSC media mixture. On thawing, it is critical to immediately wash off the freezing media and resuspend the spheroids in fresh CSC media. Gentle pipetting must be observed to minimize the disruption of spheroid structures. Moreover, it is critical to track the number of passaging to avoid bringing the cultures beyond 10–12 passages. Such a practice mitigates artifactual tissue culture adaptation of CSCs and preserves molecular characteristics of the tumor of origin (Ray et al., 2012).

Optional: Enzymatic dissociation of CSC-enriched spheroids can also represent a suitable method for passaging. However, it can affect surface protein markers and may alter specific experimental readouts upon dissociation, such as flow cytometry. For enzymatic dissociation of CSC-enriched spheroid cultures, refer to Kreso et al. (Kreso and O'Brien, 2008).

Pause point: The protocol can be paused at this point if the user opt for freezing the spheroid cultures.

Primary organoid formation in BME domes

Timing: 2 h

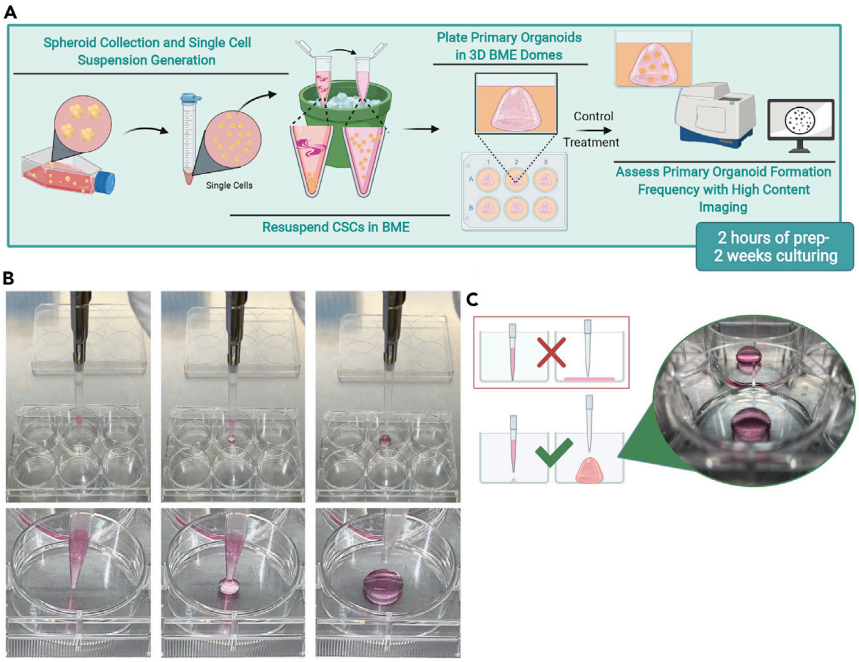

Figure 3. Organoid plating procedure from colorectal CSC-enriched spheroid cultures

(A) Schematic description of the method used to embed primary colorectal CSCs in 3D BME domes and generate a miniaturized tumor *ex vivo* system to test therapeutic interventions.

(B) Image sequence (left to right) demonstrating how to properly plate the 1:1 BME/cell mixture to form domes in a 6-well plate. The bottom panels represent a high-magnification view of the well.

(C) Schematics illustrating the appropriate positioning of the p1000 pipettor when plating BME/cell mixture to form 3D domes. Wells in which the BME/cell mixture disperses across the plastic surface should not be considered for subsequent steps.

The following steps describe the procedure to embed colorectal CSCs into 3D BME structures and grow tumor organoids, as illustrated in Figure 3A. Single cells with tumor-initiating functions have the capacity to form organoid structures once embedded in BME (Bergin *et al.*, 2021; Masibag *et al.*, 2021). Such patient-derived tumor organoids can be subjected to therapeutic interventions to further evaluate their impact on different functions *ex vivo* (Figure 3A).

21. Transfer CSC-enriched spheroids from culture flasks to 15 mL conical tubes and mechanically dissociate the spheres as above-described to generate single-cell suspensions.
    a. Typically, 1 mL of spheroid suspension is sufficient to seed a full 6-well plate with primary organoids.
    b. Make sure to pass the dissociated spheroid suspension through a 70 μm cell strainer to eliminate multicellular aggregates.

⚠ CRITICAL: If the experiment requires viral vector-based transduction (*e.g.*, lentiviral particles), it is essential to perform the transduction on the single cell suspension, prior to BME embedding. BME-embedded epithelial structures were documented as refractory to viral-based gene delivery (Maru et al., 2016). Moreover, genome engineering interventions, such as CRISPR-Cas9 based knockout or knockin systems must also be performed at that stage (Hendriks et al., 2021; Michels et al., 2020).

Note: At this point, take an aliquot of BME from −80°C and let it thaw on ice.

22. Perform a live cell count using trypan blue and a hemocytometer, or an automated cell counter. See troubleshooting 1

23. In a sterile 15 mL conical tube, prepare a 2 cells/μL dilution in CSC media.
    a. For a 6-well plate, we recommend plating 300 cells per dome. Thus, 1,800 cells should be diluted in a final volume of 900 μL of CSC media.
24. Once the BME aliquot has completely thawed, quickly add BME to the cell suspension according to a 1:1 ratio (BME:cell suspension) to obtain a final 1 cell/μL dilution.
    a. For an entire 6 well-plate, transfer 900 μL of BME to 900 μL of cell suspension.

⚠ CRITICAL: The BME solution will immediately start polymerizing at temperatures above 9°C. Immediately proceed to the next step once BME is added to the cell suspension to avoid uneven or premature polymerization before dome plating. We recommend plating no more than 6 wells at the time to avoid BME polymerization issues.

25. Using a p1000 pipettor, gently homogenize the BME/cell mixture and carefully deposit 300 μL at the center of each well of a 6-well culture plate to form dome structures, as illustrated in Figure 3B.
    a. It is important to proceed quickly to ensure BME does not polymerize before dome formation
    b. Place the tip of the p1000 at the center of the well, carefully dispense a small volume of the solution that will serve as a starting point for dome formation (Figure 3B, Methods videos S1 and S2).
    c. Carefully dispense the remainder of the 300 μL at the same position and ensure it forms a tridimensional dome and does not spread out along the plate (Figure 3C).
    d. Repeat these steps for each well into which organoids must be seeded.
26. Without disrupting the domes, place lid on the culture plate and gently transfer it to the incubator for a 10-min incubation at 37°C, 5% CO_2. See troubleshooting 3
    a. Ensure to transfer the plate very steadily without tipping or using swift movements.

⚠ CRITICAL: At this step, dome disruption results in a failed experiment since it will hinder proper 3D growth of the organoids.

27. Once the polymerization period is over, transfer the dome-containing plate back to the biological safety cabinet. Ensure BME domes have preserved their integrity and have not dispersed across the well (Figure 3C).

Note: After 10 min at 37°C, we recommend to visually inspect each well under a light microscope to ensure an even vertical distribution of the cells across the dome structures. If all cells are found at the bottom of the dome(s), it may indicate BME polymerization issues, and such domes should not be used. Contacts between the cells and the culture-treated plastic induce different cell behaviors vs. BME embedding and may alter the experimental outcomes.

28. Fill the dome-containing wells with 3 mL of CSC media.
29. Drug treatments (vs. controls) can be initiated ≥24 h post-seeding by adding the appropriate concentration of each compound in a fresh batch of CSC media (plan 3 mL per well).
    a. Carefully aspirate the media from dome-containing wells without disrupting the structures and add 3 mL of drug or control/vehicle supplemented CSC media.
    b. Terminate the drug treatments by replacing the media with fresh, drug-free CSC media at the desired period.
    c. Tumor organoid growth period can be extended beyond the drug treatments, as desired.

Note: For drug treatments, it is expected that certain compounds will exert cytotoxic effects on primary organoid growth. We recommend testing each drug at escalating concentrations

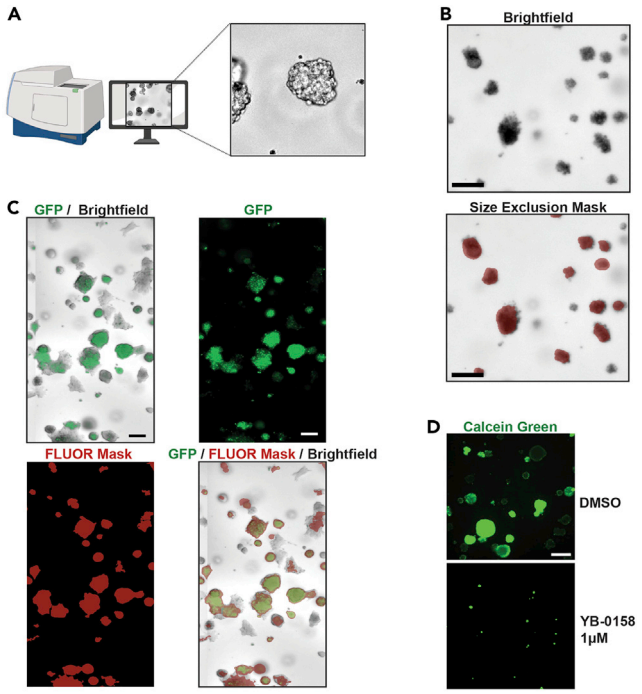

Figure 4. Examples of organoid detection methods using high content imaging

(A) 3D-embedded organoids can be detected, counted, and scored by high content imaging using ImageXpress Automated Cell Imaging Systems, or equivalent platforms.

(B) Example of organoid detection based on a size exclusion threshold, where cellular structures below a definite size are not counted from brightfield images. A color mask is applied by the analysis software over each structure considered as an organoid. Scale bar = 70 μm.

(C) Example for the use of a fluorescent (GFP) reporter system outlining a specific population of HCT116 organoids to be counted in an experiment. A color mask is applied by the analysis software over each structure presenting a fluorescence signal above a definite background threshold (FLUOR Mask). Scale bar = 70 μm.

(D) Identification of live organoid structures using the cell permeable, fluorescent dye Calcein AM (green). While several live organoids are detectable in DMSO-treated wells, only residual live cells are observed upon treatments with the anticancer small molecule YB-0158. Scale bar = 70 μm.

vs. control(s) to identify a dose range yielding sufficient residual primary organoids for subsequent evaluation of tumor-initiating activity in secondary passages.

Note: Typically, it will take 1–2 weeks to notice visible organoids, depending on the patient sample(s) used. Each patient sample gives rise to organoids presenting unique growth rate, morphological aspects, and metabolic activity. We recommend performing pilot experiments to establish patient-specific timelines and determine whether CSC media needs to be replenished over the primary organoid growth period. See troubleshooting 4

30. Use a high content imaging system (e.g., ImageXpress Pico Automated Cell Imaging System, or equivalent) to quantify changes in organoid counts and other morphological characteristics, such as size and form factor (Huang et al., 2015) in response to drug treatments. Perform brightfield scans of each well with Z-stack acquisition, ideally using 4× or 10× objective to generate merged composite images of all acquired optical sections (Figure 4A). Using a high content imaging analysis software (e.g., CellReporterXpress, Molecular Devices) it is possible to identify and count CRC tumor organoids based on a size exclusion threshold approach, as illustrated in Figure 4B. The size exclusion cut-off must be pre-determined by the user. See troubleshooting 5

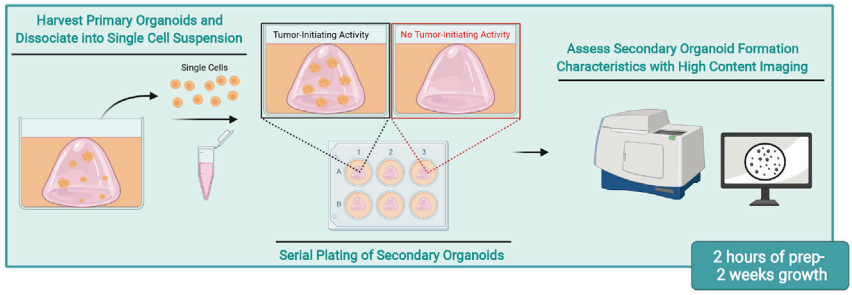

Figure 5. Schematic description of the method used to determine the impact of *ex vivo* therapeutic interventions on CSC activity based on secondary organoid formation frequency

Optional: For experiments based on fluorescence reporter systems to detect specific tumor organoids, a fluorescence z-stack acquisition scan of each well must be performed in addition to brightfield, enabling reporter-positive organoid counts (Figure 4C) (Benoit et al., 2021).

Optional: Fluorescence probes such as Calcein AM can be used to visualize live and metabolically active tumor organoids upon conversion of the non-fluorescent probe into acetoxymethyl ester hydrolyzed fluorescent Calcein (green) by intracellular esterases (Figure 4D). Only use permanent labeling reagents such as Calcein AM if these are known not to interfere with critical biological parameters of the experiment (Vilgelm et al., 2020).

Primary organoid harvest and secondary organoid plating

Timing: 2 h

The following steps describe the harvest of BME-embedded primary organoids and subsequent plating of secondary organoid series (Figure 5). To assess the impact of a drug treatment (or gene-specific silencing) on CSCs tumor-initiating capacity within CRC tumor organoids, it is essential to conduct serial/secondary plating from dissociated primary organoids. Such a rationale was initially proposed in hematopoietic stem/progenitor systems (Rizo et al., 2008) and further adapted by us for CRC (Bergin *et al.*, 2021; Masibag *et al.*, 2021). Upon the initial growth of primary organoid incubation, resulting control and drug-treated organoids will be dissociated into single-cell suspensions and embedded in a secondary series of BME domes, as detailed in steps 21–28 (starting with organoids instead of spheroids). Importantly, this secondary series of tumor organoids will not be further exposed to any drug treatments or other therapeutic interventions (Figure 5). If the tumor-initiating cell populations are altered by the treatment(s) of primary organoids, lower secondary organoid formation frequency values should be observed compared to control groups upon high content imaging of the experimental plates (Figure 5).

Note: Before starting this part, take an aliquot of BME out from −80°C and let it thaw on ice.

31. Gently aspirate the media surrounding the primary organoid-containing domes, and gently wash each well with 4 mL of sterile PBS. Repeat the aspiration step to discard the PBS.
32. Add 1 mL of Gentle Cell Dissociation reagent to chemically dissociate the BME domes and organoids.
    a. Transfer the plate(s) containing the organoids to harvest to the incubator, at 37°C, 5% CO_2 for 10 min.
    b. After the first 5 min of incubation, pipette up and down using a sterile 5 mL serological pipette to help dissociate the BME, and put the plate back to the incubator for another 5 min.

33. Once the 10-min incubation is over, execute up and down pipetting until no organoids are visible, and filter the cell suspension through a 70 μm strainer to eliminate multicellular aggregates. Collect the filtrate in a new sterile 50 mL conical tube.
34. Complete the filtrate volume to 10 mL with CSC media and preform cell counts using trypan blue and a hemocytometer, or an automated cell counter. See troubleshooting 1
35. For each secondary dome, plate a total of 15 × 10^4 cells in a volume of 300 μL of BME:cell suspension mixture (or 50 cells/μL).

Note: Since secondary organoid plating is executed using cell harvests from primary organoids, which present an heterogeneous composition with significantly lower CSC populations than spheroids (Bergin *et al.*, 2021), larger number of cells must be plated to enhance the frequency of secondary tumor organoids.

36. Proceed to BME re-embedding of primary organoid cells by executing steps 23 to 28 but using the appropriate cell number per well. See troubleshooting 3

Optional: Secondary organoid plating can be performed following a limiting dilution assay (LDA) format, by seeding decreasing number of cells (*e.g.*, 3 × 10^4, 1.5 × 10^4, 7.5 × 10^3, 3.75 × 10^3, etc.) in series of independent domes. While this approach represents a considerable investment in material and reagents, LDA plating followed by Poisson Distribution analysis can help users estimating the number of CSCs present in each experimental condition for every patient sample involved in the assay (Dorrell et al., 2014).

EXPECTED OUTCOMES

Secondary CRC organoids typically emerge after 7 days in culture, but this can vary from one patient-derived sample to another (Figure 6A). Beyond this point, secondary organoids generally become increasingly large, which may hamper subsequent high-content imaging quantification and morphological analysis (Figure 6A). Based on our experience, specific CRC patient-derived samples can initiate secondary organoid formation substantially faster than others, with variable growth rates. Thus, initial/pilot runs may enable users to document such critical patient-specific parameters to tailor subsequent experiments. In addition, secondary CRC tumor organoids can be further serially passaged and maintained under long-term tridimensional culture conditions, as documented in other systems (Leeman et al., 2019).

QUANTIFICATION AND STATISTICAL ANALYSIS

High content imaging platforms are recommended to quantify primary and secondary organoid counts, size, and other morphological features such as form factor (FF) used to score organoid shapes from circular-like to irregular (Figure 6B). An example of form factor-based scoring analysis in *ex vivo* tumor organoids was documented by Huang et al. (Huang *et al.*, 2015). Modulation of primary and secondary organoid formation can be expressed as raw counts or as relative tumor-initiation rates for a specific experimental condition vs. control. Examples of downregulation of secondary CRC tumor organoid formation using three compounds targeting colorectal CSC functions are presented in Figure 6C (Bergin *et al.*, 2021; Masibag *et al.*, 2021). In these specific cases, secondary organoids were imaged as described in step – 30, using a size exclusion threshold approach. Each dot on the violin plots represents secondary organoids counted from independent wells and two-tailed Student t-test was applied to determine the statistical significance ($p<0.05$) for each treatment vs. DMSO controls (Figure 6C). High-resolution confocal fluorescence microscopy can be used to visualize and quantify biomarkers of key functions linked to CSCs and tumor progression in primary and secondary organoids. Following formaldehyde fixation and immunofluorescence labeling, (Dekkers et al., 2019), high-resolution confocal imaging can reveal either proliferative (Ki-67^+) (Crespo *et al.*, 2017), self-renewing (Bmi1^+) (Kreso *et al.*, 2014), or differentiated (cytokeratin-20) (Sikandar et al., 2010) cells within CRC tumor organoids (Figure 6D). Confocal fluorescence microscopy also enables

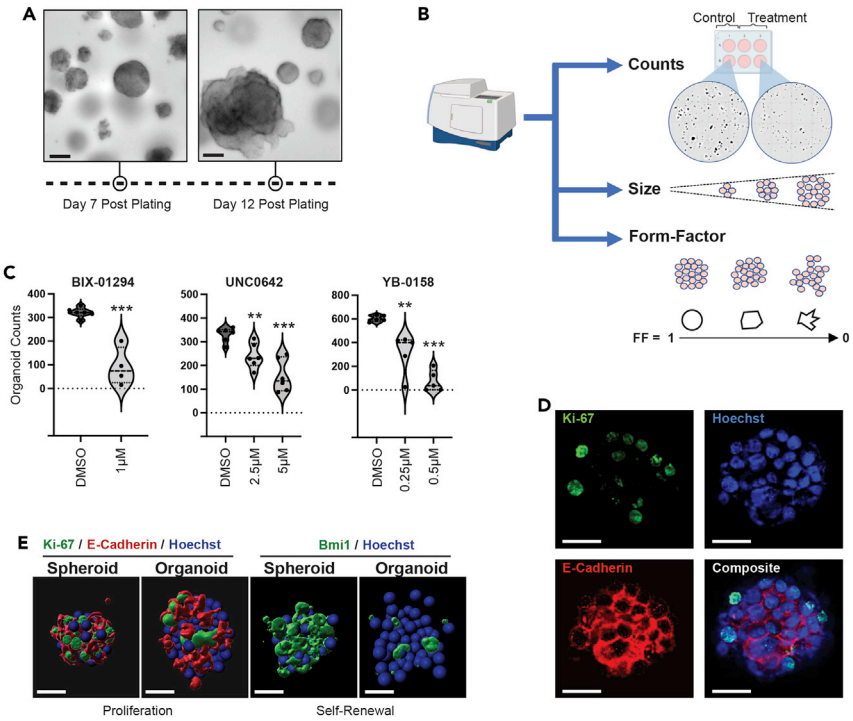

Figure 6. Schematic analysis of the colorectal tumor organoids resulting from serial passaging

(A) Brightfield images of secondary organoids depicting size difference over the growth stages. Scale bar = 70 μm.

(B) High content imaging can be used to establish organoid counts, size, and shape (form factor: FF) variations for control vs. treated conditions.

(C) Secondary organoid counts obtained from the serial passage of primary organoid cultures treated with 3 compounds targeting CSC functions in colorectal tumors (BIX01294, UNC0642: G9a inhibitor. YB-0158: Sam68 modulator). Organoid counts were established based on size exclusion (< 40 μm). $n \geq 4$ biological replicates from 3 independent patients; **: $p \leq 0.0053$, ***: $p \leq 0.0001$).

(D) Fluorescence imaging of a colorectal tumor organoid by confocal microscopy. A composite image of proliferative cells (Ki-67; green), adherens junctions (E-Cadherin; red), and nuclei (Hoechst 33342; blue) is presented. Scale bar = 30 μm.

(E) Tridimensional reconstruction of CRC patient-derived spheroids and organoids from confocal fluorescence microscopy images using the Imaris 9.5 rendering platform. For each type of multicellular structure, immunostaining of the proliferation marker Ki-67 and intestinal self-renewal marker Bmi1 are presented. Scale bar = 30 μm.

the assembly of vertical reconstruction of CSC-enriched spheroids and tumor organoids from the acquisition of serial optical sections, using ImageJ, as presented in Methods videos S3 and S4. Improved surface rendering tools such as Imaris 9.5 can be applied to serial confocal fluorescence images to enhance visualization and quantification capacity of cells expressing markers of proliferation and self-renewal in primary and secondary CRC tumor organoids (Figure 6E). In addition, extensive analysis of cellular heterogeneity can be conducted on tumor organoids by assessing the abundance and distribution various markers, using either cytometry approaches (fluorescence, CyTOF) or single-cell genomics upon completing steps - 32–34 (Brandt et al., 2019; Chen et al., 2018; Gonzalez-Exposito et al., 2019).

LIMITATIONS

The serial tumor organoid plating assays herein described does not consider the contribution of elements of the tumor microenvironment, such as stromal components and immune cells to tumor biology and drug response. To mitigate this aspect in *ex vivo* organoid systems, co-culture methods were recently developed (van Neerven et al., 2022) and could be adapted and integrated to this protocol to better represent the tumor microenvironment. Moreover, *ex vivo* drug treatments differ from *in vivo* administration, where critical pharmacological parameters such as pharmacokinetics

and pharmacodynamics (PK/PD) cannot be evaluated. Still, this method allows for rapid and cost efficient assessment of therapeutics impact on colorectal CSC activity in patient-derived tissues, and was deemed predictive of therapeutic outcomes when transposed to preclinical *in vivo* serial tumor transplantation systems (Masibag *et al.*, 2021).

TROUBLESHOOTING

Problem 1

Viable cell counts on dissociated CRC tumor fragments, spheroids, or organoids yield low values when using an automated cell counter (step-by-step method details, steps 12, 14, 22, and 34).

Potential solution

In our experience, an automated cell counter can sometimes mistakenly exclude viable cells that were freshly dissociated. If low viable cell counts are consistently observed, we recommend staining the cells with trypan blue and performing counts using a hemocytometer. It is noted that prolonged incubation with dissociation reagents can decrease cell viability. Thus, if cell viability remains an issue, dissociation time should be decreased for each type of sample.

Problem 2

No spheroids are forming from dissociated primary tumor cells in culture (step-by-step method details, step 15).

Potential solution

Unfortunately, the use of primary samples comes with patient diversity and complexity. Patient samples should be processed shortly after surgical resection. However, delayed access to the samples can be detrimental to the integrity of the tissue and associated with death and/or necrosis within tumor fragments. This, in turn, can impact the success rate of spheroid culture establishment.

Problem 3

BME fails to form robust tridimensional domes in 6-well plates (step-by-step method details, steps 26 and 36).

Potential solution

We have tested our protocol in multiple brands of culture plates, and we found that the CellTreat 6-well plates (See key resources table) are the most reliable to obtain robust BME dome formation. Moreover, we had most success using Corning Matrigel^{TM} basement membrane extract (#CB-40234) over other brands tested. Ensure to use a strict 1:1 BME:cell suspension ratio at step 24, since lower BME concentrations can result in loose polymerization and dome collapse. Increasing the final concentration of BME can also improve the polymerization of the domes but could also increase matrix rigidity and reduce drug diffusion. This option should be carefully tested by the user in pilot experiments. Finally, inadequate BME resuspension, aliquots preparation, and storage can be the root cause for inefficient dome formation.

Problem 4

Primary organoid formation frequency is low (step-by-step method details, step 29).

Potential solution

When CSCs are cultured as spheroids, they typically maintain a high rate of organoid formation (up to 90%–95%). However, patient-derived samples can differ in their respective tumor-initiating capacity. We recommend performing pilot organoid plating experiments with each patient-derived spheroid culture to determine sample-specific tumor initiation capacity. This strategy will also be useful to establish an approximate organoid growth timeline for each patient-derived culture.

Problem 5

Poor organoid resolution by high content imaging (step-by-step method details, step 30).

Potential solution

Organoids grown in BME are distributed at different points on the dome's vertical axis and it is possible for multiple organoids to be localized on top of each other. This can pose issues with imaging and quantification process. This is particularly frequent if domes are scanned at a single optical plane. We recommend using a high content imaging platform equipped with a z-stacking function, allowing to properly distinguish organoids growing on different stratums and increase the precision of the data.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Dr. Yannick D. Benoit (ybenoit@uottawa.ca).

Materials availability

This study did not generate new unique reagents

Data and code availability

This study did not generate or analyze any datasets and did not use any code.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2022.101218.

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AUTHOR CONTRIBUTIONS

C.J.B. designed and performed experiments, analyzed/interpreted data, and wrote the manuscript; Y.D.B. supervised research activities, designed experiments, performed experiments, provided results interpretation, and wrote the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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