Supplementary Information

Microfluidic single-cell culture represents a versatile approach for tumor stem cell expansion and tumor organoid generation

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Supplementary Methods

Method S1 Cell migration assay

The lower chambers of 24-well Transwell plates were pre-filled with 500 μL of DMEM containing 20% FBS. Recovered SDTs and MDTs were digested into single cells using trypsin-EDTA (0.25% EDTA), resuspended in serum-free DMEM, and adjusted to a density of 10,000 cells·mL^{-1}. Then, 200 μL of the cell suspension was added to the upper chamber. After 48 hours of incubation, the cells were stained with crystal violet. Non-migrated cells on the inner side of the upper membrane were gently removed using a cotton swab. The migrated cells on the outer surface were imaged and quantified under an inverted microscope.

Method S2 Cell invasion assay

A 30 μL aliquot of Matrigel was added to each well of a 96-well plate to form a uniform coating. After solidification, recovered SDTs and MDTs were digested into single cells and adjusted to a density of 50,000 cells·mL^{-1}. Then, 20 μL of the cell suspension was mixed with an equal volume of Matrigel (1:1 ratio), and the mixture was added onto the pre-coated Matrigel layer. Following gel solidification, 100 μL of DMEM supplemented with 10% FBS was added. After 48 hours of culture, cell invasion was observed under a microscope.

Method S3 Colony formation assay

Recovered SDTs and MDTs were digested into single cells and resuspended in DMEM supplemented with 10% FBS. The cell density was adjusted to 300 cells·mL^{-1}, and 2 mL of the suspension was seeded into each well of a 6-well plate. After two weeks of continuous culture, the cells were stained with 1% crystal violet solution, and colony

formation was documented under a camera.

Method S4 *In vitro* differentiation assay

Endothelial differentiation Cells were seeded into 6-well plates and cultured in endothelial growth medium (EGM) containing 50 $ng \cdot mL^{-1}$ VEGF to induce differentiation under adherent conditions. After 5-7 days, the cells were digested with trypsin-EDTA (0.25% EDTA), collected, and resuspended in EGM. Subsequently, 1,000 cells per well were seeded into a 96-well plate pre-coated with Matrigel. Tube formation was observed and recorded after 12-24 hours. The cells were then digested into single cells using trypsin-EDTA to dissolve the Matrigel, and the percentage of CD31-positive cells was determined by flow cytometry.

Osteogenic differentiation Osteogenic differentiation was induced using a differentiation kit (Stemcell Technologies) according to the manufacturer's instructions. Briefly, cells resuspended in stem cell medium were seeded into 24-well plates at a density of 10,000-50,000 cells per well. When the cells reached 90% confluence, the medium was replaced with osteogenic differentiation medium containing 2 mM L-glutamine (1 $mL \cdot well^{-1}$). The cells were cultured for 14 days, with medium changes every 3-4 days. Finally, the cells were fixed with 4% paraformaldehyde and stained with Alizarin Red S to assess mineralization.

Cardiomyocyte differentiation Cardiomyocyte differentiation was performed using a differentiation kit (Stemcell Technologies) with modifications. Briefly, 24-well plates were coated with Matrigel and incubated at room temperature for 1 hour, after which the excess solution was removed. Then, 50,000 cells per well were seeded and cultured in stem cell medium for 24-48 hours until 90% confluence was reached. Next, 1 mL of cardiomyocyte differentiation medium A supplemented with 1% Matrigel was added and incubated for 2 days. This was followed by sequential incubation with cardiomyocyte differentiation media B and C for 1 day each. Finally, the cells were maintained in cardiomyocyte maintenance medium for 5-7 days, with daily medium changes. The expression of the cardiomyocyte marker cTn-I was evaluated by immunofluorescence staining.

Method S5 Patient-derived tumor tissue preparation

Viable regions of the primary tumor were selected and dissected into approximately 1 cm^3 pieces, which were immediately immersed in pre-cooled Advanced DMEM/F12 medium containing 5% antibiotic-antimycotic and transported on ice to the laboratory.

The fresh tissue samples were thoroughly washed with ice-cold PBS containing 5% antibiotic-antimycotic until the supernatant became clear. After discarding the supernatant, the tissue was minced into ~1 mm^3 fragments using a scalpel. The minced tissue was then transferred to a 15 mL centrifuge tube, digested with 1 mL of collagenase type IV (2 $mg \cdot mL^{-1}$) at 37 °C for 0.5-1 h with occasional gentle agitation. Subsequently, 10 mL of ice-cold PBS was added, and the mixture was gently pipetted to dissociate the tissue fragments. The suspension was filtered through a 100 μm cell strainer to remove undigested debris. The resulting cell clusters were further dissociated into single cells for subsequent microfluidic culture.

Method S6 RNA sequencing

Total RNA was extracted and purified from the cells. The quality and integrity of the isolated RNA were initially assessed using quantitative real-time PCR (qRT-PCR), which confirmed that all RNA samples were of high quality and suitable for downstream sequencing. Subsequently, medium-throughput sequencing was employed for library preparation. The constructed libraries underwent rigorous quality control (QC) to evaluate integrity, size distribution, and uniformity. To ensure high-quality sequencing data, multiplexed sequencing was performed, and the raw data were processed using an RNA-seq analysis pipeline. Data preprocessing included normalization and quality control to ensure reliability. Differential gene expression analysis was conducted using DESeq2, while transcript quantification and differential expression analysis were performed with Cufflinks, yielding expression profiles for each gene.

Supplementary Figures

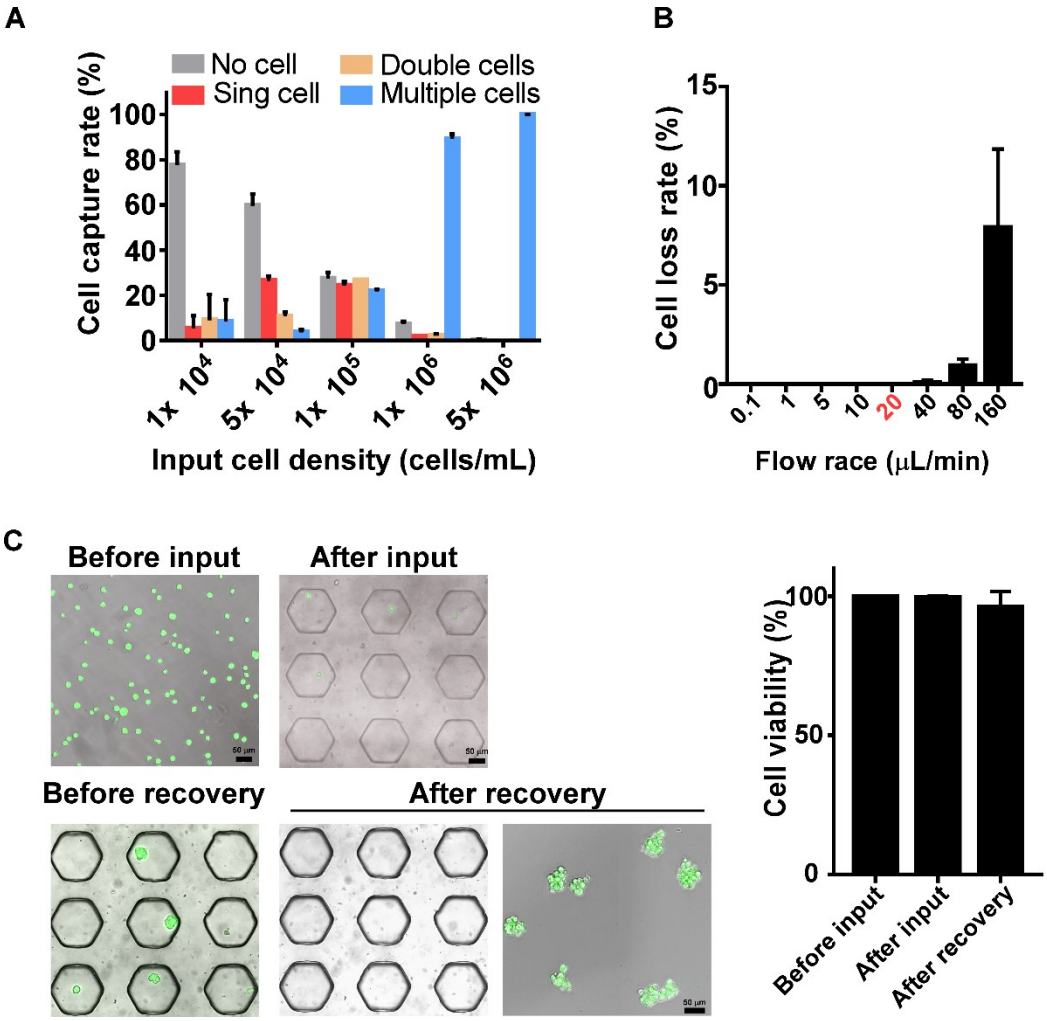


Figure S1. Microfluidic approach validation.A. Cell capturing by microwells with various input cell densities. B. Cell loss determined under different perfusion flow rate. C. Cell viabilities determined before loading, after loading, before retrieval, and after retrieval.

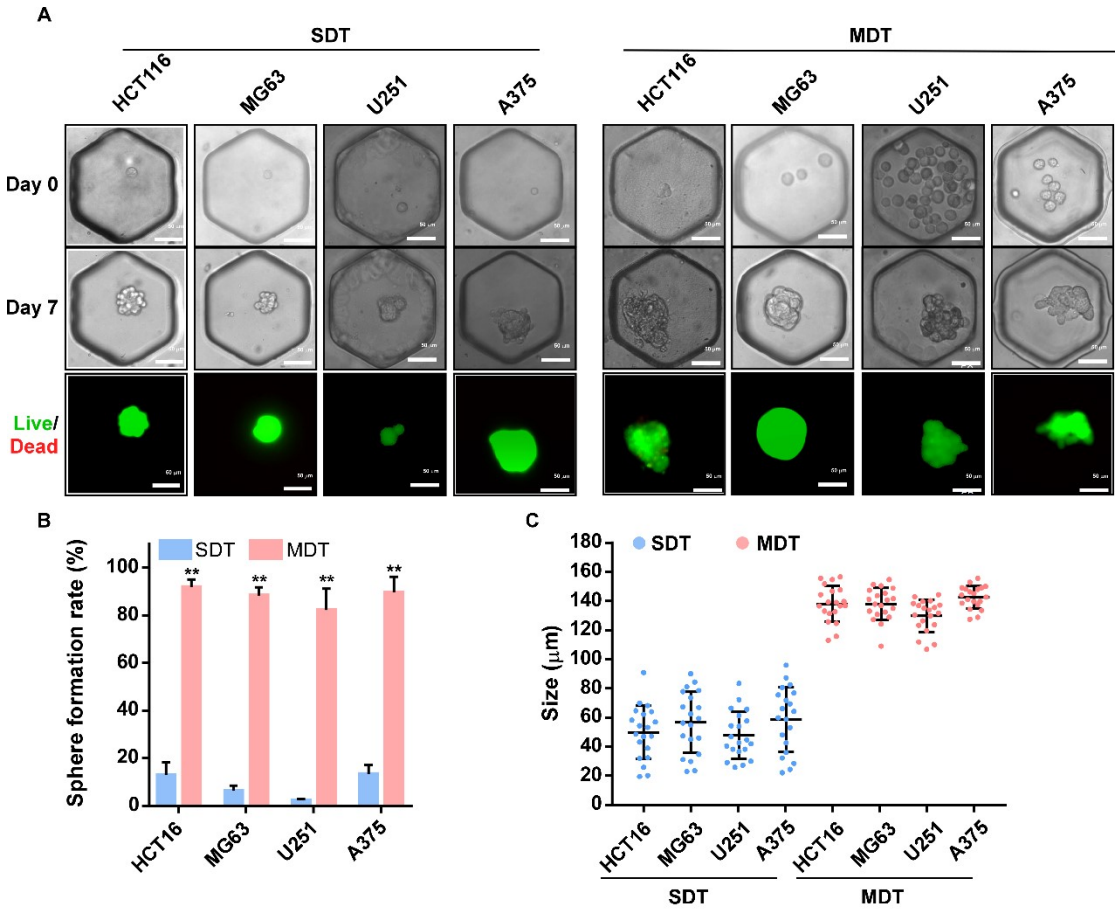


Figure S2. Formation of SDTs with various tumor cell lines.A. Representative images showing the development of SDTs and MDTs over 7 days of culture. B. Sphere formation rates of SDTs and MDTs over 7 days of culture. C. Sizes of SDTs and MDTs determined on day 7.

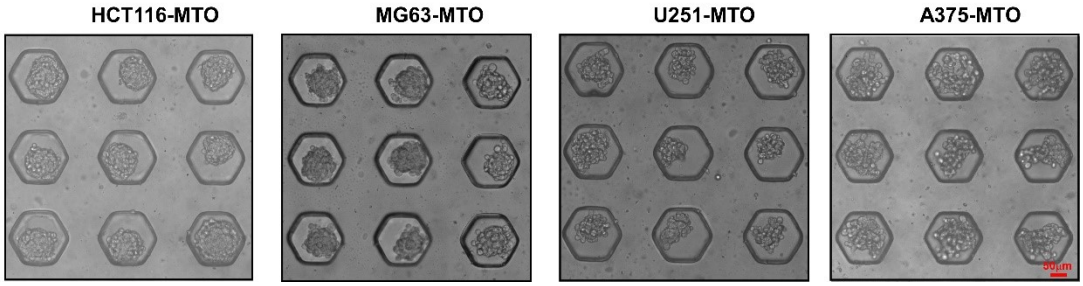


Figure S3. MTOs derived from diverse tumor cell lines following a 7-day differentiation culture period (Scale bar: 50 μm).

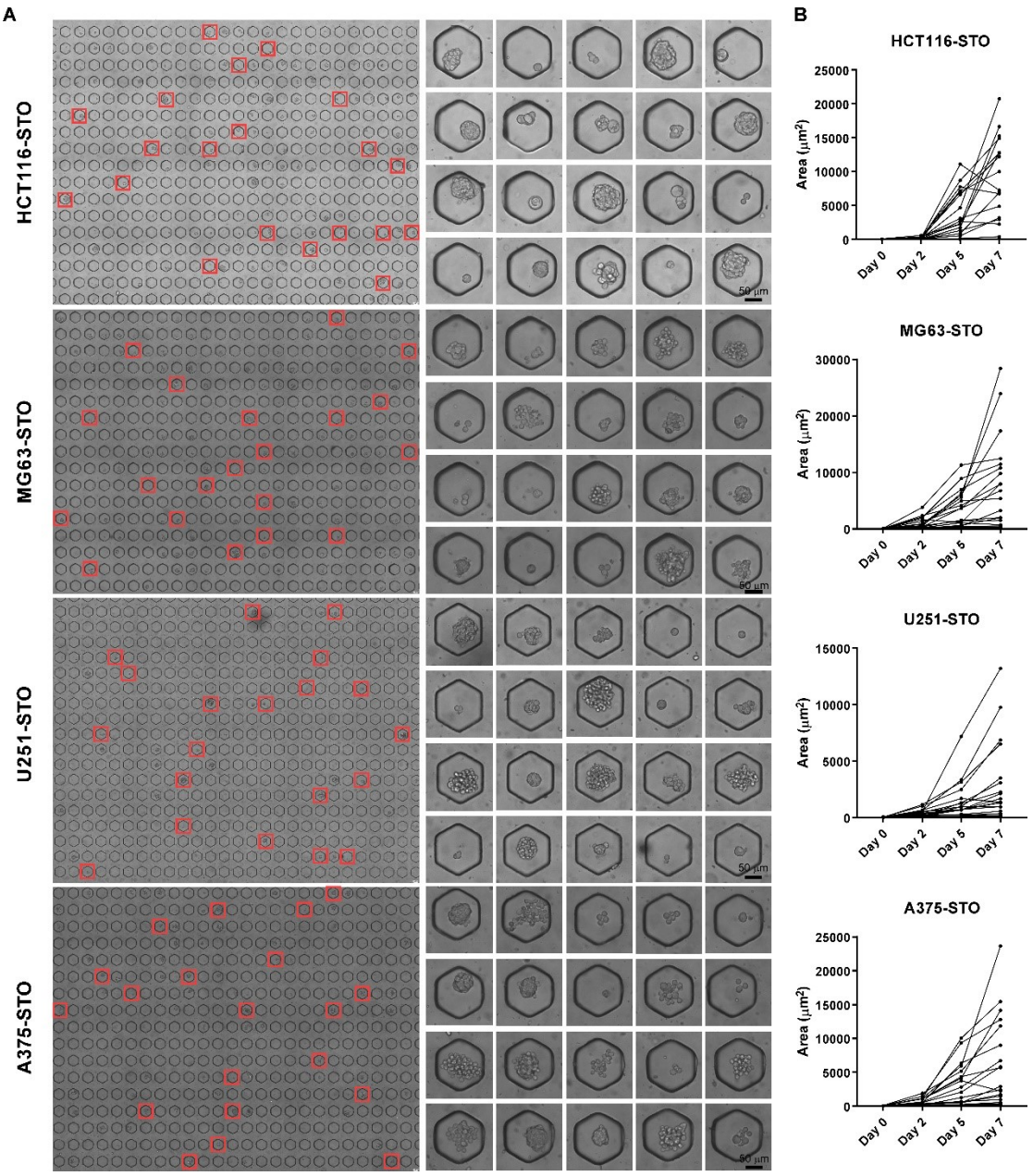


Figure S4. STOs generated from individual SDT cells expanded from various tumor cell lines.A. Representative images of STOs formed after 7 days of differentiation culture from individual SDT cells (Scale bar: 50 μm). B. Dynamic tracking of STO sizes.

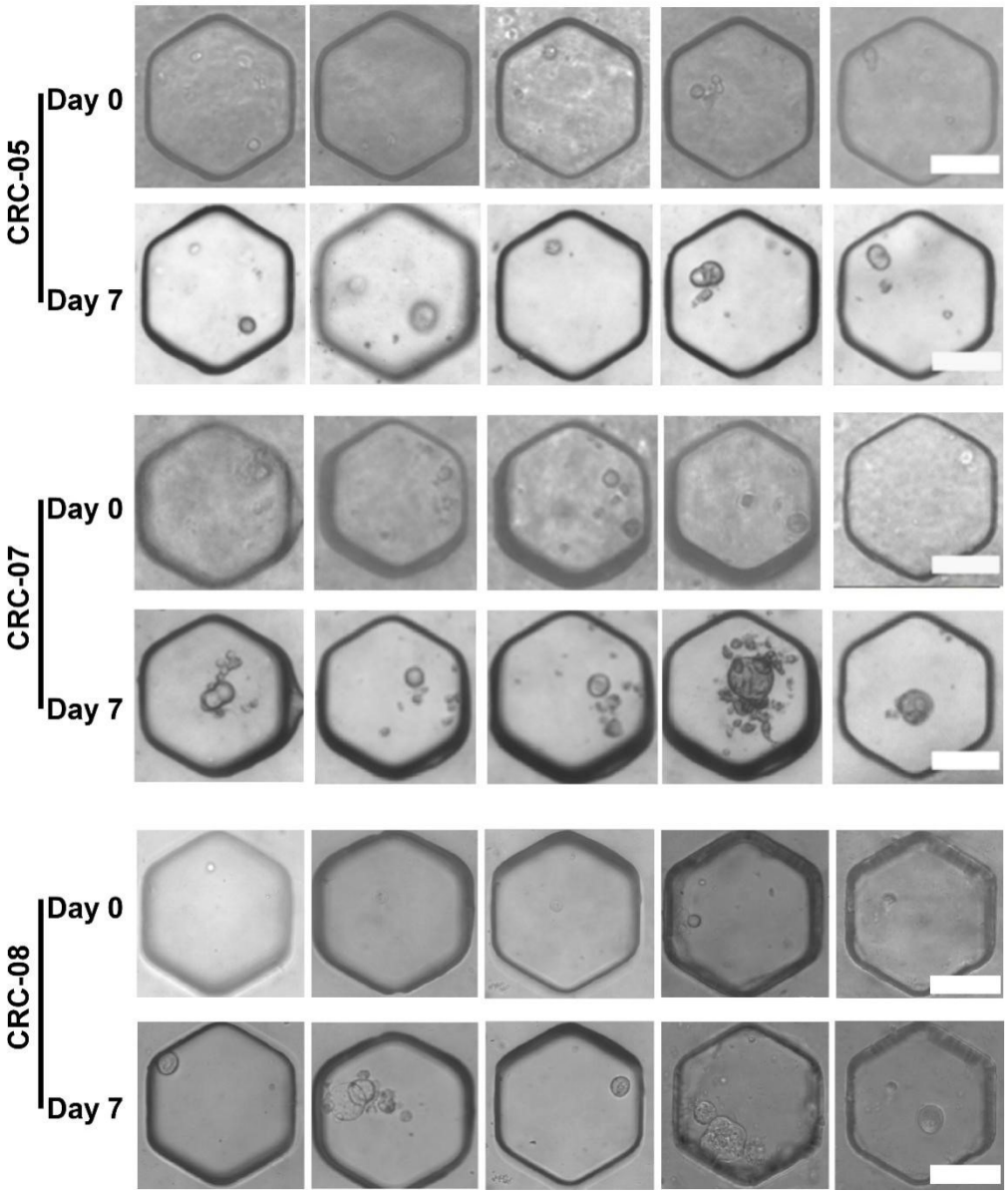


Figure S5. Representative images showing the development of SDTs over 7 days of culture (Scale bar: 50 μm)

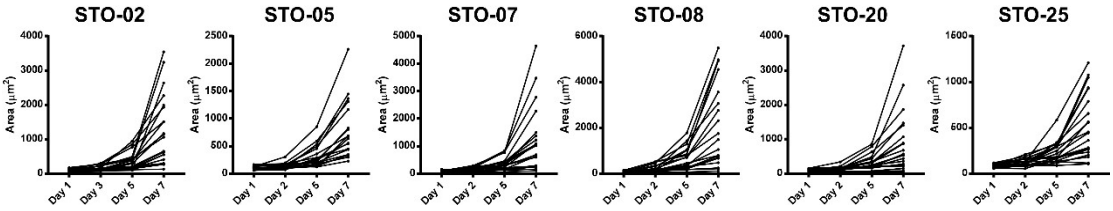


Figure S6. Dynamic tracking of sizes of STOs generated from individual SDT cells expanded from patient-derived colorectal cancer cells.

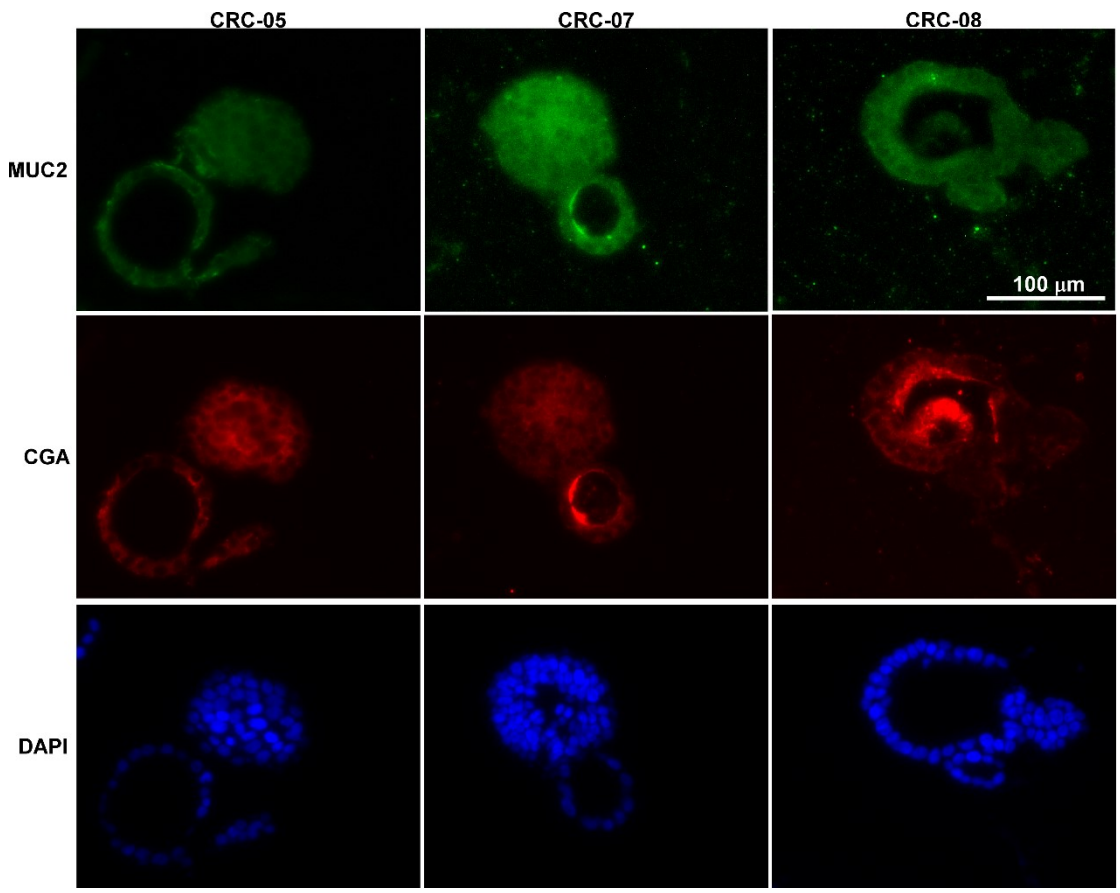
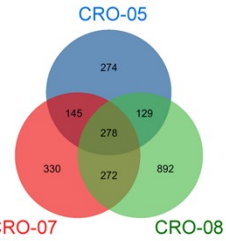
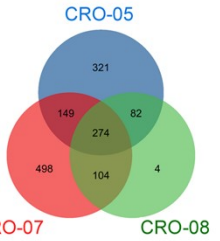


Figure S7. Immunofluorescence staining of MUC2 and CGA in patient-derived colorectal cancer STOs.(Scale bar: 100 μm)

A



B



C

Gene Sets enriched in SDT-05

Description	NES	q-value
HALLMARK_INTERFERON_ALPHA_RESPONSE	3.065617	1.53E-09
HALLMARK_INTERFERON_GAMMA_RESPONSE	2.763678	2.92E-09
HALLMARK_KRAS_SIGNALING_UP	2.318416	0.000111
HALLMARK_HYPOXIA	2.171293	0.000223
HALLMARK_APOPTOSIS	2.194957	0.000386
HALLMARK_INFLAMMATORY_RESPONSE	2.028562	0.001996
HALLMARK_COMPLEMENT	1.964034	0.004337
HALLMARK_COAGULATION	1.925147	0.007749
HALLMARK_FATTY_ACID_METABOLISM	1.854562	0.007749
HALLMARK_TNFA_SIGNALING_VIA_NFKB	1.846691	0.007749
HALLMARK_IL6_JAK_STAT3_SIGNALING	1.89655	0.012765
HALLMARK_XENOBIOTIC_METABOLISM	1.741973	0.016045

D

Gene Sets enriched in STO-05

Description	NES	q-value
HALLMARK_E2F_TARGETS	-2.767991	1.53E-09
HALLMARK_G2M_CHECKPOINT	-2.43529	1.21E-07
HALLMARK_MYC_TARGETS_V2	-2.286878	0.000219
HALLMARK_MITOTIC_SPINDLE	-1.793078	0.003725
HALLMARK_MYC_TARGETS_V1	-1.632745	0.016045

Gene Sets enriched in SDT-07

Description	NES	q-value
HALLMARK_INTERFERON_GAMMA_RESPONSE	2.323847	2.42E-05
HALLMARK_TNFA_SIGNALING_VIA_NFKB	2.216732	5.98E-05
HALLMARK_INTERFERON_ALPHA_RESPONSE	2.172085	0.000745
HALLMARK_KRAS_SIGNALING_UP	2.066096	0.001674
HALLMARK_APOPTOSIS	1.917491	0.005361
HALLMARK_KRAS_SIGNALING_DN	1.801722	0.014976
HALLMARK_P53_PATHWAY	1.721012	0.014976
HALLMARK_ESTROGEN_RESPONSE_LATE	1.600074	0.024061

Gene Sets enriched in STO-07

Description	NES	q-value
HALLMARK_E2F_TARGETS	-1.930904	0.004217
HALLMARK_MYC_TARGETS_V2	-1.862695	0.014976
HALLMARK_G2M_CHECKPOINT	-1.708525	0.014976

Gene Sets enriched in SDT-08

Description	NES	q-value
HALLMARK_APOPTOSIS	2.261599	0.000443
HALLMARK_INTERFERON_GAMMA_RESPONSE	2.224115	0.000443
HALLMARK_KRAS_SIGNALING_UP	2.240986	0.000488
HALLMARK_INTERFERON_ALPHA_RESPONSE	2.207046	0.000501
HALLMARK_TNFA_SIGNALING_VIA_NFKB	2.093652	0.001863
HALLMARK_KRAS_SIGNALING_DN	1.861166	0.009537
HALLMARK_IL6_JAK_STAT3_SIGNALING	1.827015	0.011606
HALLMARK_INFLAMMATORY_RESPONSE	1.808152	0.011812
HALLMARK_TGF_BETA_SIGNALING	1.769944	0.02468

Gene Sets enriched in STO-08

Description	NES	q-value
HALLMARK_E2F_TARGETS	-2.353872	0.000443
HALLMARK_G2M_CHECKPOINT	-2.149193	0.002942
HALLMARK_MYC_TARGETS_V2	-2.182887	0.009537
HALLMARK_APICAL_JUNCTION	-1.802823	0.011812

Figure S8. Transcriptomic profiling of patient-derived colorectal cancer SDTs and STOs. A, B. Venn diagrams displaying highly expressed genes in TSCs (A) and STOs (B) from three colorectal cancer patients. C, D. GSEA showing enriched gene sets in TSCs (C) and STOs (D).

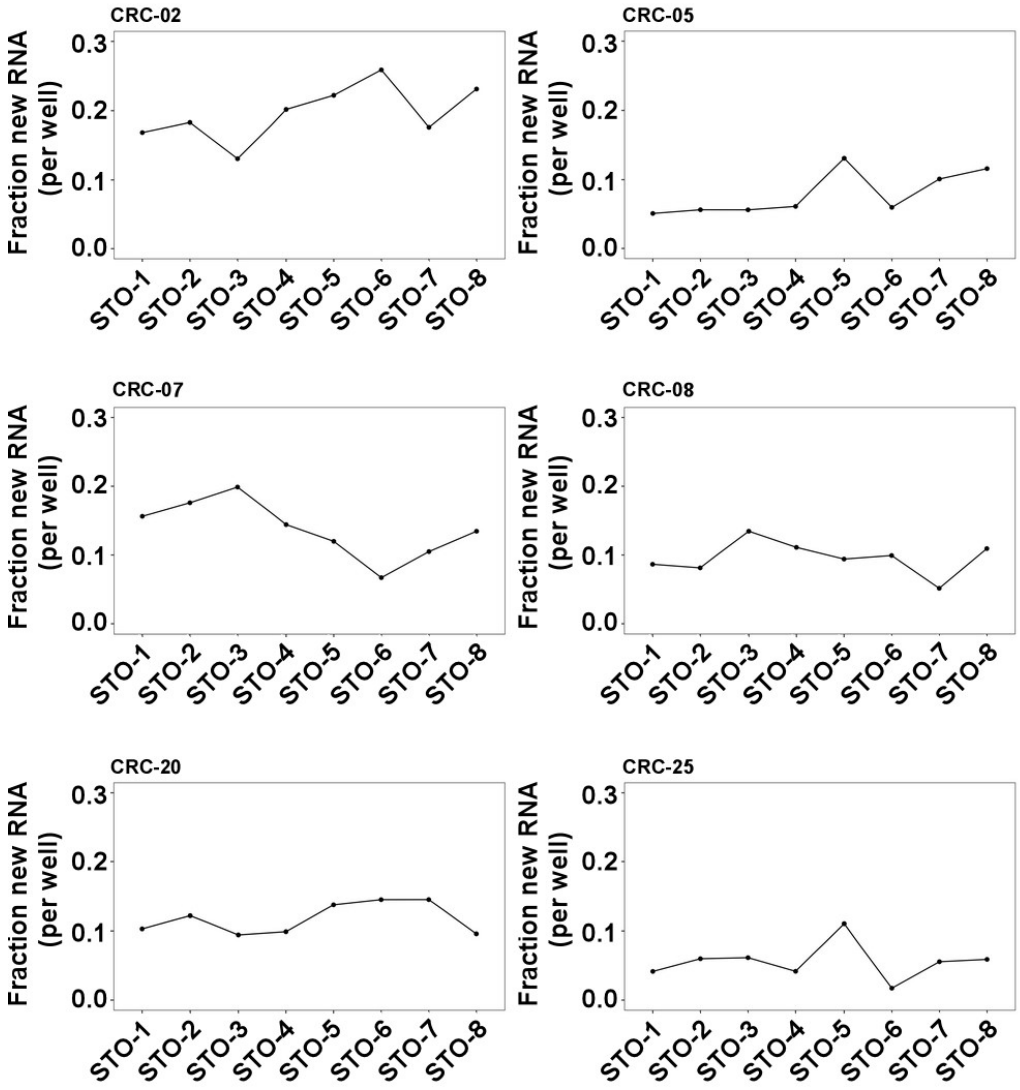


Figure S9. Dynamic single-cell RNA sequencing shows RNAs synthesized within 1 h in individual STOs after a 7-day differentiation culture.

Supplementary Tables

Table S1. Composition of colorectal cancer organoid culture medium

Component	Concentration	Supplier	Catalog Number
advanced-DMEM/F12	-	ThermoFisher	12634010
HEPES	10 mM	Aladdin	H109406
B27	1%	Gibco	17504-044
Nicotimamide	10 mM	Sigma	N0636
human EGF	50 ng·mL^{-1}	Peprotech	AF-100-15
n-Acetyl-L-Cystenine	1.25 mM	Sigma	A9165
SB202109	3 μM	MCE	72632
A8301	500 nM	MCE	HY-10432
R-Spondin	250 ng·mL^{-1}	Peprotech	120-38-1
Gastrin	10 nM	Tocris	3006
GlutaMax	1%	Gibco	35050-061
prostaglandin E2	10 nM	MCE	HY-101952
noggin	100 ng·mL^{-1}	Peprotech	120-10C
wnt-3a	50 ng·mL^{-1}	R&D Systems	5036-WN-500

Table S2. Composition of liver cancer organoid culture medium

Component	Concentration	Supplier	Catalog Number
advanced-DMEM/F12	-	ThermoFisher	12634010
HEPES	10 mM	Aladdin	H109406
N21-MAX Vitamin A Free Media Supplement (50X)	1%	R&D Systems	AR012
Nicotimamide	10 mM	Sigma	N0636
human EGF	50 $ng \cdot mL^{-1}$	Peprotech	AF-100-15
human FGF10	100 $ng \cdot mL^{-1}$	Peprotech	
n-Acetyl-L-Cystenine	1.25 mM	Sigma	A9165
A8301	500 nM	MCE	HY-10432
R-Spondin	500 $ng \cdot mL^{-1}$	Peprotech	120-38-1
GlutaMax	1%	Gibco	35050-061
prostaglandin E2	10 nM	MCE	HY-101952
noggin	100 $ng \cdot mL^{-1}$	Peprotech	120-10C
Forskolin	10 μM	MCE	HY-15371
HGF	25 $ng \cdot mL^{-1}$	Peprotech	100-39
wnt-3a	50 $ng \cdot mL^{-1}$	R&D Systems	5036-WN-500

Table S3. Antibodies used in the immunofluorescence assays

Antibody	Concentration	Supplier	Catalog Number
SOX2	1:200	Thermo Fisher	53-9811-82
CD133	1:200	Abcam	ab19898
LGR5	1:200	Abcam	ab273092
CD44	1:200	Proteintech	60224-1-ig
MUC2	1:200	Servicebio	GB14110
CGA	1:200	Servicebio	GB111316

Table S4. Clinicopathological characteristics of the tumor specimens and corresponding SDT/STO-formation outcomes

Tumor type	Patient	Gender	Age	Histological type	SDT formation rate (%)	SDT size (μm)	STO formation rate (%)	STO size (μm)
colorectal cancer	CRC-01	Male	82	Adenocarcinoma	0	-	0	-
	CRC-02	Female	54	Mucinous adenocarcinoma	5.81±1.97	11.63±4.45	34.23±7.33	37.28±16.14
	CRC-03	Male	38	Adenocarcinoma	0	-	0	-
	CRC-04	Male	73	Adenocarcinoma	0	-	0	-
	CRC-05	Female	66	Adenocarcinoma	11.43±4.22	12.06±6.19	53.18±5.89	29.68±9.57
	CRC-06	Male	53	Adenocarcinoma	0	-	0	-
	CRC-07	Female	78	Adenocarcinoma	3.79±1.15	10.45±2.55	41.17±9.43	36.12±17.49
	CRC-08	Male	85	Adenocarcinoma	3.83±1.69	11.59±6.61	25.02±15.14	44.89±23.72
	CRC-09	Female	77	Adenocarcinoma	0	-	0	-
	CRC-10	Female	52	Adenocarcinoma	0.31±0.45	10.25±6.12	0	-
	CRC-11	Female	69	Adenocarcinoma	0	-	0	-
	CRC-12	Male	34	Adenocarcinoma	0	-	0	-
	CRC-13	Male	81	Adenocarcinoma	0.69±0.73	10.92±3.67	0	-
	CRC-14	Female	53	Adenocarcinoma	1.26±0.44	12.51±4.28	0	-
	CRC-15	Male	59	Adenocarcinoma	0	-	0	-
	CRC-16	Male	72	Adenocarcinoma	0	-	0	-
	CRC-17	Male	66	Adenocarcinoma	0.25±0.31	10.62±3.13	0	-
	CRC-18	Male	67	Adenocarcinoma	0.04±0.03	9.92±6.27	0	-
	CRC-19	Female	71	Adenocarcinoma	0.02±0.02	10.17±5.01	0	-
	CRC-20	Male	55	Mucinous adenocarcinoma	17.77±4.69	12.10±5.90	65.30±6.78	30.08±15.79
	CRC-21	Male	79	Adenocarcinoma	0.07±0.05	10.24±3.06	0	-

	CRC-22	Male	75	Adenocarcinoma	0.12±0.06	9.41±4.17	0	-
	CRC-23	Female	69	Adenocarcinoma	0.02±0.03	10.03±6.52	0	-
	CRC-24	Male	42	Adenocarcinoma	0.05±0.05	11.06±4.15	0	-
	CRC-25	Female	73	Adenocarcinoma	7.47±1.90	10.76±3.41	41.02±8.61	24.64±8.72
	CRC-26	Male	29	Adenocarcinoma	0	-	0	-
	LIHC-01	Female	51	Hepatocellular carcinoma	0	-	0	-
	LIHC-02	Male	46	Hepatocellular carcinoma	0.12±0.13	11.37±5.22	0	-
Liver cancer	LIHC-03	Female	75	Hepatocellular carcinoma	0	-	0	-
	LIHC-04	Male	63	Cholangiocellular carcinoma	0	-	0	-
	LIHC-05	Male	68	Hepatocellular carcinoma	0	-	0	-
Osteosarcoma	BT-01	Male	7	Osteosarcoma	0	-	0	-
	BT-02	Male	10	Ewing's sarcoma	0	-	0	-
Glioma	GO-01	Male	53	Glioma	0	-	0	-
	GO-03	Male	41	Glioma	0	-	0	-