User Guide

3dGRO® Colorectal Organoids

SCC721-SCC743

Pack Size: ≥1500 viable organoids/vial

Store in liquid nitrogen.

FOR RESEARCH USE ONLY

Not for use in diagnostic procedures. Not for Human or Animal Consumption.

Product Overview

Patient derived organoids (PDOs) are novel *in vitro* 3D cell models that have tissue-like functionality and are an alternative to traditional 2D cell lines and PDX animal models. PDOs can be derived from adult patient biopsies or resected tissues containing native LGR5+ stem cell populations and cultured within an ECM-rich substrate using specialized organoid media. Adult tissue derived organoids are thought to have more mature phenotypes than iPSC derived organoids and have been shown to predict patient responses to chemotherapeutics.

Our 3dGRO® colorectal organoid biobank is a collection of tissue-derived human colorectal organoids for disease modeling research. The colorectal organoid biobank contains over 20 highly characterized organoid lines from colorectal adenocarcinoma diseased patients, representing both primary and metastatic tumors, *Kras* driver mutations, and matched normal adjacent tissues.

ID	Age	Sex	Kras Status	Disease Type or Normal	Split Ratio	Media	Catalog Number
CSC.240	83	M	Wild-Type	Adenocarcinoma	1:7	A+S	SCC721
CSC.421	83	F		Adenocarcinoma	1:5	B	SCC723
CSC.449	40	M	Wild-Type	Adenocarcinoma	1:12	A+S	SCC724
CSC.406N	69	F	Wild-Type	Normal (CRC tissue adjacent)	1:3	B	SCC725
CSC.421N	83	F		Normal (CRC tissue adjacent)	1:3	B	SCC726
CSC.449N	40	M	Wild-Type	Normal (CRC tissue adjacent)	1:3	B	SCC727
POP.024.G4	66	M		Adenocarcinoma, metastatic (liver)	1:5	A+S	SCC728
CSC.073.G1	67	M	G12A	Adenocarcinoma, metastatic (lung)	1:12	A+S	SCC729
POP.074.G4	86	M		Adenocarcinoma	1:10	A	SCC730
POP.112.G2	74	M	G12A	Adenocarcinoma	1:3	A	SCC731
POP.161.G1	60	F		Adenocarcinoma, metastatic (lung)	1:3	A+S	SCC732
POP.141.G2	57	M	G12D	Adenocarcinoma, metastatic (liver)	1:7	A	SCC733
POP.174.G1	56	M	G13D	Adenocarcinoma, metastatic (lung)	1:9	A	SCC734
POP.170	61	M		Adenocarcinoma, metastatic (liver)	1:6	A	SCC735
POP.066.G4	46	F		Adenocarcinoma, metastatic (liver)	1:9	A	SCC736

ID	Age	Sex	Kras Status	Disease Type or Normal	Split Ratio	Media	Catalog Number
POP.092.G2	46	F		Adenocarcinoma	1:10	A	SCC737
POP.160.G2	69	M		Adenocarcinoma, metastatic (liver)	1:4	A+S	SCC738
CSC.171C.G1	47	F	G12V	Adenocarcinoma	1:9	A	SCC739
CSC.171L.G1	47	F	G12V	Adenocarcinoma, metastatic (liver)	1:6	A	SCC740
CSC.413	43	M	Wild-Type	Adenocarcinoma	1:10	A	SCC741
CSC.456	68	M	Wild-Type	Adenocarcinoma, metastatic (liver)	1:10	A	SCC742
CSC.463	43	F	Wild-Type	Adenocarcinoma, metastatic (lung)	1:9	A	SCC743

Materials Provided

3dGRO® Colorectal Organoids (SCC721-743): One (1) vial containing ≥1500 viable organoids/vial. Store in liquid nitrogen.

Materials Required (Not provided)

Matrigel® Growth Factor Reduced (GFR) Basement Membrane Matrix (CLS356231), TrypLE™ Express Enzyme (1X) phenol red (Thermo Fisher, 12605-010), cultureware and media components (see Protocols).

Quality Control Testing

- Viability: ≥1500 viable organoids/vial
- Organoid Growth: Pass
- Negative for infectious diseases by a Human Essential CLEAR panel by Charles River Animal Diagnostic Services.
- Verified to be of human origin and negative for mouse, rat, Chinese hamster, Golden Syrian hamster, and non-human primate interspecies contamination, as assessed by a Contamination Clear panel by Charles River Animal Diagnostic Services.
- Mycoplasma Contamination: Negative
- STR Profile: Pass

Storage and Stability

Store in liquid nitrogen. The organoids can be cultured for at least 10 passages after initial thawing without significantly affecting the cell marker expression and functionality.

Protocols

All protocols are performed within a Class II laminar flow biohood and with an aspirator unless otherwise specified. Incubators are humidified and are set to 37 °C and 5% CO_2. PPE should be worn such as gloves, lab coat, and safety glasses. See page 3 for an example protocol for culturing Human Colorectal Organoids.

Preparing 1X Human Colorectal Organoid Media.

Colorectal Organoid Medium A

Component	Volume for 100 mL	Volume for 1 L	Final Concentration
DMEM/F12 Plus Basal Medium	94.4 mL	944 mL	1X
B-27™ Supplement (50X)	2 mL	20 mL	1X
Ala-L-Glutamine Solution (100X), 200 mM	1 mL	10 mL	2 mM
HEPES Solution, 1 M in water	1 mL	10 mL	10 mM
N-Acetyl-L-cysteine, prepared as 500 mM solution in water	250 µL	2.5 mL	1.25 mM
[Leu^{15}]-Gastrin I, reconstituted to 100 µM in PBS/0.1% BSA	10 µL	100 µL	10 nM
Recombinant human Noggin, reconstituted to 100 µg/mL in PBS/0.1% BSA	100 µL	1 mL	100 ng/mL
A-83-01, reconstituted to 1 mM in DMSO	50 µL	500 µL	0.5 µM
Penicillin-Streptomycin, 100x solution	1 mL	10 mL	1X
Human EGF, reconstituted to 100 µg/mL in PBS/0.1% BSA	50 µL	500 µL	50 ng/mL

Colorectal Organoid Medium A + S

Medium comprises all components of Colorectal Organoid Medium A plus SB202190

Component	Volume for 100 mL	Volume for 1 L	Final Concentration
SB202190, reconstituted to 20 mM in DMSO	50 µL	500 µL	10 µM

Colorectal Organoid Medium B

Component	Volume for 100 mL	Volume for 1 L	Final Concentration
DMEM/F12 Plus Basal Medium	44.5 mL	445 mL	1X
B-27TM Supplement (50X)	2 mL	20 mL	1X
Ala-L-Glutamine Solution (100X), 200 mM	1 mL	10 mL	2 mM
HEPES Solution, 1 M in water	1 mL	10 mL	10 mM
N-Acetyl-L-cysteine, prepared as 500 mM solution in water	250 µL	2.5 mL	1.25 mM
[Leu15]-Gastrin I, reconstituted to 100 µM in PBS/0.1% BSA	10 µL	100 µL	10 nM
Recombinant human Noggin, reconstituted to 100 µg/mL in PBS/0.1% BSA	100 µL	1 mL	100 ng/mL
A-83-01, reconstituted to 1 mM in DMSO	50 µL	500 µL	0.5 mM
Penicillin-Streptomycin, 100x solution	1 mL	10 mL	1X
Human EGF, reconstituted to 100 µg/mL in PBS/0.1% BSA	50 µL	500 µL	50 ng/mL
SB202190, reconstituted to 20 mM in DMSO	50 µL	500 µL	10 µM
CHIR99021, reconstituted to 10 mM in DMSO	25 µL	250 µL	2.5 µM
Rspo1 conditioned medium	10 mL	100 mL	10% (v/v)
Wnt3a conditioned medium	40 mL	400 mL	40% (v/v)

1. Combine media components except EGF.
   Note: EGF is not stable long term and thus should be added fresh with each media change.
2. Filter combined components with 0.2 µM filter. Aliquots of Colorectal Organoid Media may be stored at least 6 months at -20 °C. Fresh or thawed media is stable for at least 2 weeks at 4 °C.

Thawing Organoids

1. Prepare sufficient growth-factor-reduced Matrigel® Matrix (CLS356231; diluted to 8 mg/mL) for eight 25 µL domes. Set aside on ice.
2. Thaw a vial of human colorectal organoids by submerging 3/5 of vial into a 37 °C water bath until only a sliver of ice remains. Spray the outside of the vial with 70% ethanol or isopropanol.
3. In a sterile tissue culture hood, quickly and gently transfer the vial contents into a 15 mL conical tube containing 4 mL of cold 1X Human Colorectal Organoid Media and 10 µM Y-27632 (ROCK inhibitor).
4. Centrifuge the 15 mL conical tube for 5 minutes at 500 x *g*.
5. Carefully aspirate the media with a pipet, being cautious to avoid disturbing the organoid pellet. Organoids should appear as an opaque layer at the bottom of the tube. If a clear layer of residual Matrigel® Matrix is visible overlaying the organoid pellet, remove as much as possible without disturbing the organoid pellet.

   Optional: Resuspend pellet and residual Matrigel® Matrix in 1 mL Cell Recovery Solution (Corning, 354253) and incubate on ice for 30 minutes. Swirl the conical tube twice during the incubation. Centrifuge for 5 minutes at 500 x *g*. Aspirate Cell Recovery Solution, being careful not to disturb the organoid pellet.
6. Transfer 200 µL of thawed Matrigel® Matrix to the organoid pellet. Quickly and gently resuspend the organoid pellet by pipetting up and down 5-10 times with a P-200 micropipette set to 180 µL, being careful to avoid causing air bubbles.
7. Dispense 25 µL domes into one well of a 6-well plate or one dome per well of a 24-well plate. Work quickly and minimize formation of air bubbles during pipetting.
8. Allow the domes to incubate for 10 minutes in a 37 °C humidified incubator with 5% CO_2.
9. Transfer the plate containing the organoid domes to a sterile tissue culture hood. Gently add 3 mL of 1X Human Colorectal Organoid Medium containing 10 µM ROCK inhibitor to the well of the 6-well plate or 1 mL of medium to each well of the 24-well plate.

   Note: ROCK inhibitor is added only at the time of thawing and on the day of passaging. Subsequent media changes do not require ROCKi supplementation.
10. Incubate plate in a 37 °C humidified incubator with 5% CO_2.
11. After 24 hours, check for recovery by observation by bright field microscope. Live organoids should have well-defined edges.
12. Exchange media every other day with 1X Human Colorectal Organoid Media (No ROCKi added). Passage 4-5 days after thaw. Passage ratio should be kept at a conservative 1:1.25 to 1:2 for the first 1-2 passages. Once organoids have been established, they may be passaged using the optimal split ratio as specified in the table.

Passaging Organoids

Once established, human colorectal organoids should be passaged every 7 days, except for SCC723 CSC.421 which should be passaged every 9-10 days. Passage of organoids after 7 days is not recommended unless specified for a certain organoid line.

1. Prepare enough GFR Matrigel® Matrix for a volume of 25 µL per dome. To maintain approximate equal density, split colorectal organoids according to the ratio recommended in the table for each individual line.
2. Prepare ice-cold 1X PBS + 10 µM ROCK inhibitor.
3. Aspirate medium from wells.
4. Add 1 mL TrypLE™ Express + 10 µM ROCK inhibitor to each well of 24-well plate or 3 mL to each well of 6-well plate.
5. Detach Matrigel® domes in each well using a P-1000 pipette. Gently break up the domes several times with P-1000 pipette and return plate to incubator.
6. Incubate plate for 10 minutes. After 10 minutes resuspend several times with P-1000 pipette and checking dissociation with a bright field microscope. Incubate plate for another 10 minutes, repeating resuspension and breaking up any large clumps of cells.

   Note: Do not allow incubation time to exceed 20 minutes as this may lower the cell viability of the organoids.
7. Once suspension consists entirely of single cells and small clumps of 2-3 cells, transfer organoid cell suspension to a 15-mL conical tube.
8. To wash, add equal volume of cold 1X PBS + 10 µM ROCK inhibitor to well and transfer to conical tube. Centrifuge the 15 mL conical tube for 5 minutes at 500 x g.

9. Carefully aspirate the media, being careful not to disturb the organoid cell pellet. If a clear layer of Matrigel® is present overlaying the organoid cell pellet, remove as much Matrigel® Matrix as possible. If the Matrigel® layer is not clear but appears opaque, do not attempt to remove. An opaque Matrigel® Matrix layer indicates that the smaller sized organoids that are difficult to pellet may be present in the layer.
10. Transfer the appropriate volume of thawed GFR Matrigel® Matrix to the organoid cell pellet with a P-1000 micropipette. Quickly and gently resuspend the organoid cell pellet about 5-10 times with a P-1000 tip set to 20 µL below the volume transferred. Avoid causing formation of air bubbles.
11. Dispense 25 µL domes into wells of the culture plate. After each 1 minute of dispensing, place suspension on ice for 5 minutes to cool and prevent solidification of the Matrigel® Matrix.
12. Allow the domes to incubate for 10 minutes in a 37 °C humidified incubator with 5% CO_2.
13. Remove plate from incubator and add 1X Human Colorectal Organoid Medium containing 10 µM ROCK inhibitor. Exchange media every 2 days with fresh media without ROCK inhibitor.

    Note: ROCK inhibitor is added only at the time of thawing and on the day of passaging. Subsequent media changes do not require ROCKi supplementation.

Cryopreservation of Organoids

Important Notes Before Starting:

- We recommend freezing 2 x 25 domes per cryovial. Each dome should contain at least 700-1000 organoids.
- It is important **not** to dissociate the organoids prior to freezing (either by enzymatic dissociation or by mechanical methods). Do not use enzymatic dissociation to detach the organoids during banking. The Matrigel® Matrix is minimally disrupted with a pipette tip only enough so that the pieces can be suspended in freeze medium. Organoids should be frozen a couple of days prior to the normal day of passage. For example, if you normally passage at day 7, then freezing should occur at Day 5.
- Patient-derived organoids may be frozen in 1X Human Colorectal Organoid Media + 10% DMSO or in 3dGRO® Organoid Freeze Medium (SCM301).
- Prepare a Mr. Frosty® container and have it ready for storing organoids for freezing down at -80 °C.

1. Chill sufficient 1X PBS + 10 µM ROCK inhibitor on ice for at least 30 minutes before starting.
2. Remove plate or dish containing organoid domes from 37 °C incubator and place in a tissue culture hood. Aspirate media.
3. Add sufficient volume of cold PBS + 10 µM ROCK inhibitor to the culture dish containing the organoids.
4. Gently disrupt the organoid domes with a P-1000 micropipette set to 900 µL using chilled 1X PBS + 10 µM ROCK inhibitor.

   Important: Keep organoids as intact as possible. Use the expulsion of the cold PBS/ROCKi liquid from the P-1000 pipette to dislodge the Matrigel® domes. Transfer organoid suspension to a new 15 mL conical tube.
5. Add additional cold 1X PBS + 10 µM ROCK inhibitor to wash the plate or dish to collect residual organoids and transfer the organoid suspension to the 15 mL conical tube.
6. Centrifuge the 15-mL conical tube containing the organoid suspension for 5 minutes at 500 x *g*.
7. Carefully aspirate the media, avoiding disruption of the organoid pellet. If a clear layer of Matrigel® Matrix is present overlaying the organoid cell pellet, remove as much Matrigel® Matrix as possible. If the Matrigel® layer is not clear but appears opaque, do not attempt to remove. An opaque Matrigel® layer indicates that smaller sized organoids that are difficult to pellet may be present in the layer. The residual Matrigel® Matrix should not affect cryopreservation.
8. Resuspend the organoid pellet with freeze medium comprised of 1X Human Colorectal Organoid Medium containing 10% DMSO at 2 domes per mL.
9. Quickly transfer 1 mL into a cryovial and repeat until all suspension are transferred into cryovials.
10. Quickly transfer cryovials to a Mr. Frosty® container and place in a -80 °C freezer for 24 hours.
11. After 24 hours, transfer the cryovials to liquid nitrogen (-135 °C) for long-term storage.

Whole Mount Immunocytochemistry of Organoids

Important Notes Before Starting:

The following protocol is meant to serve as a guidance for first time users and is based on organoids cultured in 24-well plates. The protocol may be modified and adapted once users are more familiar with the process.

- We recommend using a pair of scissors that have been sterilized with 70% ethanol or isopropanol to cut the ends of P-1000 tips to enlarge the opening. Modified P-1000 tips are used to transfer fixed organoids without shearing them. Do not use serological pipettes as they are too bulky to handle small volumes and organoids may stick to the side of the pipettes.
- During PBS washes, gravity is used to collect organoids. Do not use centrifugation as the centrifugal force will result in mis-shaped organoids.
- The 4% paraformaldehyde performs the dual function of fixing the organoids and to help partially dissolve the Matrigel® Matrix and release the organoids. It is important to remove as much of the Matrigel® Matrix as possible from the organoids. Matrigel® Matrix may result in increased background autofluorescence. The more confluent the organoids are inside the domes at the time of fixing, the more readily the Matrigel® Matrix will dissolve.

1. Prepare a 4% paraformaldehyde (PFA) solution by diluting an 8% PFA Solution (Electron Microscopy Sciences 157-8-100) 1:1 with 1X PBS.
2. Prepare modified P-1000 and P-200 pipette tips by cutting the ends with a sterilized scissor.
3. Aspirate the medium from each well containing an organoid dome. Wash each well twice with 1 mL 1X PBS. Aspirate between PBS washes.
4. Add 1 mL of the 4% PFA solution to each well. Incubate 45-60 minutes at room temperature on a gently rocking or shaking platform. The shaker/rocker will help expedite detaching the Matrigel® domes and the release of the organoids from the Matrigel® Matrix.

   Note: GFR Matrigel® domes will partially dissolve when fixed in PFA. At the end of the incubation period, you will notice that many (but not all) the domes are dislodged and that some of the organoids (but not all) will have been released from the domes.
5. Using the modified P-1000 pipette tips, collect any released organoids along with the fixative solution and transfer the contents to a 50 mL conical tube. Allow the organoids to settle to the bottom of the conical tube by gravity (~10-15 minutes). DO NOT CENTRIFUGE.
6. In the meantime, add 1 mL 1X PBS per well to the 24-well plate containing the organoid domes. Incubate 10-15 minutes at room temperature. This is done to dilute the PFA in the dome.
7. Carefully aspirate the fixative from the conical tube containing the released organoids (from step 5) and leave a small amount of liquid behind. This will ensure that the organoid pellet will not be aspirated off.
8. Using modified P-1000 pipette tips, collect any released organoids along with the PBS solution from each well (from step 6) and transfer the contents to the 50 mL conical tube. Allow the organoids to settle to the bottom of the conical tube by gravity (~10-15 minutes).
9. Repeat steps 5-8 two more times.
10. Add 0.8 mL of 1X PBS into each well that contains residual organoid domes.
11. Carefully aspirate the supernatant from the conical tube containing released organoids. Leave a small amount of liquid behind. This will ensure that the organoid pellet will not be aspirated off.
12. Add 4.8 mL 1X PBS to the organoid pellet. Swirl the conical tube to resuspend the organoid pellet. Using a modified P-1000 tip, transfer 200 µL of the organoid suspension into each well containing the 0.8 mL volume of residual organoid domes (from step 10).

    Note: Some organoids may stick to the modified P-1000 tip.
13. If staining will not be performed immediately, seal the 24-well plate containing fixed organoids with Parafilm® and store in the refrigerator at 2-8 °C for up to 1 month.
14. When ready to perform ICC, transfer the 24-well plate containing the fixed organoids to a dissecting microscope.
15. Using modified P-200 tips (from step 2), pipette 1-4 organoids into each well of an 8-well chamber slide. Remove any residual PBS using an unmodified P-200 pipette tip. Avoid accidentally pipetting up the organoids and shearing them through the P-200 tip.

16. Add 0.4 mL Blocking Buffer (5% horse serum plus 0.5% Triton® X-100 in 1X PBS) to each well of an 8-well chamber slide containing the fixed organoids. Block at 2-8 °C overnight or at room temperature for 2-4 hours.

    Note: Use the serum from the same species as the host secondary antibody.
17. Using an unmodified P-200 pipet, remove the blocking buffer while tilting the chamber slide. Avoid pipetting the organoids through the P-200 tip.
18. Prepare primary antibodies or directly conjugated antibodies (300-500 µL) in Blocking Buffer.
19. Add primary antibodies. Incubate overnight at 2-8 °C on a gently shaking or rotating platform.
20. Next day, wash 3X with 1X PBS for 10-15 minutes each on the shaking/rotating platform.

    Note: Do not use centrifugation. Remove the PBS with a P-200 pipet after each wash while tilting the chamber slide.
21. Prepare secondary antibodies (300-500 mL) in Blocking Buffer.
22. Add secondary antibodies. Incubate overnight at 2-8 °C on a gently shaking or rotating platform.
23. Next day, wash with 1X PBS for 10-15 minutes on the shaking/rotating platform.

    Note: Do not use centrifugation. Remove the PBS with a P-200 pipet after each wash while tilting the chamber slide.
24. Counterstain with DAPI (5 µg/mL in 1X PBS) for 15-20 minutes.
25. Wash 3X with 1X PBS for 10-15 minutes each on the shaking/rotating platform.

    Note: Do not use centrifugation. Remove the PBS with a P-200 pipet after each wash while tilting the chamber slide.
26. Add 300-400 µL of 1X PBS into each well. Samples are now ready to be imaged on a confocal microscope.

Morphology and Characterization

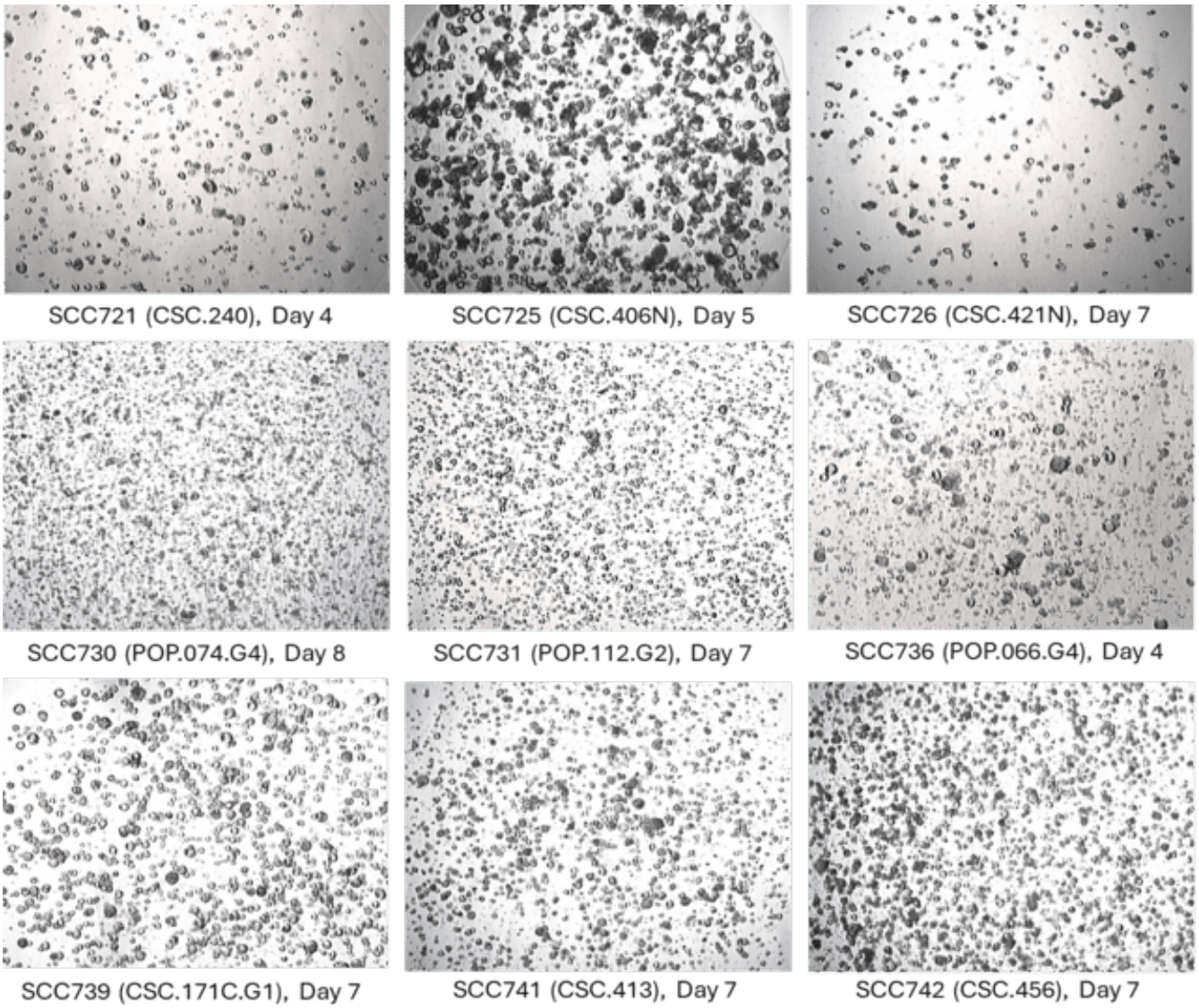

Figure 1. Various 3dGRO® Human Colorectal Cancer Organoids grown in Matrigel® domes, 2X magnification brightfield images.

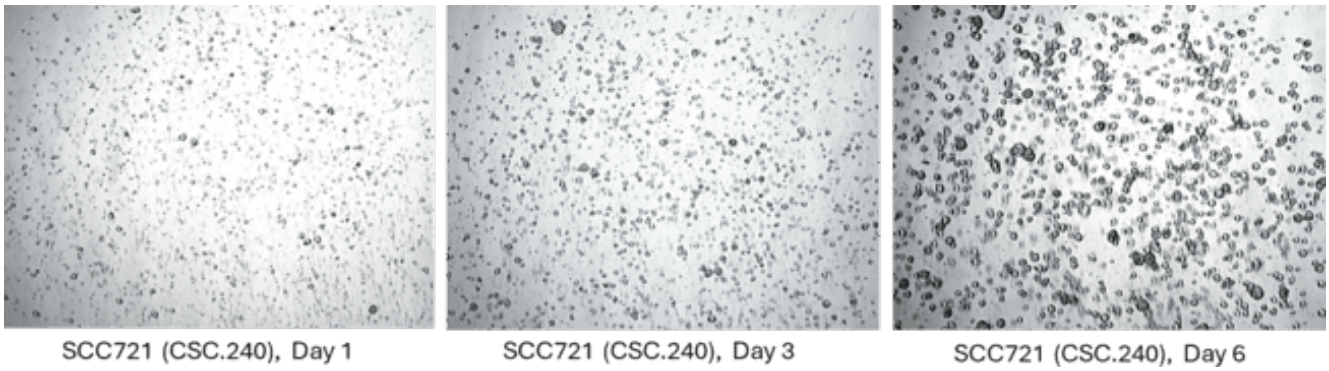

Figure 2. Time course of CRC organoid growth over a 6-day time period shows positive cell proliferation (SCC721).

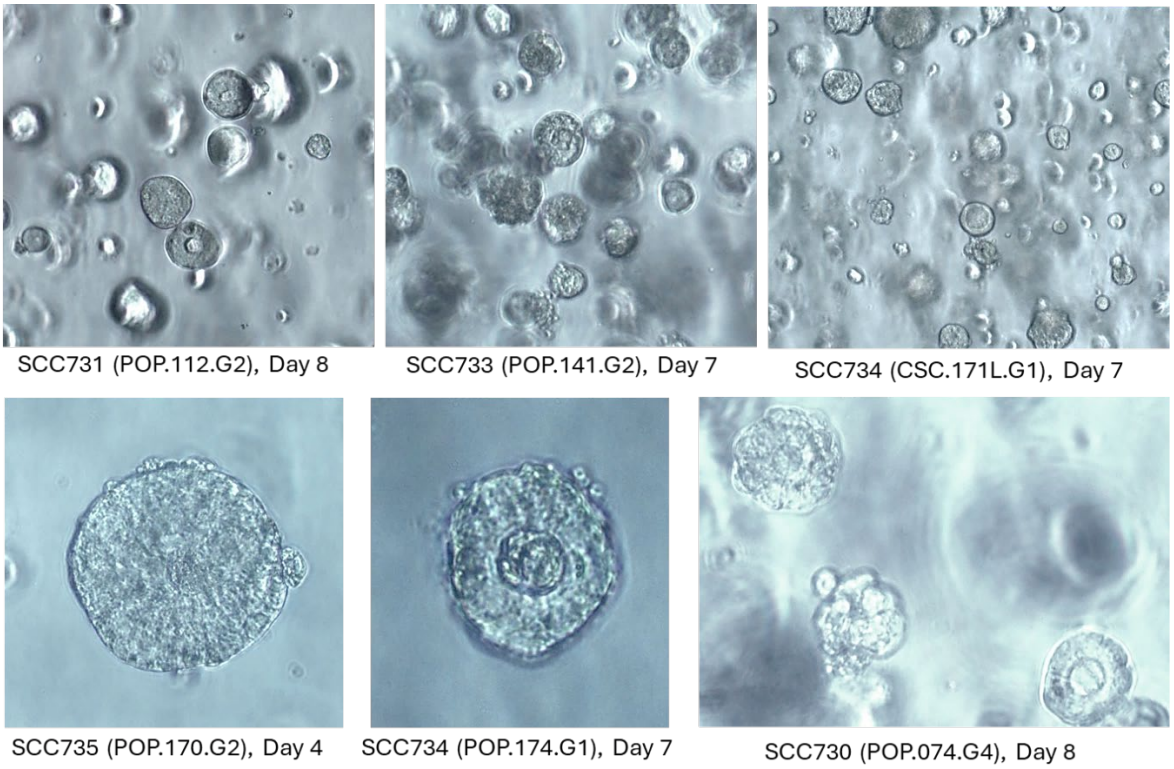

Figure 3. Various 3dGRO® Human Colorectal Cancer Organoids grown in Matrigel® domes, 10X (top panel) and 20X (bottom panel) magnification brightfield images.

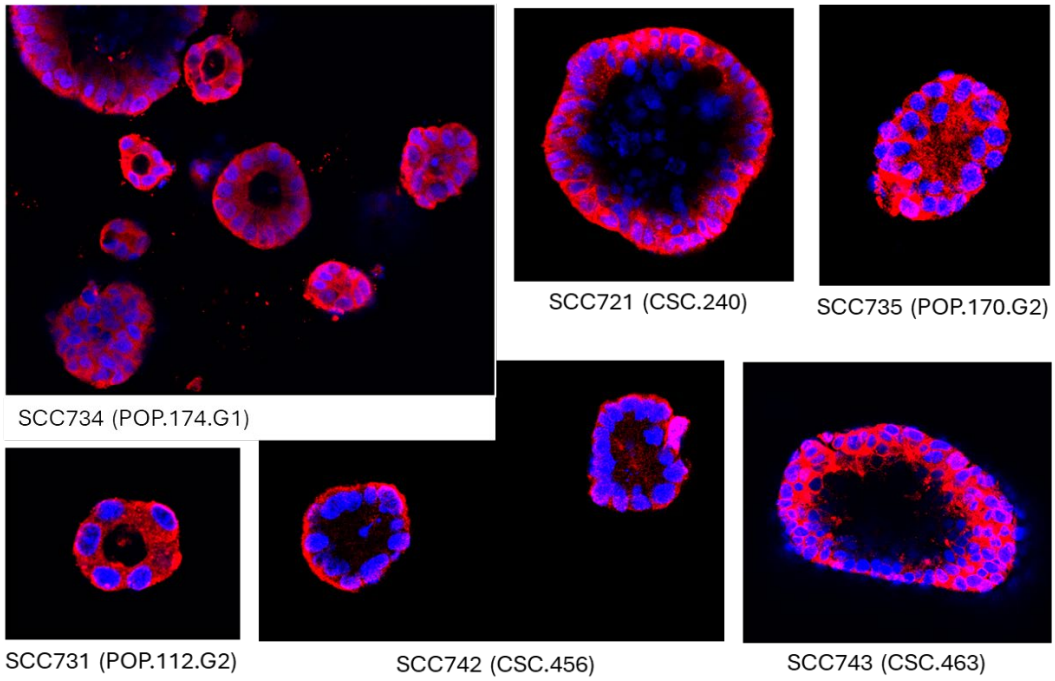

Figure 4. Immunocytochemical characterization of human colorectal cancer organoids. Colorectal adenocarcinoma organoids express the cancer marker carcinoembryonic cell adhesion molecule 5 (CEACAM5). Red, CEACAM5 (Abcam EPCEAR7); blue, DAPI. 20x magnification confocal images.

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