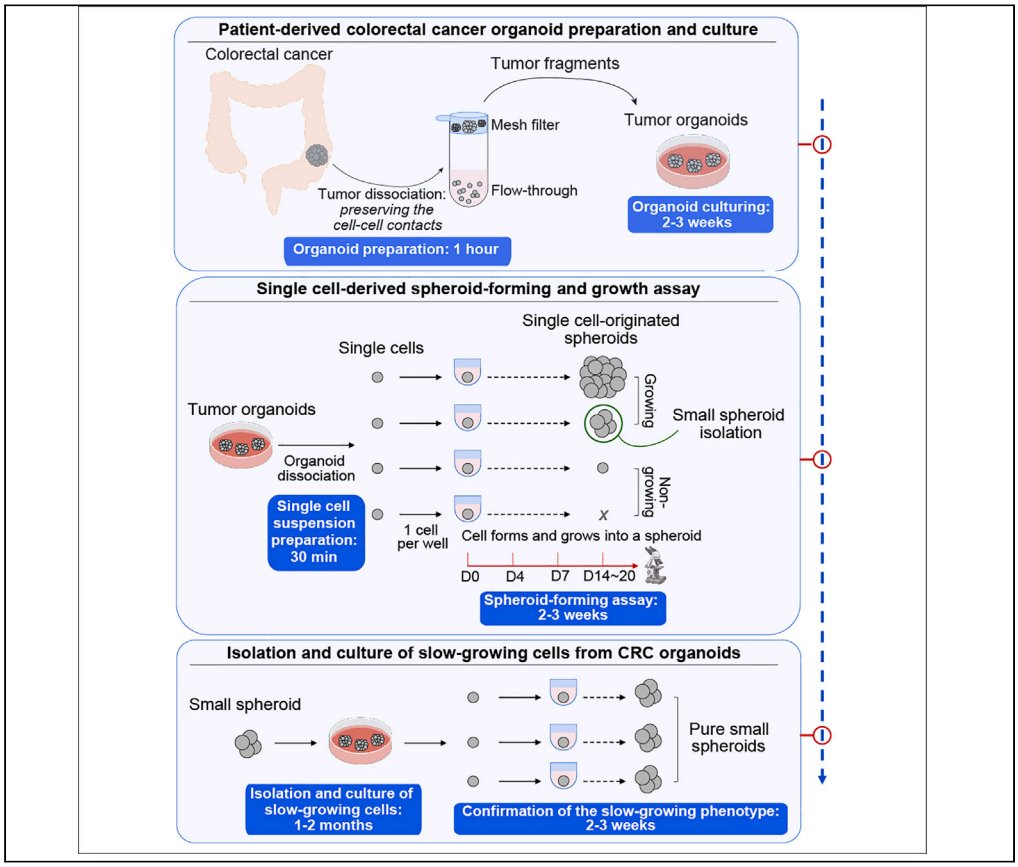
STAR Protocols

CellPress
OPEN ACCESS

Protocol

Tracking the growth fate of single cells and isolating slow-growing cells in human colorectal cancer organoids



Patient-derived tumor organoids are three-dimensionally cultured cancer cells that enable a suitable platform for studying heterogeneity and plasticity of cancer. We present a protocol for tracking the growth fate of single cells and isolating slow-growing cells in human colorectal cancer organoids. We describe steps for organoid preparation and culturing using the cancer-tissue-originating spheroid method, maintaining cell-cell contact throughout. We then detail a single-cell-derived spheroid-forming and growth assay, confirming single-cell plating, monitoring growth over time, and isolating slow-growing cells.

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

Roberto Coppo,
Jumpei Kondo,
Kunishige Onuma,
Masahiro Inoue

roberto.coppo.87@gmail.com (R.C.)
masa_inoue@kuhp.kyoto-u.ac.jp (M.I.)

Highlights

Rapid preparation of colorectal cancer organoids by preserving cell-cell contacts

Tracking the growth ability of the cancer cells at single-cell resolution

Stable culture of isolated slow-growing cancer cell subpopulation

The system allows us to phenotypically study intra-tumor cancer cell heterogeneity

Coppo et al., STAR Protocols 4, 102395
September 15, 2023
https://doi.org/10.1016/j.xpro.2023.102395

STAR Protocols

CellPress
OPEN ACCESS

Protocol

Tracking the growth fate of single cells and isolating slow-growing cells in human colorectal cancer organoids

Roberto Coppo,[1,3,*] Jumpei Kondo,[1,2] Kunishige Onuma,[1] and Masahiro Inoue[1,4,*]

[1]Department of Clinical Bio-resource Research and Development, Graduate School of Medicine, Kyoto University, Kyoto, Japan

[2]Present address: Division of Health Sciences, Department of Molecular Biology and Clinical Investigation, Graduate School of Medicine, Osaka University, Yamadaoka, Suita City, Osaka, Japan

[3]Technical contact

[4]Lead contact

*Correspondence: roberto.coppo.87@gmail.com (R.C.), masa_inoue@kuhp.kyoto-u.ac.jp (M.I.)
https://doi.org/10.1016/j.xpro.2023.102395

SUMMARY

Patient-derived tumor organoids are three-dimensionally cultured cancer cells that enable a suitable platform for studying heterogeneity and plasticity of cancer. We present a protocol for tracking the growth fate of single cells and isolating slow-growing cells in human colorectal cancer organoids. We describe steps for organoid preparation and culturing using the cancer-tissue-originating spheroid method, maintaining cell-cell contact throughout. We then detail a single-cell-derived spheroid-forming and growth assay, confirming single-cell plating, monitoring growth over time, and isolating slow-growing cells.

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

BEFORE YOU BEGIN

Accumulating evidence suggests that colorectal cancer (CRC) cells represent phenotypically dynamic heterogeneous cell populations.[2,3] Over the last decade, three-dimensional cultures utilizing patient-derived tumors have been developed for various cancer types, including CRC, while preserving the key characteristics of the original tumor.[4] Herein, we used the cancer tissue-originated spheroid (CTOS) method, in which cell–cell contact is maintained throughout organoid preparation, culture, and passaging.[5] The growth of each CRC organoid within the same line was heterogeneous,[6] suggesting that CRC organoids prepared using the CTOS method retain heterogeneous populations of cancer cells. We also found that CRC organoids could predict responses to chemotherapy.[6,7] Furthermore, we demonstrated that a small subset of cells within CRC organoids can initiate regrowth after exposure to high-dose radiation, and that cell fate is non-genetically and reversibly determined.[8] Recently, we revealed the existence of phenotypically heterogeneous cell subpopulations in CRC organoids with distinct but interchangeable growth patterns and drug sensitivity.[1]

To study the heterogeneity and plasticity of CRC cell subpopulations, it is critical to analyze cells at the single-cell level.[9] Recently, single-cell transcriptome analyses have been performed to study the characteristics of CRC cells.[10] However, such a "snapshot" analysis involving the isolation of CRC cells using specific markers[11] or dye retention[12] is limited when applied to a continuously changing process. Therefore, it is necessary to employ a phenotypically trackable cell culture system with a single-cell resolution.

Here, we provide protocols for the preparation of patient-derived CRC organoids by preserving cell–cell contact[5] and the culture system, allowing the tracking of cell growth at single-cell resolution.[1] The single-cell-derived spheroid-forming and growth (SSFG) assay includes strict confirmation of single-cell plating in a well, culture under growth-permissive conditions, and a time-course assessment of growth in each well. This method allowed us to precisely track the capacity for spheroid formation as well as the growth fate of each cell. We found that CRC cells are composed of distinct subpopulations: cells generating small spheroids (S-cells) and cells generating large spheroids (L-cells). Moreover, we successfully isolated and cultured subpopulations of S-cells exhibiting a drug-tolerant persister (DTP) phenotype, which is a nongenetically reversible state of drug resistance.[1] Because current cancer therapies have been designed and developed mainly against fast-growing cells, isolated S -cells could be a novel platform for developing treatments targeting slow-growing cells.

Institutional permissions

All procedures outlined in this protocol were carried out according to the ethical regulations approved by the Institutional Ethics Committees of Osaka International Cancer Institute (1803125402) and Kyoto University (R1575, R1671). Fresh surgical samples from patients with CRC were obtained after obtaining written informed consent.

Reagent stocks preparation

Timing: 15 min

Reagent	Final stock concentration	Amount	Volume
Liberase™ DH	5 mg/mL	50 mg	10 mL DMEM/F-12
DNase I	10 mg/mL	100 mg	10 mL MilliQ
Y-27632 (Rho-kinase inhibitor)	10 mM	10 mg	3.12 mL MilliQ

Store at −20°C for up to 2 months. Prepare aliquots to avoid repeated freeze/thaw cycles.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological samples		
Human: patient-derived CRC organoids (For the mutational status of the CRC organoids used in this study, please refer to Coppo et al. (2023)[1]) Experimental model details: CRC C45 line; Homo sapiens; APC p.Glu1374*, TP53 p.Glu1374*, KRAS p.Gly12Asp, BRAF wt, PIK3CA wt, AKT1 wt, SMAD4 wt, CTNNB1 wt, MLH1 wt, FBXW7 wt; 80s; TNM stage IVA; male.	Osaka International Cancer Institute and Kyoto University	N/A
Human: single cell-originated spheroids derived from CRC organoids	Osaka International Cancer Institute and Kyoto University	N/A
Chemicals, peptides, and recombinant proteins		
D-MEM/Ham's F-12 with L-Glutamine, Phenol Red, HEPES and Sodium Pyruvate	FUJIFILM Wako Pure Chemical Corporation	042-30555
DNase I	Roche	11284932001
HBSS(+) without phenol red	FUJIFILM Wako Pure Chemical Corporation	084-08965
Liberase™ DH Research Grade, high Dispase concentration	Roche	5401089001
Matrigel® Growth Factor Reduced (GFR) Basement Membrane Matrix	Corning	354230

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Penicillin-Streptomycin (10,000 U/mL)	Thermo Fisher Scientific	15140122
StemCell Keep	BioVerde	BVD-VPL-A1-20
StemPro™ hESC SFM https://www.thermofisher.com/order/catalog/product/A1000701	Thermo Fisher Scientific	A1000701
Trypsin-EDTA (0.25%)	Thermo Fisher Scientific	25200072
Y-27632 (Rho-kinase inhibitor)	Selleckchem	S1049
Experimental models: Organisms/strains		
NOD/Scid mice	CLEA Japan	NOD/ShiJic-scidJcl
Software and algorithms		
GraphPad Prism 9	GraphPad Software Inc.	https://www.graphpad.com/
ImageJ (Fiji)	https://doi.org/10.1038/nmeth.2019	https://imagej.net/software/fiji
LuminaVision	Mitani	https://www.mitani-visual.jp/download/catalogs/
Other		
90 mm Petri dish, non-treated (BIO-BIK I-90)	INA • OPTIKA	6-8626-01
6-well plate, flat bottom, non-treated	Iwaki	1810-006
24-well plate, flat bottom, non-treated	Iwaki	1820-024
Cellstar® 96U-well plate	Greiner Bio-One	650185
ClipTip™ 384 125	Thermo Fisher Scientific	94410153
E1-ClipTip™ Equalizer	Thermo Fisher Scientific	4672060BT
Falcon® 5 mL Round Bottom Polystyrene Test Tube, with Cell Strainer Snap Cap	Corning	352235
Reagent Reservoirs, Polystyrene, Sterile	Corning	53504-035
Stainless steel wire mesh (hole size: 250 and 500 μm)	Tianhao Wire Mesh	https://www.tianhaofiltermesh.com/product-category/uncategorized/
Falcon® Cell Strainers	Corning	352340; 352350; 352360
PrimeSurface® 384U	Sumitomo Bakelite	MS-9384U
Syringe 27Gx1/2 (1 mL)	Terumo	SS-10M2713
15 mL conical tube	Thermo Fisher Scientific	339650
50 mL conical tube	Thermo Fisher Scientific	339652
Eppendorf Safe-Lock Tubes, 1.5 mL	Eppendorf	61-0168-86
LEICA DMI4000B microscope	Leica Microsystems	N/A

MATERIALS AND EQUIPMENT

Tumor dissociation solution

Reagent	Final concentration	Amount
D-MEM/Ham's F-12 with L-Glutamine, Phenol Red, HEPES and Sodium Pyruvate	N/A	9.79 mL
Penicillin-Streptomycin (10,000 U/mL)	100 U/mL	100 μL
Liberase™ DH (5 mg/mL)	0.26 U/mL	100 μL
DNase I (10 mg/mL)*	10 μg/mL	10 μL
Total	**N/A**	**10 mL**

Freshly prepare before use. *: to add before use, depending on incubation time (see step 7).

HBSS buffer

Reagent	Final concentration	Amount
HBSS(+) without Phenol Red	N/A	493 mL
Bovine serum albumin (BSA) 25% (A1000701)	0.1%	2 mL

(*Continued on next page*)

Continued

HBSS buffer

Reagent	Final concentration	Amount
Penicillin-Streptomycin (10,000 U/mL)	100 U/mL	5 mL
Total	**N/A**	**500 mL**

Store at 4°C for up to 1 month.

CTOS organoid medium

Reagent	Final concentration	Amount
DMEM/F-12 with 1× GlutaMAX medium (A1000701)	N/A	445 mL
StemPro™ hESC Supplement 50× (A1000701)	1×	10 mL
Bovine serum albumin (BSA) 25% (A1000701)	1.8%	40 mL
Penicillin-Streptomycin (10,000 U/mL)	100 U/mL	5 mL
Total	**N/A**	**500 mL**

Store at 4°C for up to 1 month.

SSFG medium

Reagent	Final concentration	Amount
CTOS organoid medium	N/A	39.16 mL
Matrigel® Growth Factor Reduced (GFR) Basement Membrane Matrix	2%	800 µL
Y-27632 (Rho-kinase inhibitor) 10 mM	10 µM	40 µL
Total	**N/A**	**40 mL**

Freshly prepare at the time of use and keep it on ice. The required volume may vary depending on the experiment; please refer to step 28.

STEP-BY-STEP METHOD DETAILS

Isolation of patient-derived CRC organoids from primary tumor tissues

Timing: 1 h

In this section, we describe the method used to isolate and prepare tumor organoids from freshly resected human CRC tissues (Figure 1A). The following protocol is an adaptation of our previously developed CTOS method, in which cell–cell contacts are maintained throughout organoid preparation, culture, and passage.[5]

Note: Fresh primary CRC tissues obtained from the surgical samples or xenograft tumor were preserved on ice in a 50 mL conical tube (or equivalent) containing 15 mL of fresh DMEM/Ham's F12 medium or a physiological saline solution (such as HBSS buffer), supplemented with 100 U/mL of penicillin-streptomycin (150 µL).

1. Collect a fresh surgical CRC sample as described above and immediately proceed to tissue dissociation in a biological safety cabinet.
2. Transfer the CRC sample to a 90 mm petri dish and, utilizing tweezers to immobilize the tumor fragment; apply a scalpel to cut the tumor fragment into pieces less than 2 mm in size (Figure 1B).

⚠ CRITICAL: Tumor samples were cut into smaller pieces to prevent excessive tissue damage.

3. Wash the minced tumor fragments with 20 mL of HBSS buffer, gently mix up and down a couple of times, and transfer to a new 50 mL conical tube.

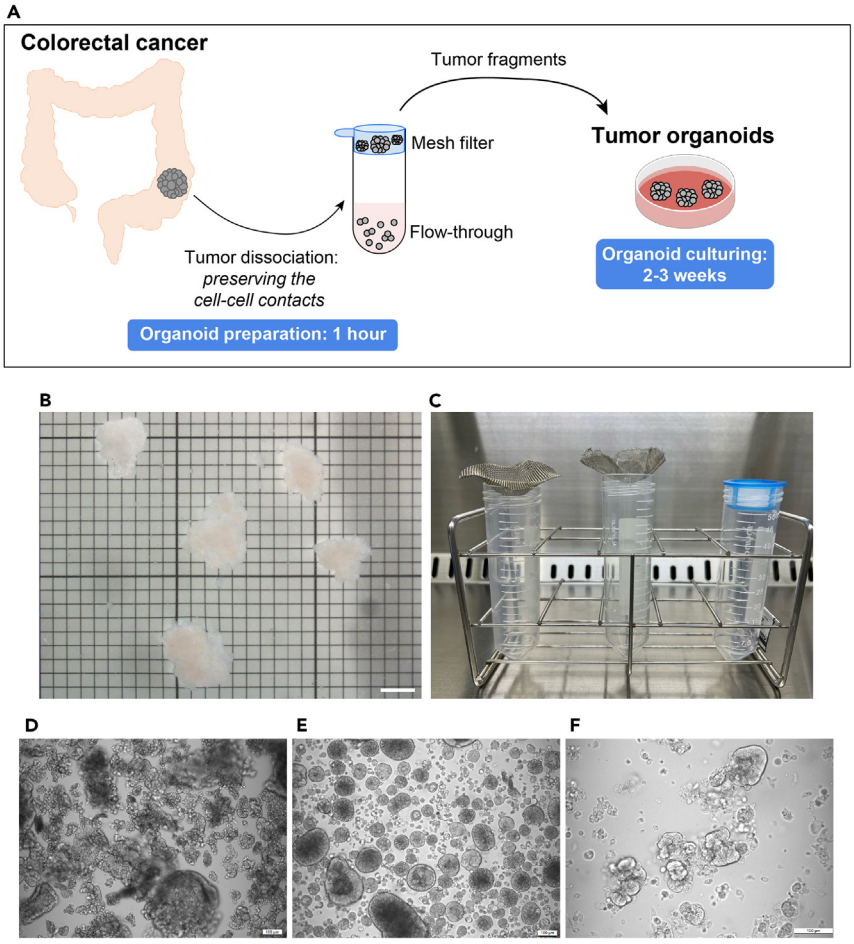


Figure 1. Preparation of patient-derived colorectal cancer organoids from primary tumor tissue

(A) Schematic overview of the cancer tissue-originated spheroid method.

(B) Images of tumor fragments dissected into small pieces.

(C) Image of the mesh filters (hole size 500, 250, and 40 μm, respectively) and the 50 mL conical tubes.

(D–F) Representative phase-contrast images of colorectal cancer (CRC) tumor organoids growing in suspension. (D) Image of the tumor fragments immediately after preparation. (E) Image of CRC organoids one day after preparation. (F) Image of CRC organoids immediately after disruption. Scale bar = 100 μm.

4. After waiting approximately 1 min for the tumor fragments to sink to the bottom, discard the supernatant (composed mainly of blood cells, single cells, and debris).
5. Resuspend the tumor fragments with 10 mL of the tumor dissociation solution.

   Note: To successfully maintain the cell–cell contact, we used Liberase™ DH as a blend of digestion enzymes.

6. Transfer the minced tumor fragment suspension to a 100 mL sterile conical flask with a magnet bar.
7. To digest the tumor tissue, incubate the sample for ~15–30 min in a 37°C water bath with constant stirring (700 rpm); adding 10 μL of DNase I for the last 15 min of incubation.

   ⚠ CRITICAL: Digestion time depends on the CRC sample. In some cases, it can be up to 60 min. Excessive digestion decreases the yield of the organoid preparations. The decision to stop the digestion step is based on visual inspection by the researchers and their

judgement regarding the size of the tumor clusters by checking under a microscope and observing the conical flask containing the tumor fragment suspension. Therefore, researchers should carefully monitor the progression of digestion. In the CTOS method, avoiding complete tissue dissociation into single cells is essential because over-digestion can cause significant cell damage.

8. Serially strain the suspension of digested tumor fragments using mesh filters and 50 mL conical tubes (Figure 1C).
   a. Pass the digested tumor suspension through a sterile stainless-steel wire mesh (hole size: 500 μm), and transfer the flow-through into the next mesh filter.
   b. Wash the tumor fragments trapped in the mesh with 5 mL of HBSS buffer.
   c. Repeat these steps for each filtration step.

Note: The following mesh filters can be applied in the following order. Stainless-steel wire mesh (hole size: 250 μm); 100-μm cell strainer (Falcon® Cell Strainers); 40-μm cell strainer (Falcon® Cell Strainers).

⚠ CRITICAL: When the number of tumor fragments is low, fewer filters should be used; for example, the 500- and 40-μm filters can be used to reduce material loss during the passage procedure.

9. Individually recover the tumor fragments, with a P1000 Pipette, in the fraction of 250–500, 100–250, and 40–100 μm with 1–2 mL of the CTOS organoid medium:
   a. Transfer the mesh filter containing the tumor fragments to a non-treated 60 mm Petri dish.
   b. Add 1–2 mL of the CTOS organoid medium to the filter.
   c. Using a P1000 Pipette, scratch and collect the tumor fragments, and proceed to step 10.

Pause point: The protocol can be paused at this point if the user makes freeze-stocks of the collected tumor fragments. To freeze-stock the tumor organoids, use the StemCell Keep reagent according to the manufacturer's protocol (https://www.diagnocine.com/Content/Upload/Product/datasheets/VPL-A1_Diagnocine_Manual.pdf).

Note: If the sample is frozen after step 9 of the protocol, proceed to step 10 after thawing and follow the protocol as described.

10. Transfer the collected tumor fragments to a non-treated 6-well plate dish containing 2–3 mL of CTOS organoid medium per well (Figure 1D).

Note: If only a few tumor fragments are obtained, a smaller plate such as a 12-well plate is used. Confluency is a crucial factor for organoid growth since it affects the nutrient and oxygen availability in cells. Approximately 50–100 fragments are seeded in 2–3 mL of CTOS organoid medium. However, optimal confluency may vary for each CRC organoid. It is recommended to optimize it based on the growth rate, the size of the organoids, and/or the colour change of the medium.

11. Incubate the culture in a 5% CO_2-humidified chamber at 37°C for 24 h. See troubleshooting 1.

Note: After 12 h of culture, the tumor fragments became transparent and spherical with a smooth surface (Figure 1E).

Maintenance of the CRC organoids

Timing: 2–3 weeks

This section describes the steps required to efficiently maintain, culture, passage, and expand CRC organoids for establishing CRC organoid lines. The following protocol is an adaptation of the methods described by Kondo et al.[5]

12. The day after organoid preparation, CRC organoids should be washed twice with HBSS buffer to remove cellular debris.
    a. Collect the CRC organoids and transfer into a 1.5 mL tube.
    b. Using a P1000 Pipette, mix the suspension up and down ten times, and wait approximately 1–2 min for the CRC organoids to sink to the bottom. Then, discard the supernatant (mainly composed of debris and/or dead cells).
    c. Wash the CRC organoids with 1 mL of HBSS buffer.
    d. Repeat steps b and c.
    e. Add 1 mL of CTOS organoid medium to the CRC organoids, and transfer them to a non-treated 6-well plate dish containing 2–3 mL of CTOS organoid medium per well.
    f. Incubate the organoid culture in a 5% CO_2-humidified chamber at 37°C for 6 days. See troubleshooting 2.

Note: Once established, CRC organoid cultures are monitored daily and passaged once a week to expand and maintain their viability. CRC organoids can be cultured in suspension. Some CRC organoid lines might require the addition of Matrigel GFR and/or growth factors (e.g 100 μg/mL of bFGF and/or 10 μg/mL of Rho-kinase inhibitor) in the CTOS organoid medium to promote their growth and/or survival rate. The organoids may be embedded in Matrigel GFR (5 μL of Matrigel GFR containing 1×10^2 organoids) in the CTOS organoid medium, depending on their viability and growth rate.

13. For passaging, CRC organoids should be dissociated using the syringe disruption method once a week[13]:
    a. One week after the organoid preparation or the previous disruption step, or when the diameter of the spheroids reaches 200–300 μm, collect the CRC organoids and transfer into a 1.5 mL tube.
    b. Using a P1000 Pipette, mix the suspension up and down ten times. After waiting approximately 1–2 min for the CRC organoids to sink to the bottom, discard the supernatant liquid (composed mainly of debris and/or dead cells).

Note: If the CRC organoids do not settle at the bottom, one can subject them to centrifugation at 400 × *g* for 3 min at 25°C.

    c. Wash the CRC organoids with 1 mL of HBSS buffer.
    d. Repeat steps b and c.
    e. Add 200 μL of the CTOS organoid medium to the CRC organoids.
    f. Disrupt the CRC organoids into smaller fragments by passing them through a 1 mL syringe with a 27 G needle at a high flow rate (~0.5 mL/s) ~5–10 times. See troubleshooting 3.

⚠ CRITICAL: The number of syringe strokes required to disrupt the organoids may depend on the CRC organoid line. Excessive disruption leads to decreased organoid viability. To ensure proper disruption, bubble formation should be avoided during this step.

    g. Resuspend the disrupted CRC organoids in a non-treated 6-well plate containing 2–3 mL of CTOS organoid medium per well (Figure 1F).

Note: CRC organoids are spontaneously re-formed from these fragments within hours.

    h. Incubate the culture in a 5% CO_2-humidified chamber at 37°C for 1 week.

Note: Although this step depends on the seeding density of the CRC organoids, it is safer to replace the medium once every 3–4 days as a standard. If the organoids are in suspension, the plate can be swirled clockwise to allow them to gather at the centre of the plate. Using a P1000 pipette, carefully aspirate the old medium from the edge of the plate and replace it with fresh medium. Alternatively, one can pellet the organoids using centrifugation at 400 × *g* for 3 min at 25°C. After pelleting the organoids, carefully aspirate the old medium and resuspend them in a freshly prepared medium.

⚠ CRITICAL: The CRC organoids should be subjected to further experiments at least one day after passaging to avoid the influence of organoid disruption and remodelling.

Note: Organoids prepared from freshly harvested primary CRC tumors should be cultured and expanded for 2–3 weeks before being subjected to SSFG assay. This step avoids contamination with stromal cells.

14. Within 2–3 passages, organoids should be freeze-stocked with StemCell Keep according to the manufacturer's protocol (https://www.diagnocine.com/Content/Upload/Product/datasheets/VPL-A1_Diagnocine_Manual.pdf).

Note: A CRC organoid 'line' is defined by the following criteria: i) growing continuously in culture, ii) generating xenograft tumor (at least two passages *in vivo*), and iii) being sufficiently freeze-stocked in order to reproduce the experiments.[5]

Single-cell-derived spheroid-forming and growth (SSFG) assay

Timing: 2–3 weeks

In this section, we describe a phenotypically trackable cell culture system with single-cell resolution (Figure 2A). The SSFG assay allows us to precisely track not only the capacity of spheroid formation but also the cell fate of growth of each spheroid-forming cell in CRC organoids with single-cell resolution. For the complete details of this protocol, please refer to Coppo et al.[1]

15. Collect one hundred to a thousand CRC organoids and transfer into a 15 mL conical tube containing 10 mL of HBSS buffer.

Note: The SSFG assay can also be performed using a small number of organoids (e.g ten CRC organoids). Four hundred cells at least are required for the assay and a CRC organoid with a diameter of 100 μm approximately consists of ~100 cells.[5]

16. Centrifuge the CRC organoids at 400 × *g* for 3 min at 25°C.
17. Discard the supernatant and wash the organoid pellet with 10 mL of HBSS buffer.
18. Centrifuge the CRC organoids at 400 × *g* for 3 min at 25°C, and then discard the supernatant.
19. Add 2 mL of 0.25% trypsin-EDTA to the organoid pellet. Using a P1000 Pipette, mix the suspension up and down ten times, and incubate for ~5–10 min at 37°C in a water bath.

⚠ CRITICAL: The incubation time with 0.25% trypsin-EDTA required to dissociate organoids into single cells may vary among CRC organoid lines. Excessive treatment impairs cell viability.

20. Mix the cell suspension by gently pipetting a hundred times to promote organoid dissociation into single cells. See troubleshooting 4.

⚠ CRITICAL: The number of pipetting steps required to promote cell dissociation varies among CRC organoid lines. Thus, the number of pipetting steps can be reduced to prevent cell death.

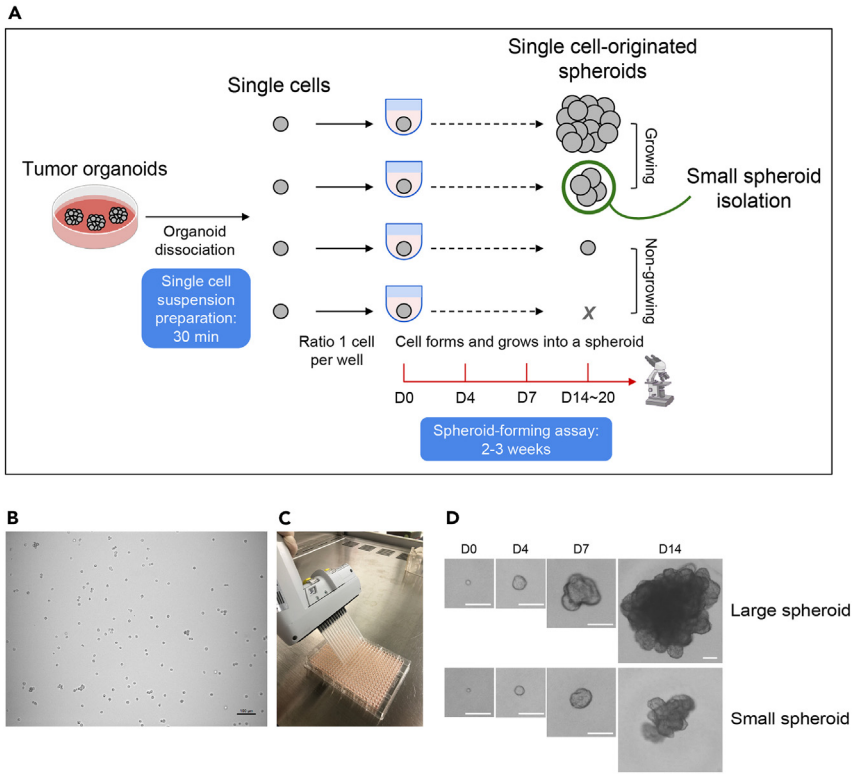


Figure 2. Phenotypically trackable cell culture system with single-cell resolution

(A) Schematic overview of the single-cell-derived spheroid-forming and growth (SSFG) assay.

(B) Representative phase-contrast image of the colorectal cancer cells after dissociation into single cells and filtration through a 35-μm cell strainer. Scale bar = 100 μm.

(C) Plating procedure of a single-cell suspension in a non-treated 384-well plate using an electronic pipette.

(D) Representative phase-contrast images of the single cell-originated spheroids at different days (D) of culture. Scale bar = 100 μm. Figure adapted from Coppo et al. (2023).[1]

21. Add 6 mL of the CTOS organoid medium to inactivate trypsin.
22. Add 8 μL of DNase I (10 μg/mL) and incubate for 1 min at 25°C.
23. Centrifuge the cell suspension at 400 × *g* for 3 min at 25°C.
24. Discard the supernatant liquid and wash the cell pellet with 10 mL of HBSS buffer.
25. Centrifuge the cell suspension at 400 × *g* for 3 min at 25°C, and then discard the supernatant.

⚠ CRITICAL: It is essential to carefully remove the supernatant to avoid the loss of the cell pellet, which is invisible when working with a low number of CRC organoids.

26. Resuspend the cell pellet in 1 mL of HBSS buffer and filter into a 5 mL round bottom tube with a 35-μm cell strainer cap in order to remove cell clusters (Figure 2B).
27. Keep the cell suspension on ice and determine the cell count.

⚠ CRITICAL: To increase the probability of seeding cells at a ratio of one cell per well, it is essential to count the number of cells precisely. In addition, it is crucial to examine the single-cell status under a microscope. If organoid dissociation into single cells is incomplete and there are many small clusters, dissociation using steps 23–26 should be repeated.

28. Dilute ~300–400 cells in a 20 mL cold SSFG medium. Keep the cell suspension on ice.

Note: The amount of medium used depends on the experiment. For example, 20 mL of SSFG medium is required to seed a 384-well plate with 50 μL of medium per well. The amount of medium required may vary depending on the number of 384-well plates to be prepared, the number of cells to be seeded, or both (approximately 300–400 cells per plate).

29. Dispense the cell suspension into a sterile reagent reservoir and gently mix.
30. Using an E1-ClipTip electronic pipette (or equivalent), seed the cell suspension into a non-treated 384-well plate with a ratio of one cell per well (50 μL/well) (Figure 2C).
31. Centrifuge the plate at 400 × *g* for 3 min at 25°C.

Note: Centrifugation of the cell plate is used to concentrate the cells at the bottom of the U-bottom wells and eliminate any bubbles to facilitate picture acquisition in step 33.

32. Incubate the plate in a 5% CO_2-humidified chamber at 37°C and culture for 2–3 weeks.

Note: The growth rate of single cells may differ between different CRC organoid lines. The culture timing should be adjusted for each line.

33. Within 2 h of cell seeding (D0), the presence of one cell per well is strictly confirmed by image acquisition using a LEICA DMI4000B microscope equipped with Lumina Vision (or equivalent).

Note: Lumina Vision equipment allows automatic image acquisition of the entire 384-well plate. Alternatively, the YAMAHA CELL HANDLER™ (https://global.yamaha-motor.com/business/hc/) is an automated system that can be used to detect, select, and seed individual cells as well as for image acquisition.

34. After 7 days of culture (D7), add 30 μL of fresh SSFG medium (without Y-27632) per well (total amount: 12 mL) using an E1-ClipTip electronic pipette (or equivalent) and a sterile reagent reservoir. Add the medium to the top of the 50 μL previously seeded (D0).
35. The growth of single cells into spheroids are monitored by image acquisition on different culture days (D0, D4, D7, and D14), as shown in Figure 2D. See troubleshooting 5.

Optional: The spheroids are further cultured after D14 by adding the SSFG medium without Y-27632 (30 μL/well) until D20 when the growth rate of the CRC organoid line is low.

Note: The spheroid-forming capacity (percentage of single cells which can grow and form spheroids) ranged from ~19–59% in different CRC organoid lines.[1]

Isolation and culture of slow-growing cells from CRC organoids

Timing: 1–2 months

This section describes the application of the SSFG assay to identify, isolate, and culture slow-growing cells from CRC organoids (Figure 3). The SSFG assay revealed that the CRC organoids are composed of two types of cells: S-cells, generating small spheroids, and L-cells, generating large spheroids (Figure 2D). For complete details regarding the use and execution of this protocol, please refer to Coppo et al.[1]

Note: To identify small and large spheroids, the results of the SSFG assay were quantified as described in the Quantification and Statistical Analysis section. Because each spheroid was strictly derived from a single cell, it can be called a clone.

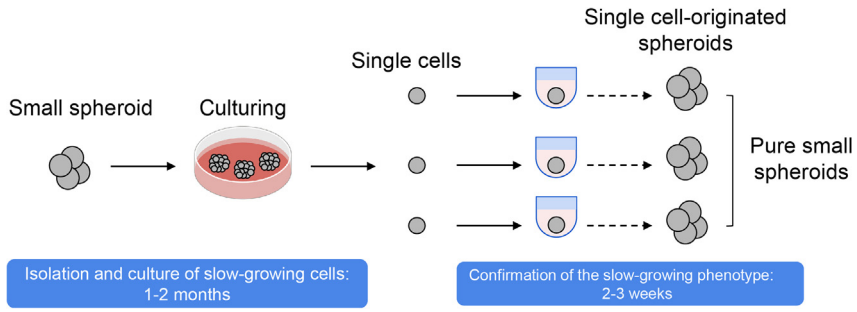


Figure 3. Isolation and culture of slow-growing cells from colorectal cancer organoids

Schematic overview of the experiments to confirm the slow-growing phenotype of the isolated small spheroid clones.

36. At the end of the SSFG assay (D14 or D20), pick-up the small spheroids and individually transfer (one clone per well) into a well of a non-coated 96-well plate containing 100 μL of the CTOS organoid medium supplemented with 2% Matrigel GFR.
37. Incubate the spheroids in a 5% CO_2-humidified chamber at 37°C and culture for 2 weeks.

Note: The slow-growing spheroids might be monitored daily, and the CTOS organoid medium supplemented with 2% Matrigel GFR changed after one week of culture. The medium is replaced by carefully aspirating the old medium from the top using a P200 pipette, ensuring that the spheroids are not aspirated or lost. Following aspiration, fresh medium is added to each well.

38. When the slow-growing spheroids reach a size of 3–4 × 10^5 μm^2, individually dissociate them by the syringe disruption method as described in the section maintenance of the CRC organoids, step 13.
39. Culture the disrupted spheroids (one clone per well) in 500 μL of the CTOS organoid medium supplemented with 2% Matrigel GFR into a well of a non-coated 24-well plate.
40. Incubate the spheroids in a 5% CO_2-humidified chamber at 37°C for 1 week.

Note: The growth rates of slow-growing spheroids may differ among CRC organoid lines. The culture timing should be adjusted for each line. The organoids should be expanded to perform the next assays and to prepare sufficient freeze stocks.

41. Once isolated the slow-growing spheroids, a subsequent round of SSFG assay should be performed to confirm the slow-growing pattern.
    a. One hundred organoids from a slow-growing clone are subjected to the SSFG assay, as described in the Single-cell-derived spheroid-forming and growth assay section.
    b. The results are quantified as described in the section quantification and statistical analysis section, confirming the slow-growing phenotype.

EXPECTED OUTCOMES

Patient-derived CRC organoids can be obtained using the CTOS method with a high success rate and yield.[1,5,6] The SSFG assay revealed the heterogeneous growth of cells in CRC organoids at the single-cell level (Figure 2D). The spheroid-forming capacity varied from approximately 19%–59% in different CRC organoid lines. The non-growing cells, the area of which is below 2.5 × 10^3 μm^2, are a mixture of early death, late death, growth arrest, and a decline in spheroid size.[1] The growing-cells within cancer cells show a wide range of spheroid growth abilities. The putative threshold between the fast- and slow-growing phenotypes was set based on the maximum size of the selected slow-growing clone at the end of the SSFG assay. The S-cells were isolated. The slow-growing pattern of S-cells was confirmed by additional rounds of the SSFG assay. A clone was defined as slow-growing when the progeny of single cells generated only small spheroids (Figure 3).[1]

QUANTIFICATION AND STATISTICAL ANALYSIS

To quantify the results of the SSFG assay (refer to the single-cell-derived spheroid-forming and growth assay section), one cell per well was confirmed at the beginning of the assay by analyzing the captured images at D0 and discarding the wells containing multiple cells (Figure 4A). Next, the area of every single cell was calculated using ImageJ Fiji software. After setting the measurement scale, the cell area was traced with a line, and the outlined surface was measured using ImageJ Fiji software (as described in https://imagej.nih.gov/ij/docs/pdfs/ImageJ.pdf). Single cells with an area greater than 300 μm^2 (possibly cell doublets or small clusters) on D0 were excluded from the subsequent analyses (Figure 4A). The size of the spheroids was evaluated on D14 and/or D20 by measuring the area of each single-cell-derived spheroid using the acquired images and ImageJ Fiji software, as described above. First, non-growing spheroids were identified. To define non-growing spheroids, the time course of spheroid growth was monitored. Spheroids that were unable to grow (spheroid size decreased or did not increase) were categorized as non-growing spheroids. The maximum area of non-growing spheroids was observed on D14 (Figure 4B). Second, growing spheroids with a size greater than the threshold were identified (Figure 4C) and the growing spheroids were subgrouped into small and large spheroids (Figure 2D and 4D). Cells that generated small and large spheroids were categorized as S- and L-cells, respectively. In some organoid lines, the spheroid size distribution was bimodal, whereas others did not show an apparent threshold. The putative threshold of the spheroid area is 1.0×10^5 μm^2 determined by one CRC organoid line (C45). The threshold in each organoid line can be determined by a second-round SSFG assay because the cells derived from small spheroids generate only small spheroids (see below). The spheroid-forming capacity was calculated as the percentage of single cells that were able to grow and form spheroids (growing spheroids) on day D14, as shown in Figure 4F.

To isolate the single cell-derived slow-growing spheroids (small spheroids) described in the isolation and culture of slow-growing cells from CRC organoids, the slow-growing spheroid clones were individually collected, expanded in culture, and subjected to the next round of the SSFG assay. When all the single cells grow into small spheroids (S-pattern), the subclone can be called a 'S-pattern clone' (Figure 4E). The growth pattern was distinct from that of cells from large spheroids which generated both small and large spheroids (D-pattern) in the second-round SSFG assay. For the complete details of this protocol, please refer to Coppo et al.[1]

LIMITATIONS

Although the SSFG assay revealed the existence of distinct but interchangeable subpopulations of CRC cells with different growth fates, the model is not without limitations. *In vitro* assays generally run the risk of allowing cells to adapt to culture conditions. In this study, the patient- or PDX-derived CRC organoids were cultured in a medium containing the StemPro™ hESC SFM supplement, which was originally formulated for the growth of human-induced pluripotent and embryonic stem cells. Consequently, this organoid medium may allow selective expansion of cancer stem-like cells, promoting their spheroid-forming capacity. We have previously reported the ability to generate differentiated-like cells and heterogeneity in CRC organoids when cultured in StemPro™ hESC SFM medium. For example, the CRC organoids cultured in StemPro™ hESC SFM or the organoid-derived xenograft tumors preserve their 3D structure and retain the differentiation characteristics of the original cancer tissue.[5,14,15] We reported that the growth of each CRC organoid within the same line is heterogeneous, suggesting that CRC organoids cultured in StemPro™ hESC SFM medium retain heterogeneous cell populations.[6] This led us to apply the SSFG assay to CRC organoid lines and demonstrate heterogeneous cell growth ability at single-cell resolution.[1] These results suggest that the organoids cultured in the StemPro™ hESC SFM medium substantially preserve the differentiation status and the heterogeneity in the original tumors. However, the characteristics of these cells with a slow-growing fate remain to be elucidated in both patient tumors and PDX. In addition, some CRC cells are dependent on niche signals, such as WNT, for the self-renewal of stem-like cells, whereas others are independent. The absence of these environmental factors in our SSFG assay may have affected the growth patterns of the CRC cells.

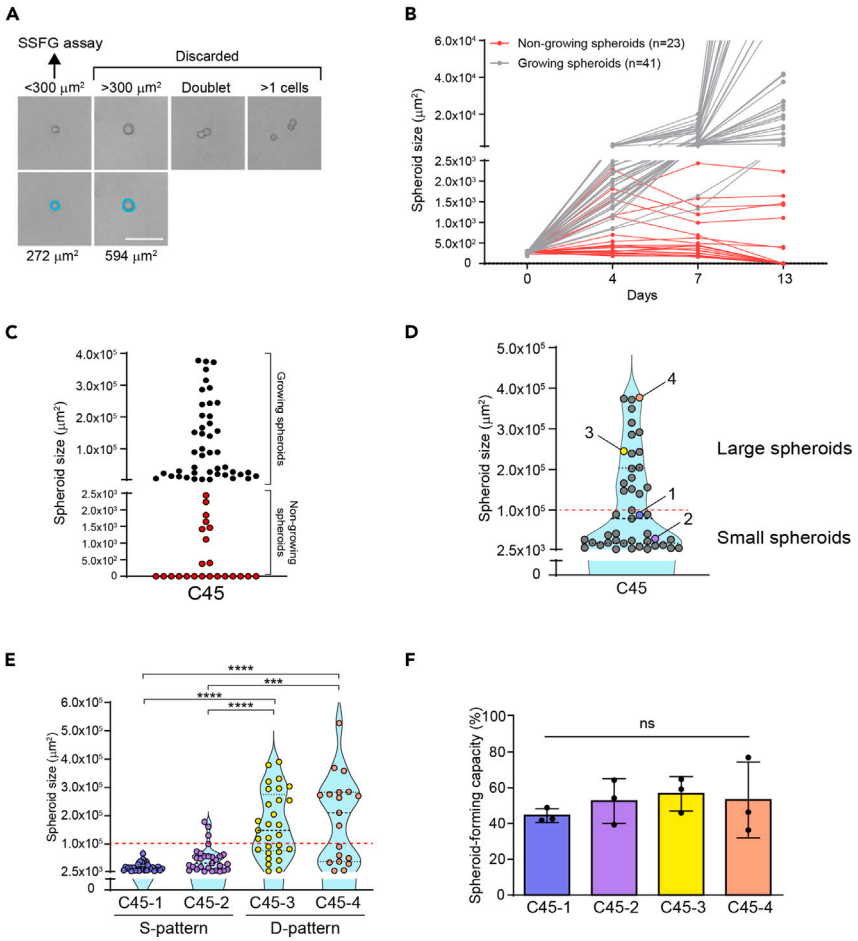


Figure 4. Quantification of the data obtained with the single-cell-derived spheroid-forming and growth (SSFG) assay

(A) Phase-contrast images of the SSFG assay at D0. The blue circles indicate the projected area of single cells. Scale bar = 100 μm.

(B) Growth curve of the growing (gray) and the non-growing (red) single cell-originated spheroids.

(C) Size distribution of the single-cell-derived spheroids in the SSFG assay. Black and red dots represent the growing and the non-growing spheroids, respectively.

(D) Violin plots of the SSFG assay for the C45 CRC organoid line. Four indicated spheroids are collected for the next round of the SSFG assay. The red dashed line indicates a rounded value (1.0×10^5 μm^2) of the area at D14, for the C45-1 line, and it is indicative of a putative threshold between the small and the large spheroids in the C45 line.

(E) Violin plots of the second round of SSFG assays for the indicated clones in (D). The red dashed line indicates the putative threshold value (1.0×10^5 μm^2).

(F) Spheroid-forming capacity of the indicated CRC organoid lines. The mean ± SD is shown. (A–F) Figures adapted from Coppo et al. (2023).[1]

TROUBLESHOOTING

Problem 1

Low yield of organoids during preparation from primary tumors (step-by-step method details, steps 1–11).

Potential solution

- Fresh primary CRC tissues obtained from the surgical samples of patients or PDX tumors exhibit heterogeneity and complexity. The ratio of stromal and tumor cells varies among the samples, which affects the yield of the organoid preparation.

- The tumor sample needs to be cut into smaller pieces as sharply as possible to reduce cell damage (step 2). Moreover, excessive enzymatic digestion (step 7) of the CRC sample completely disrupts cell–cell contacts, decreasing the yield of organoids (step 9). Reducing the digestion time to 10–15 min is recommended for soft tumors.
- When the number of digested tumor fragments is low, the use of fewer mesh filters during organoid collection is recommended to reduce material loss during filtering (steps 8 and 9).

Problem 2

Poor growth of CRC organoids in culture (step-by-step method details, step 12).

Potential solution

Although the CTOS organoid medium generally allows the growth of CRC organoids in suspension, some CRC organoids require the culture condition optimization to achieve growth and survival *in vitro*. Some CRC organoid lines require interaction with the basement membrane matrix (for example, Matrigel GFR) for efficient growth and survival. Culturing CRC organoids under both conditions in parallel increases the success rate of organoid culture establishment. CRC organoids (1×10^2) can be embedded in a droplet of 100% Matrigel GFR (5 μL) and, after the gel solidification, cultured in the CTOS organoid medium. In exceptional cases, as reported by Fujii et al.,[16] the necessary supplements can vary. It is recommended to perform a preliminary experiment to determine the necessity or the optimal timing of replenishing the growth factors for each case, if necessary. For example, the growth factors (such as bFGF) are generally added every other day or every 3–4 days; however, the effect of the growth factors can be optimized based on the growth and the survival rate of CRC organoids.

Problem 3

Excessive cell death occurred in the organoids at passage (step-by-step method details, step 13).

Potential solution

The number of passages through the syringe to disrupt organoids should be optimized for each CRC organoid line. Therefore, it is recommended to reduce the number of passages through the syringe and/or decrease the flow rate. Excessive disruption damages the cells in organoids, leading to an increase in cell death.

Problem 4

Excessive cell death following dissociation into single cells (step-by-step method details, steps 19 and 20).

Potential solution

The incubation time with 0.25% trypsin-EDTA required to dissociate the organoids into single cells varies among the CRC organoid lines. Excessive treatment increases cell death and decreases the spheroid-forming capacity of single cells. It is recommended to reduce the incubation time by up to ~2–5 min when excessive cell death is observed. Moreover, the number of pipetting steps required to promote cell dissociation increases cell death. It is recommended that the number of pipetting steps be reduced to approximately 20–50 to prevent cell death.

Problem 5

The spheroid-forming capacity was extremely low (step-by-step method details, step 35).

Potential solution

The spheroid-forming capacity (the percentage of single cells which can grow and form spheroids) in the SSFG assay ranges from approximately 19%–59% in different CRC organoid lines.[1] A pilot spheroid-forming cell assay is recommended to determine the sample-specific spheroid formation capacity in the SSFG medium. Adding growth factors (such as 100 μg/mL bFGF) or increasing the

amount of Matrigel GFR or both in the SSFG medium may improve the spheroid-forming capacity. Using a low concentration of Matrigel GFR can increase the formation and growth of spheroids from single cells compared with suspension culture. Matrigel GFR provides an extracellular matrix (ECM) such as laminin, collagen IV, and entactin/nidogen, which stimulates organoid growth. However, using 2% Matrigel GFR rather than a higher concentration is advantageous for the SSFG assay, as the less viscous medium facilitates handling, such as seeding and microscopic observation. Moreover, the spheroids form a more uniform spherical structure at lower Matrigel GFR concentrations than at higher concentrations. This can be beneficial for applications such as drug screening or imaging, where uniformity facilitates evaluation. Therefore, using 2% Matrigel GFR instead of a higher concentration or a suspension culture can balance the growth advantage of Matrigel as a scaffold and the feasibility of the SSFG assay.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Masahiro Inoue (masa_inoue@kuhp.kyoto-u.ac.jp).

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.

ACKNOWLEDGMENTS

This work was supported by a Grant-in-Aid from P-CREATE, a Japan Agency for Medical Research and Development, Japan, 20cm0106203h0005 (M.I., R.C., J.K., K.O.); Japan-UK Research Cooperative Program between JSPS and The Royal Society (M.I.); Grant-in-Aid for Scientific Research, 21H02714 (B) (M.I.); and the International Research Fellow of Japan Society for the Promotion of Science, 22F22754 (M.I.). R.C. was supported by Postdoctoral Fellowships for Research in Japan.

AUTHOR CONTRIBUTIONS

R.C. and M.I. designed the experiments and wrote the manuscript. R.C. conducted the experiments and analyzed the data. R.C. and J.K. interpreted the data obtained. K.O. and M.I. supervised the project.

DECLARATION OF INTERESTS

J.K., K.O., and M.I. belong to the Department of Clinical Bio-resource Research and Development at Kyoto University, which is sponsored by KBBM, Inc. M.I. is an inventor of the patents related to the CTOS method.

REFERENCES

1. Coppo, R., Kondo, J., Iida, K., Okada, M., Onuma, K., Tanaka, Y., Kamada, M., Ohue, M., Kawada, K., Obama, K., and Inoue, M. (2023). Distinct but interchangeable subpopulations of colorectal cancer cells with different growth fates and drug sensitivity. iScience *26*, 105962. https://doi.org/10.1016/j.isci.2023.105962.

2. Batlle, E., and Clevers, H. (2017). Cancer stem cells revisited. Nat. Med. *23*, 1124–1134. https://doi.org/10.1038/nm.4409.

3. Hirata, A., Hatano, Y., Niwa, M., Hara, A., and Tomita, H. (2019). Heterogeneity in colorectal cancer stem cells. Cancer Prev. Res. *12*, 413–420. https://doi.org/10.1158/1940-6207.CAPR-18-0482.

4. Drost, J., and Clevers, H. (2018). Organoids in cancer research. Nat. Rev. Cancer *18*, 407–418. https://doi.org/10.1038/s41568-018-0007-6.

5. Kondo, J., Endo, H., Okuyama, H., Ishikawa, O., Iishi, H., Tsujii, M., Ohue, M., and Inoue, M. (2011). Retaining cell-cell contact enables preparation and culture of spheroids composed of pure primary cancer cells from colorectal cancer. Proc. Natl. Acad. Sci. USA *108*, 6235–6240. https://doi.org/10.1073/pnas.1015938108.

6. Kondo, J., Ekawa, T., Endo, H., Yamazaki, K., Tanaka, N., Kukita, Y., Okuyama, H., Okami, J., Imamura, F., Ohue, M., et al. (2019). High-throughput screening in colorectal cancer tissue-originated spheroids. Cancer Sci. *110*, 345–355. https://doi.org/10.1111/cas.13843.

7. Tashiro, T., Okuyama, H., Endo, H., Kawada, K., Ashida, Y., Ohue, M., Sakai, Y., and Inoue, M. (2017). In vivo and ex vivo cetuximab sensitivity assay using three-dimensional primary culture

system to stratify KRAS mutant colorectal cancer. PLoS One *12*, e0174151. https://doi.org/10.1371/journal.pone.0174151.

8. Endo, H., Kondo, J., Onuma, K., Ohue, M., and Inoue, M. (2020). Small subset of Wnt-activated cells is an initiator of regrowth in colorectal cancer organoids after irradiation. Cancer Sci. *111*, 4429–4441. https://doi.org/10.1111/cas.14683.

9. Kuo, C.J., and Curtis, C. (2018). Organoids reveal cancer dynamics. Nature *556*, 441–442. https://doi.org/10.1038/d41586-018-03841-x.

10. Dalerba, P., Kalisky, T., Sahoo, D., Rajendran, P.S., Rothenberg, M.E., Leyrat, A.A., Sim, S., Okamoto, J., Johnston, D.M., Qian, D., et al. (2011). Single-cell dissection of transcriptional heterogeneity in human colon tumors. Nat. Biotechnol. *29*, 1120–1127. https://doi.org/10.1038/nbt.2038.

11. Shiokawa, D., Sato, A., Ohata, H., Mutoh, M., Sekine, S., Kato, M., Shibata, T., Nakagama, H., and Okamoto, K. (2017). The induction of selected Wnt target genes by Tcf1 mediates generation of tumorigenic colon stem cells. Cell Rep. *19*, 981–994. https://doi.org/10.1016/j.celrep.2017.04.017.

12. Richichi, C., Brescia, P., Alberizzi, V., Fornasari, L., and Pelicci, G. (2013). Marker-independent method for isolating slow-dividing cancer stem cells in human glioblastoma. Neoplasia *15*, 840–847. https://doi.org/10.1593/neo.13662.

13. Piulats, J.M., Kondo, J., Endo, H., Ono, H., Hagihara, T., Okuyama, H., Nishizawa, Y., Tomita, Y., Ohue, M., Okita, K., et al. (2018). Promotion of malignant phenotype after disruption of the three-dimensional structure of cultured spheroids from colorectal cancer. Oncotarget *9*, 15968–15983. https://doi.org/10.18632/oncotarget.24641.

14. Okuyama, H., Kondo, J., Sato, Y., Endo, H., Nakajima, A., Piulats, J.M., Tomita, Y., Fujiwara, T., Itoh, Y., Mizoguchi, A., et al. (2016). Dynamic change of polarity in primary cultured spheroids of human colorectal adenocarcinoma and its role in metastasis. Am. J. Pathol. *186*, 899–911. https://doi.org/10.1016/j.ajpath.2015.12.011.

15. Onuma, K., Sato, Y., Okuyama, H., Uematsu, H., Homma, K., Ohue, M., Kondo, J., and Inoue, M. (2021). Aberrant activation of Rho/ROCK signaling in impaired polarity switching of colorectal micropapillary carcinoma. J. Pathol. *255*, 84–94. https://doi.org/10.1002/path.5748.

16. Fujii, M., Shimokawa, M., Date, S., Takano, A., Matano, M., Nanki, K., Ohta, Y., Toshimitsu, K., Nakazato, Y., Kawasaki, K., et al. (2016). A colorectal tumor organoid library demonstrates progressive loss of niche factor requirements during tumorigenesis. Cell Stem Cell *18*, 827–838. https://doi.org/10.1016/j.stem.2016.04.003.