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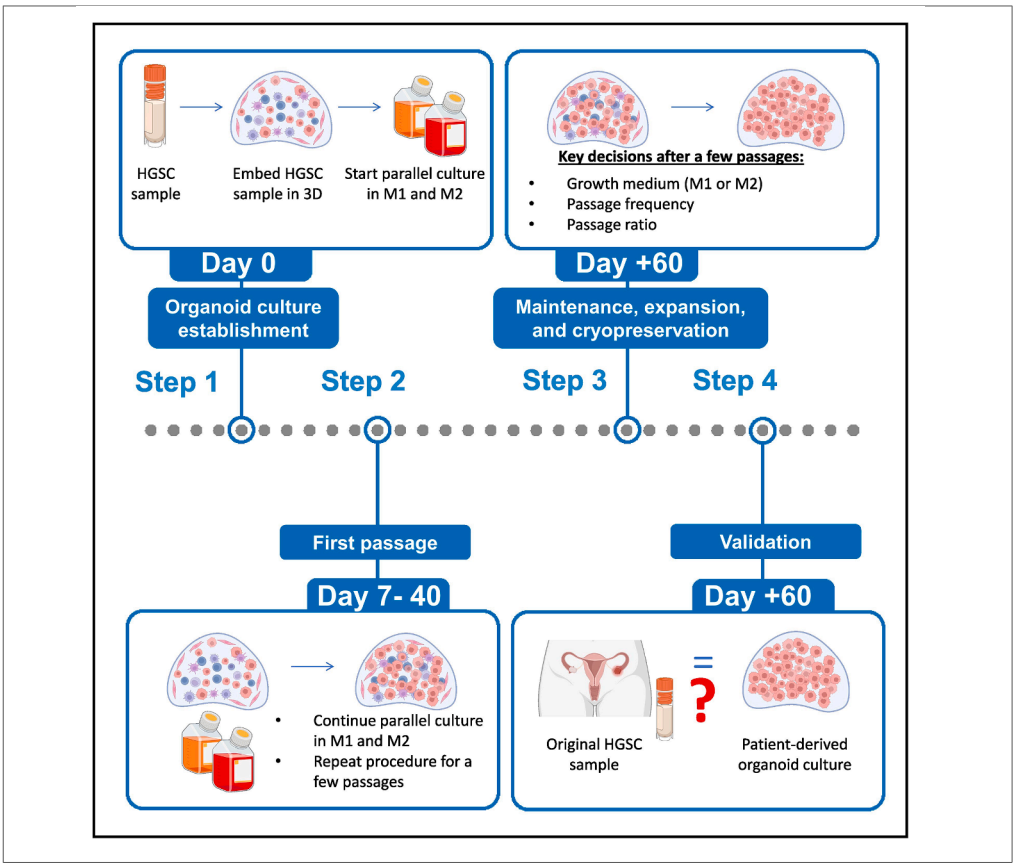
Protocol for generating and culturing high-grade serous ovarian carcinoma organoids from fresh or cryopreserved patient samples



Stable long-term high-grade serous ovarian carcinoma (HGSC) organoids can be difficult to develop due to limited primary sample growth in 3D conditions. Here, we present a detailed protocol for the development of stable HGSC organoids, including all steps from seeding primary cells through culture monitoring, passaging, and expansion to viable cryopreservation and resuscitation. We provide guidance on initial passaging, passaging ratios, and criteria for successful culture establishment. Moreover, we include a troubleshooting section tackling recurrent problems in HGSC organoid development.

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

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Highlights

Detailed protocol for establishing robust long-term HGSC organoids

Guidance on passaging, expansion, cryopreservation, and resuscitation

Real examples guide culture establishment and quality assessment

Extensive troubleshooting for common HGSC organoid culture issues

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Protocol for generating and culturing high-grade serous ovarian carcinoma organoids from fresh or cryopreserved patient samples

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SUMMARY

Stable long-term high-grade serous ovarian carcinoma (HGSC) organoids can be difficult to develop due to limited primary sample growth in 3D conditions. Here, we present a detailed protocol for the development of stable HGSC organoids, including all steps from seeding primary cells through culture monitoring, passaging, and expansion to viable cryopreservation and resuscitation. We provide guidance on initial passaging, passaging ratios, and criteria for successful culture establishment. Moreover, we include a troubleshooting section tackling recurrent problems in HGSC organoid development.

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

BEFORE YOU BEGIN

High-grade serous ovarian carcinoma (HGSC) organoids have proven difficult to develop, especially from cryopreserved patient samples. Common problems include a lack or limited sample growth in 3D, presence, or overgrowth of stromal and immune cells, or a lack of proliferation after an initial period of cell expansion, resulting in unstable cellular growth rates and inability to establish stable long-term cultures.[1] The use of optimized medium formulations and culture protocols is essential to minimize some of these difficulties. In the past years, multiple approaches have been developed.[2,3]

Here, we provide detailed protocols for developing organoid cultures from fresh or pre-processed cryopreserved HGSC samples using the "M1/M2 method". In this method, the primary HGSC sample is cultured in parallel in two optimized medium formulations – Medium 1 (M1), which is lower in growth stimulants, and Medium 2 (M2), which is richer in growth stimulants, until sustained growth of cancer cells as organoids occurs in one of the media. The simultaneous use of two media formulations for the same patient sample aims to increase the likelihood of developing a successful organoid culture. Other protocols to generate long-term HGSC organoids rely on a single medium formulation[4,5] and only culture patient samples with multiple formulations in the short-term culture.[6,7] Additionally, our protocol represents an improvement for the establishment of long-term organoids from cryopreserved samples, demonstrating a higher success rate than other protocols (53% vs 23–38%).[1]

Assessing whether M1 or M2 will promote the growth of the organoids is an empirical process that can last from a few weeks to several months, as it requires consistent passages and diligent culture monitoring. In our experience, developing a stable long-term organoid culture from an HGSC sample takes, on average, around 3 months.[1]

This section covers the preparation of culture media and the extracellular matrix, which are required before starting an organoid culture. The step-by-step protocols focus on the establishment, development, maintenance, and validation of organoids. Next, the expected outcomes section provides detailed guidelines about critical steps in the organoid development process, including when to passage organoids, and how to determine the passage ratio. Finally, the troubleshooting section addresses frequent problems in organoid development, including sample-related issues as well as other technical aspects. We remind the readers that all procedures with organoids must be conducted in sterile conditions.

Innovation

Compared to other publications with HGSC organoids focusing on downstream applications, the novelty of this protocol comes from providing detailed guidelines for organoids establishment from cryopreserved patient samples, from embedding in 3D to long-term expansion, supported by real examples. The use of two separate, highly optimized, tissue-type-specific media for culturing each HGSC sample maximizes the likelihood of successful establishment of long-term organoid cultures.[1] This protocol describes organoid generation in great technical detail, including examples of both successful and failed cultures, showing different stages of the establishment process and including an extended troubleshooting section.

Institutional permissions

All organoid cultures included in the protocol were derived from human patient samples in accordance with local ethical and regulatory standards.

The patients whose samples were used to develop organoids were treated at the Department of Gynecology and Obstetrics at Turku University Hospital, Finland. Patients signed an informed consent for the use of the samples in research. In some cases, the consent also included permission for long-term biobanking. Fresh samples were obtained during standard-of-care procedures performed for clinical treatment, including laparoscopic biopsies, tumor debulking surgeries, or ascites paracenteses and pleural puncture. Samples were processed on the day of collection to obtain a cell suspension, which was then cryopreserved. The collection and storage of the samples were approved by the Ethics Committee of the Hospital District of Southwest Finland (ETMK) from Turku (Appr. No.: ETMK 53/180/2009 § 238).

We remind the readers that working with human samples requires specific informed consent from the patients and approval from ethical committees and/or other relevant institutions.

Preparation of organoid culture media (M1 and M2)

Timing: 1 h and 20 min (including thawing of reagents)

Note: In the "M1/M2 method", two optimized culture medium formulations, M1 and M2, are used. As M2 consists of M1 supplemented with 4 additional ingredients, M1 is always prepared first. The basis for the M1 medium is the commercial Advanced DMEM/F12 medium. The volume of M1 and M2 to be prepared must be adjusted to the user's needs.

⚠ CRITICAL: It is essential that all reagents for medium preparation are in an optimal condition. Therefore, it is crucial to avoid using reagents that have undergone excessive freezing

and thawing cycles. We recommend adjusting the volumes of the aliquoted reagent in advance, so that each aliquot is used only once (see aliquot preparation for exceptions).

⚠ CRITICAL: Always use freshly prepared culture medium when working with HGSC organoids (not older than 10 days).

1. Determine the volumes of culture medium required for the next 7–10 days.
2. Thaw the reagents for media preparation at 18–24 °C. See reagents for preparation of medium.
3. In the meantime, weigh the N-acetyl-L-cysteine and nicotinamide powders.
4. Prepare M1 following the order and volumes described in the medium 1 formulation table.
5. After adding all the components, mix vigorously to dissolve and homogenize all medium components.
6. Aliquot the desired volume of M1 in a separate sterile bottle to begin M2 preparation. Measure the volume with a serological pipette. Alternatively, use the full M1 bottle (521 mL) to prepare M2.
7. Add the reagents for M2 preparation following the volumes described in the medium 2 formulation table.
8. Swirl the bottle to mix the medium.
9. Store the media in the fridge at 4°C.

Preparation of BME hydrogel

Timing (When working with a full vial): 20–30 min (including thawing of reagents)

Note: Our protocols for HGSC organoid generation and culture development involve a basement membrane extract (BME) hydrogel. The descriptions below apply to Cultrex Reduced Growth Factor Basement Membrane Extract, Type 2, Pathclear (R&D Systems, #3533), which from here on is referred to as "BME hydrogel". This hydrogel type is liquid when cold (+4°C) and forms a semi-solid gel at temperatures above 15°C. It may be possible to use other types of hydrogels, e.g., Matrigel (Corning) or Geltrex (Gibco), but some experimental procedures may require adjustment.

Note: Protein concentration in BME hydrogel is different in each product lot (8-12 mg/mL). For reproducibility and optimal results, we recommend working with a protein concentration of 7.5–8 mg/mL. Stock BME hydrogel can be diluted with PBS (usually 10-30 v/v%, depending on the BME hydrogel lot) to achieve this concentration and obtain the ready-to-use hydrogel. Before starting, obtain the protein concentration of your BME hydrogel lot from the supplier and calculate the dilution factor. For example, for a BME hydrogel lot with original protein concentration of 11 mg/mL that has to be diluted to 8 mg/mL, the dilution factor would be 0.73 (8 mg/mL divided by 11 mg/mL). Thus, to prepare 1 mL of 8 mg/mL BME hydrogel, 730 μL of 11 mg/mL BME hydrogel should be mixed with 270 μL of sterile, cold PBS.

Note: We recommend the culture of organoids in 6-well cell culture-treated plates. Polystyrene cell-culture treated plates have a hydrophilic surface that improves the attachment of the BME hydrogel. We do not recommend using hydrophobic surfaces, such as non-treated or low-attachment. We seed 10 BME hydrogel domes of 20 μL each per well (Figure 1). Thus, seeding one well requires 200 μL of ready-to-use BME hydrogel. This volume refers to a seeding conducted with a mechanical pipette.

⚠ CRITICAL: If using an electronic repeater pipette for seeding organoids, the volume of ready-to-use BME hydrogel needed per well is slightly higher due to the dead volume and the dome size (by default, larger than if seeded with a manual pipette). For instance, for seeding 1 well, around 280 μL (±10 μL) instead of 200 μL may be needed.

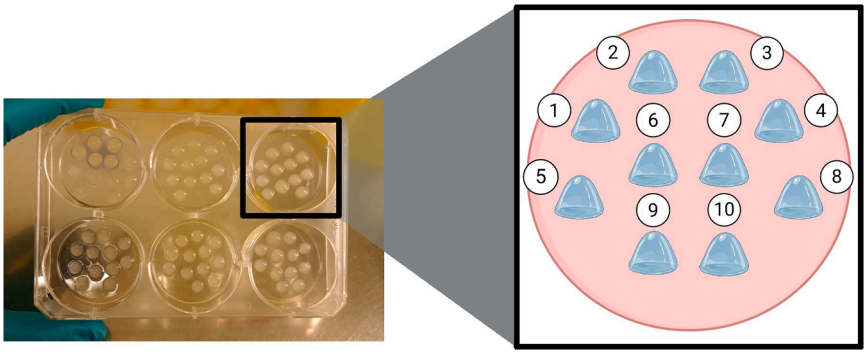

Figure 1. Seeding of BME hydrogel domes

BME hydrogel domes seeded in a 6-well plate and seeding procedure.
Created in BioRender. Gall Mas, L. (2025) https://BioRender.com/djmyqkx.

Note: Preparation of the ready-to-use BME hydrogel in advance is essential to ensure that it is homogenous and free from air bubbles, as they can compromise dome stability. We recommend preparing it as soon as the BME hydrogel is thawed.

Before starting, calculate the required amount of ready-to-use BME hydrogel (see protocols for establishment or passage of organoid cultures). Due to the high viscosity of the hydrogel, we recommend preparing an additional volume of 20–30% depending on the type of pipette used for seeding.

Thaw the BME hydrogel at 18–24 °C, gently rotating the vial every few min (to prevent parts of the BME hydrogel from polymerizing at 18–24 °C), and immediately transfer it to ice when thawed. This can take up to 20 min. Alternatively, thaw the BME hydrogel overnight in a fridge at 4°C on ice.

10. Place 2 tubes on ice and aliquot cold PBS in one of them.
11. To ensure that the BME hydrogel is homogeneous before using it, gently mix it with the P1000 pipette in its original vial without introducing air bubbles. Avoid mixing by inversion or shaking.
12. Transfer the desired amount of the BME hydrogel into the cold, empty tube (on ice).
13. Add cold PBS to achieve the desired BME hydrogel protein concentration.
14. Gently mix the BME hydrogel with the P1000 pipette to homogenize the solution and let it rest on ice. This allows air bubbles to dissipate and improves hydrogel consistency.

Note: Always homogenize the ready-to-use BME hydrogel solution before using it by gently resuspending it with the P1000. Avoid introducing air bubbles.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological samples		
High-grade serous ovarian cancer samples	Turku University Hospital	NA
Chemicals, peptides, and recombinant proteins		
Advanced DMEM/F12 (1X) (Gibco)	Thermo Fisher Scientific	12634010
Primocin	InvivoGen	ant-pm-05 ant-pm-1, ant-pm-2
HEPES (1M) (Gibco)	Thermo Fisher Scientific	15630080
GlutaMAX (100X) (Gibco)	Thermo Fisher Scientific	35050061
B-27 Supplement (50X), serum free (Gibco)	Thermo Fisher Scientific	17504044
N-acetyl-L-cysteine (Sigma-Aldrich)	Merck	A7250

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Nicotinamide (Sigma-Aldrich)	Merck	N0636
17β-Estradiol (Sigma-Aldrich)	Merck	E8875
SB202190	MedChemExpress	HY-10295
A83-01 (Sigma-Aldrich)	Merck	SML0788
Recombinant human FGF-4 (PeproTech)	Thermo Fisher Scientific	100-31
Recombinant human FGF-10 (PeproTech)	Thermo Fisher Scientific	100-26
Recombinant human heregulin 1-β (PeproTech)	Thermo Fisher Scientific	100-03
Animal-free recombinant human EGF (PeproTech)	Thermo Fisher Scientific	AF-100-15
Forskolin	MedChemExpress	HY-15371
Hydrocortisone (Sigma-Aldrich)	Merck	H0888
Y-27632	MedChemExpress	HY-10071
Fungin	InvivoGen	ant-fn-1, ant-fn-2
Amphotericin B (Gibco)	Thermo Fisher Scientific	15290018, 15290026
Other		
125 mL Square PET Storage Bottles	Corning	431530
250 mL Square PET Storage Bottles	Corning	431531
TrypLE Express Enzyme (1×), no phenol red	Thermo Fisher Scientific	12604013
Cell lifter	Corning	3008
14 mL Round Bottom High Clarity PP Test Tube (Falcon)	Corning	352059
Cultrex Reduced Growth Factor Basement Membrane Extract, Type 2, Pathclear	R&D systems	3533
Multipette E3/Multipette E3×	Eppendorf	4987000029
Combitips advanced 1.0 mL	Eppendorf	0030089430
Nunc Cell-Culture Treated 6-well plates	Thermo Fisher Scientific	140685
STEM-CELLBANKER GMP grade	Amsbio	11924
Digital camera for light microscope	Leica	DFC340 FX

MATERIALS AND EQUIPMENT

Reagents for preparation of medium

- We recommend preparing aliquots of all the reagents in advance according to the manufacturer's instructions and storing them in a −20°C freezer except for N-acetyl-L-cysteine and nicotinamide powders.
- Nicotinamide powder can be weighed in advance and stored at 18–24 °C. N-acetyl-L-cysteine must be weighed fresh each time.
- Commercially ready reagents (Primocin and B-27 supplement) come in exact amounts for preparing 500 mL of M1 (1 mL and 10 mL, respectively). 1M HEPES and 100X GlutaMAX stocks should be aliquoted beforehand into 5 mL aliquots and stored in a −20°C freezer. If preparing lower volumes of media, adjust the aliquot sizes.
- Growth factors and hormones (FGF-4, FGF-10, EGF, neuregulin-1, hydrocortisone, and β-estradiol) should be prepared as single-use aliquots. Small molecule inhibitors (SB202190, A83-01, and forskolin) aliquots can be frozen and thawed up to 3 times. Reconstitute reagents according to the manufacturer's instructions.

Formulation of Medium 1 (M1)

Note: The weights of N-Acetyl-L-Cysteine and nicotinamide powders are expressed as a range rather than absolute values, as some error is tolerable.

Note: For M1 preparation, all the reagents are calculated considering a final volume of 500 mL of Advanced DMEM/F12. For simplicity, we disregard the added 21 mL from Primocin (1 mL),

B-27 supplement (10 mL), HEPES (5 mL), and GlutaMAX (5 mL), as we have not observed any differences in organoid growth in M1 when correcting for the added volume.

⚠ CRITICAL: Store the medium (M1 and M2) in the fridge at 4°C for no longer than 10 days. Using the medium beyond its lifespan can negatively impact the successful development of organoid cultures. The same applies to the aliquots used in the preparation of the medium.

Reagent	Final concentration	Amount
Advanced DMEM/F12	–	500 mL
Primocin	100 µg/mL	1 mL
HEPES	10 mM	5 mL
GlutaMAX	1 ×	5 mL
N-Acetyl-L-Cysteine	1 mM	86 mg ± 4 mg
Nicotinamide	5 mM	305 mg ± 4 mg
B-27 supplement	1×	10 mL
β-estradiol	100 nM	5 µL
SB202190	0.5 µM	25 µL
A83-01	0.5 µM	25 µL
FGF-4	10 ng/mL	50 µL
FGF-10	10 ng/mL	50 µL
Store in the fridge at 4°C for up to 10 days.		

Formulation of Medium 2 (M2)

Note: For M2 preparation, measure a precise amount of M1. If preparing a full bottle of M2, consider 521 mL of M1.

Reagent	Final concentration	Amount
Medium 1 (M1)	–	300 mL
Neuregulin-1	5 nM	30 µL
EGF	5 ng/mL	15 µL
Forskolin	5 µM	150 µL
Hydrocortisone	500 ng/mL	75 µL
Store in the fridge at 4°C for up to 10 days.		

Y-27632: Rho-associated protein kinase inhibitor

- Prepare 10 mM stocks in DMSO.
- Use at a final concentration of 5 µM in growth medium when washing cryopreserved samples and for 2 to 3 days when passaging organoids to prevent anoikis.

STEP-BY-STEP METHOD DETAILS

Establishment of organoid cultures from fresh or cryopreserved patient samples

Timing (For 1 sample): 1 h and 40 min

Patient cells are counted and embedded in BME hydrogel. Seeded plates are incubated for 45 min at 37°C until the BME hydrogel domes solidify. Culture media are added.

Note: This protocol utilizes pre-processed patient samples (dissociated tissue to small cell clusters or single cells, dissociation method described previously in Senkowski et al., 2023), either cryopreserved (in STEM-CELLBANKER) or fresh.

Note: When starting a new organoid culture with the "M1/M2 method", the same sample is cultured in parallel in M1 and M2. This means that each patient sample must be seeded in at least two wells of a 6-well plate (one well per medium), if enough material is available. We recommend seeding 20.000–100.000 live cells per dome. If the number of viable cells is insufficient to seed two wells with 10 domes per well (one full well per medium), we recommend reducing the number of domes per well, rather than omitting any of the media. A minimum of five domes should always be seeded, as working with low volumes of BME hydrogel increases the risk of cell loss during passages. In the case of a surplus of cells, two wells, one for each medium, with 10 domes per well, can be seeded. This allows for more flexibility when passaging the samples, as domes with a few growing organoids can be later pooled to concentrate the cells. See the BME hydrogel preparation to determine how much BME hydrogel volume to use per well.

1. Place a 6-well plate in the cell incubator at 37°C.

Optional: Steps 2–10 apply to cryopreserved patient samples only.

2. Warm up 20 mL of M1 in the water bath at 37°C for sample washes. Each wash requires 10 mL, and each sample is washed twice.
3. Aliquot 10 mL of warm M1 in a 15 mL tube.
4. Thaw the sample in the water bath at 37°C.
5. Using a P1000 pipette, transfer 1 mL of warm M1 into the cryovial with the sample, gently mix and transfer the cells back to the tube with the warm medium. Rinse the cryovial 1-2 times with 1 mL of M1 from the same tube to recover any remaining cells.
6. Gently mix the cell suspension 2-3 times with the P1000 pipette.
7. Centrifuge the cells at 200 × *g* for 5 min.
8. Aspirate the supernatant gently, without disrupting the cell pellet.
9. Add Y-27632 to the tube with the remaining 10 mL of M1 to a final concentration of 5 μM.
10. Transfer the 10 mL of M1 with Y-27632 into the tube containing the cell pellet and gently resuspend it.
11. Count the live cells with a dye exclusion method (e.g., Trypan Blue) to determine the number of live cells. An automated cell counter can be used.
12. Aliquot the number of live cells needed for starting the organoid culture in a centrifuge tube. Leftover patient cells can be cryopreserved (see cryopreservation protocol).
13. Centrifuge the cells at 200 × *g* for 5 min.
14. Carefully aspirate the supernatant, without disrupting the cell pellet.

Note: If necessary, use a mechanical pipette to remove the remaining supernatant. It is important that the supernatant is thoroughly removed to avoid overdiluting the gel.

15. Gently resuspend the pellet in ready-to-use BME hydrogel (see BME hydrogel preparation) to achieve a final concentration of at least 1 × 10^6 live cells/mL to be able to seed 20.000 cells/dome.

⚠ CRITICAL: Stop mixing when the cell suspension appears homogeneous. Avoid over-pipetting, as the presence of small cell clusters is beneficial for initial sample growth.

⚠ CRITICAL: If there is a large amount of supernatant that cannot be aspirated without losing cells, use 100% BME hydrogel instead of the ready-to-use hydrogel. This will avoid

further diluting the hydrogel, which can impair dome stability. In addition, adjust the amount of used hydrogel according to the available cell number.

16. Seed the BME hydrogel domes on pre-warmed 6-well plates.

Note: Although we recommend using an electronic repeater pipette for seeding BME hydrogel domes, a manual P200 pipette can be convenient when seeding small volumes. Importantly, the cell suspension remaining as a dead volume in the repeater pipette tip should also be dispensed as a separate hydrogel dome.

17. Incubate the plates in the cell incubator at 37°C for 45 min to allow the BME hydrogel domes to solidify.
18. In the meantime, warm up M1 and M2 supplemented with Y-27632 at a final concentration of 5 μM.

Note: Each well of a 6-well plate requires 3 mL of medium.

19. Once the BME hydrogel domes are solidified, add 3 mL of warm culture medium to each well with a serological pipette.

⚠ CRITICAL: Add the medium by gently releasing it towards the wall of the well, as pipetting the medium directly on the BME hydrogel domes could damage them.

20. Keep the cultures in the cell incubator at 37°C. Change media every 2-3 days (see change of medium). Do not add Y-27632 in any of the following medium changes.

Optional: Take pictures for monitoring culture growth (see monitoring of organoid cultures).

21. Passage the organoid culture when confluent (see passage protocol).

Note: The timespan between seeding and passaging a newly started organoid culture varies between samples. This issue is covered in detail in the expected outcomes section.

Monitoring of organoid cultures

Timing (For 1 plate): 5 min

Organoid cultures are inspected with a light microscope and images of the cultures are acquired at 2.5× and 10×.

22. Inspect the organoid cultures with a light microscope.
23. Acquire pictures with a digital microscope camera.

Note: Use phase-contrast settings at a magnification of 2.5× to capture the entire dome and monitor changes in culture confluence (Figure 2A). Use phase-contrast settings at a magnification of 10× to capture the organoid-like structures and assess the culture quality (Figure 2B). Use bright field settings at a magnification of 20× to capture the morphology of single clusters (Figure 2C).

Optional: We recommend taking pictures on the day of starting the culture, and before and after the passages. However, pictures can be acquired more frequently if needed.

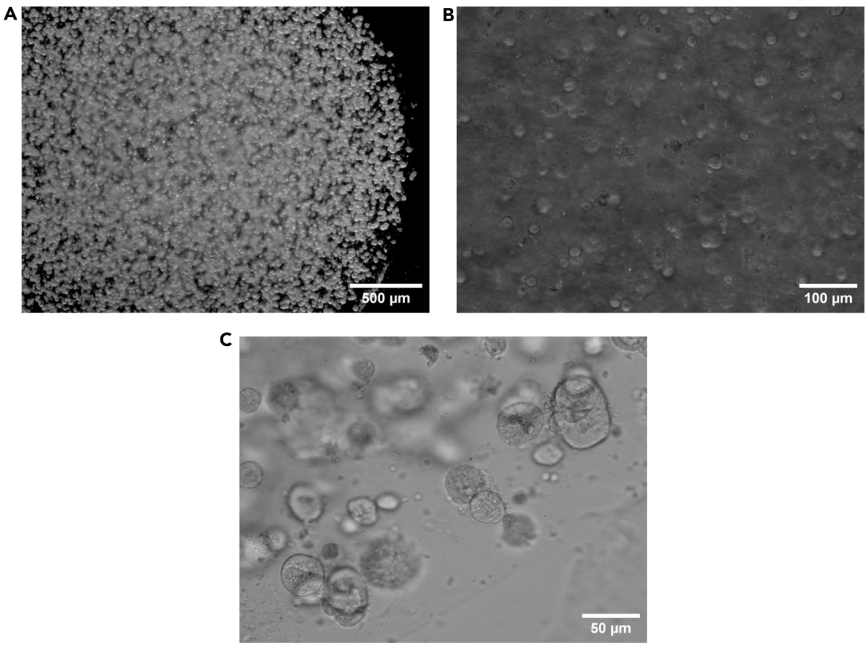

Figure 2. Microscopy images of a stable organoid culture acquired with different magnifications and settings
(A) Image of a BME hydrogel dome acquired with a 2.5X objective in phase-contrast. Scale bar: 500 µm.
(B) Image of organoid structures acquired with a 10X objective in phase-contrast. Scale bar: 100 µm.
(C) Image of organoid structures acquired with a 20X objective in bright field. Scale bar: 50 µm.

Change of medium

Timing (For 1 plate): 15 min (including reagent warming)

The old medium is aspirated, the organoids are rinsed with warm PBS, and fresh medium is added.

Note: Medium must be changed every 2 to 3 days. Each well of a 6-well plate requires 3 mL of growth medium and 1–1.5 mL of PBS.

⚠ CRITICAL: When adding PBS or culture medium to the wells, use a serological pipette attached to an electronic pipette controller. Always release the liquids on the wall of the well carefully, as adding them directly into the BME hydrogel domes or using a high flow speed can disrupt them.

24. Calculate the amount of medium and PBS needed.
25. Warm up the PBS, M1, and M2 in a water bath at 37°C.
26. Start by removing the old medium in all the wells by aspiration. Slightly tilt the plate and place the tip towards the wall of the well.

⚠ CRITICAL: Avoid touching the BME hydrogel domes. If by mistake a dome is touched, change the tip to avoid sample cross-contamination.

27. Add pre-warmed PBS into the wells to wash BME hydrogel domes. The PBS should cover the BME hydrogel domes.
28. Aspirate the PBS as in step 3. No incubation with PBS is needed; it can be aspirated right away.
29. Add pre-warmed medium into the wells.
30. Keep the cultures in the cell incubator at 37°C.

Passage of organoid cultures

Timing (For 1 or more wells in a 6-well plate): 1 h and 40 min

BME hydrogel domes are rinsed with PBS before mechanical and enzymatic digestion for 15 min. Cell digest is collected, spun down, and the pellet is resuspended in BME hydrogel. BME hydrogel domes are seeded and, after solidifying, are covered with fresh medium. Microscopy images can be taken before and after passages to monitor the culture growth.

⚠ CRITICAL: One of the aims of passaging organoids is to keep the cultures at a cell density that stimulates growth and allows for a reproducible passaging ratio and interval. Importantly, an optimal growth density is an individual characteristic of each culture and needs to be found in an empirical process (see: Determining passage ratio, below). For HGSC cells to re-grow as organoids after a passage, we recommend dissociating the organoids into smaller clusters. Over-pipetting and dissociating to single cells can be detrimental, especially when cultures are newly started. Therefore, it is important to handle the cultures gently. Performing this procedure reproducibly between passages is crucial for maintaining the passage ratio and frequency.

⚠ CRITICAL: Considering that over-pipetting is detrimental, we do not recommend counting cells when passaging organoid cultures, as it requires a homogenous cell suspension. Instead, we recommend determining a passage ratio based on a visual assessment of the culture's confluence. More information about this is provided in the expected outcomes section.

31. Photograph the cultures (optional) (see monitoring of organoid cultures).
32. Warm up PBS in a water bath at 37°C and place an empty 6-well cell culture plate in the cell incubator.
33. Remove the old medium by aspiration.
34. Wash the BME hydrogel domes with pre-warmed PBS (see change of medium).
35. Aspirate the PBS.
36. Add 2 mL of TrypLE Express into each well.
37. Scrape the BME hydrogel domes off the well surface with a cell lifter.

⚠ CRITICAL: After this step, all the BME hydrogels should be floating in TrypLE Express, and no remaining fragments should be attached to the bottom of the plates. If so, scrape until all the BME hydrogel is detached.

38. Pre-wet a P1000 pipette tip in TrypLE Express or 1% BSA solution in PBS (aspirate and expel 1 mL of fluid). This reduces the adhesion of the organoids to the inside of the tip and minimizes cell loss.
39. Mechanically digest the BME hydrogels in the well by aspirating and releasing them with the P1000. Repeat 10–14 times, until no large BME hydrogel fragments are visible.
40. Incubate the plate for 15–20 min in the cell incubator at 37°C.
41. After the incubation, pre-wet a P1000 pipette tip in TrypLE Express or BSA solution.
42. Collect the cell digest.

Note: To minimize potential cell loss from adhesion to the inside of the tip, start by collecting some clear liquid (50–100 μL) from the well and proceed with the layer of digested cells. Stop when all visible cells are collected, even when the full capacity of the pipette has not been reached.

⚠ CRITICAL: Avoid resuspending the cell digest – only aspirate it.

43. Transfer the cell digest to a centrifuge tube.

Note: The tube of choice will depend on the pipette used for seeding (mechanical or electronic repeater pipette) (see establishment of organoid cultures).

44. With the same tip, wash the well with the remaining liquid and transfer the content into the same tube.

⚠ CRITICAL: It is important to collect the remaining cells that might be attached to the bottom of the well.

45. Rinse the well with 1 mL of PBS and collect the wash in the same tube. Make sure no cells are attached to the bottom of the well.

Note: At this point, the tube should contain around 3 mL of liquid per digested well.

46. Centrifuge the cell digest at 300 × *g* for 5 min.
47. After the centrifugation, check that the cells have pelleted correctly and that a compact pellet, attached to the bottom of the tube, is visible. If not, check the troubleshooting section.

Note: In early organoid cultures, pellets can be small, barely visible or not visible by eye. If using U-bottom centrifuge tubes, the pellet can be spread out in the bottom of the tube.

48. Aspirate the supernatant carefully.

⚠ CRITICAL: Avoid leaving a lot of liquid above the pellet, as this can later dilute the BME hydrogel and prevent it from solidifying correctly. For improved accuracy, use a mechanical pipette instead of a vacuum system to aspirate the supernatant.

49. Mix the ready-to-use BME hydrogel gently and take up the desired amount.
50. Resuspend the pellet in the ready-to-use BME hydrogel until the cell suspension appears homogeneous.

⚠ CRITICAL: Avoid over-pipetting (10–20 times is usually enough) and introducing air bubbles into the suspension.

Note: While achieving a homogenous cell suspension is recommended, the presence of cell clusters is generally beneficial for the regrowth of the cultures. Thus, over-pipetting (that is, continuing resuspension even after the suspension is visibly homogeneous) can be detrimental to the culture.

51. Seed 10 domes of 20-µL per well into the pre-warmed 6-well cell culture plate.
52. Incubate the plates in the cell incubator at 37°C for 45 min.
53. In the meantime, warm up 3 mL per well of M1 and/or M2 containing Y-27632 at a final concentration of 5 µM.
54. After the incubation, gently add 3 mL of the culture medium to each well.
55. Keep the cultures in the cell incubator at 37°C. Change the medium every 2 to 3 days (see change of medium).
56. Take pictures of the organoids after the passage to monitor the growth (optional) (see monitoring of organoid cultures).
57. Passage the organoid culture when required (see expected outcomes).

Cryopreservation of organoid cultures

Timing (For 1 or more wells in a 6-well plate): 30 min

Organoids are digested as in a normal passage. The cell pellet is transferred into a cryovial and preserved in STEM-CELLBANKER.

⚠ CRITICAL: When cryopreserving organoids, it is important to determine the passage ratio for re-establishing the culture. Because the viability of organoids decreases due to cryopreservation, we recommend reducing the normal passage ratio of the culture by 50%. For instance, if a culture on the day of freezing would have been expanded from 1 well to 3 wells (1:3), when re-establishing the culture from 1 well of frozen organoids, only 1.5 wells (15 domes) instead of three should be seeded.

Note: To cryopreserve organoids, follow the instructions for passage of organoid cultures until step 18.

58. After aspirating the supernatant, add STEM-CELLBANKER to the pellet without mixing.

Note: The amount of STEM-CELLBANKER added to the pellet depends on the number of wells pooled in a single cryovial. As a reference, for 1-3 confluent wells, use 1 mL of the cryopreserving agent.

59. Collect the pellet and STEM-CELLBANKER solution with a P1000 pipette tip and transfer it into a cryovial.
60. Re-suspend the pellet by pipetting up and down 5–10 times, until there are no visible large pellet fragments.
61. Transfer the cryovial to a −80°C freezer for short-term storage. For long-term storage (>2 months), after initial freezing for 1–7 days at −80°C, transfer the cryovials to liquid nitrogen storage tank.

Note: STEM-CELLBANKER is a chemically defined and FBS-free freezing solution. Cryovials can be directly transferred to a −80°C freezer without being placed in cell freezing containers.

Re-establishment of organoid cultures from cryopreserved organoids

Timing (For 1 cryovial): 1 h and 20 min

Organoids are washed twice in M1 and embedded in BME hydrogel. Seeded plates are incubated for 45 min in a cell incubator at 37°C until the BME hydrogel domes solidify. Culture medium is added.

Note: This protocol is identical to the establishment of organoid cultures from patient samples, with the following adjustments:

62. Step 7: Centrifuge the cells at 300 × *g* for 5 min instead of 200 × *g*.
63. Steps 11 to 12: Skip, as these cells do not need to be counted. Cryopreserved cultures are re-established following the passage ratio as described in cryopreservation of organoid cultures.
64. Step 13: Centrifuge the cells at 300 × *g* for 5 min instead of 200 × *g*.

Note: The re-establishment of a culture from cryopreserved organoids is considered a passage. Therefore, the passage number of a re-established organoid culture is one passage

higher than the passage at cryopreservation. For example, if a culture is cryopreserved at the end of passage 10, the re-established culture starts at passage 11.

⚠ CRITICAL: After re-establishing cryopreserved organoids, the regrowth capacity and confluence of the cultures during the first 1 to 2 passages can differ from the typical for the culture. Therefore, one might need to modify the passage ratio and frequency for the cultures to resume normal growth. Overall, there are two situations one can encounter: 1) The organoid density is lower than the usual density of the culture, and as a result, the culture is growing more slowly. In such a case, at the first passage after thawing, the passage ratio should be decreased, and the time until the next passage increased, to concentrate the organoids. In our experience, organoids should resume their standard growth rate before the second or third passage after thawing. If not, continue passaging them, adjusting the passage ratio and frequency to return to the optimal cell density of the culture. 2) The organoid density is higher than the usual density of the culture. In this case, increase the passage ratio to decrease the cell density. Regardless of the situation, we recommend performing at least one recovery passage (passaging the culture once) before using it in any downstream application.

EXPECTED OUTCOMES

Definition of an organoid culture

We define HGSC organoids as long-term, self-renewing, patient-derived 3D cancer cell cultures, which recapitulate genomic features of the tumor of origin and grow stably over time.[7] To designate an HGSC patient-derived 3D cell culture as a stable and robust organoid culture, we consider that the following criteria must be fulfilled: 1) Organoid cultures must enter an exponential growth phase, where growth becomes stable, making it possible to expand the organoids of the culture following a specific passage ratio (e.g., from 1 well to 2 wells) and stabilized frequency (e.g., 10–12 days). The growth can be continued indefinitely, without slowing down or culture senescence. 2) Organoid cultures can be cryopreserved, and the re-established culture must eventually resume its normal growth rate. 3) The genomic identity of the organoid culture must be confirmed, and it must be demonstrated that the culture is derived solely from the tumor of origin. 4) The tumor purity of the culture must be close to 100% (e.g., no major contamination with normal cells is observed).

The time required to develop a stable organoid culture varies between patient samples, as each of them has a unique cellular composition in terms of varying fractions of cancer cells and other tumor-associated cell types (see success of an organoid culture).

The development of stable HGSC organoid cultures focuses on expanding the fraction of cancer cells, while depleting the other cell types present in the patient sample. To do so, it is essential to determine the optimal culture media for each sample (M1 or M2) and passage the cultures regularly. Mild enzymatic digestion and fragmentation of the 3D cell clusters during passaging is important to assess the regrowth capacity of the organoid-initiating cells, expand the cancer cell fraction, maintain an optimal cell density, and eliminate undesired cell types and cellular debris. Deciding when to passage a newly started culture for the first time, which cell density is optimal, and which medium is appropriate for a specific sample can be challenging. The following sections cover and discuss these aspects.

When to passage a newly started organoid culture for the first time

The rate at which a newly cultured HGSC samples form organoid-like structures in 3D and ultimately become stable cultures can differ (Figure 3). In some samples, organoid-like structures develop after 12–19 days of culture (Figure 4A), but in others, it can take up to 33–40 days (Figure 4B).

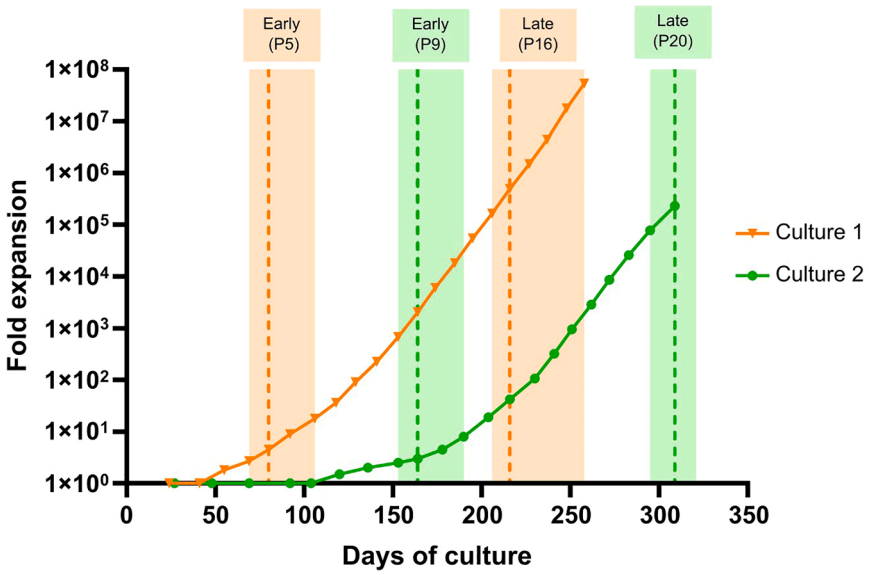

Figure 3. Growth curves during organoid development

The curves show the cumulative fold expansion over time for two successful organoid cultures (Culture 1 and 2) developed from different patient samples in M2. The passage ratio was calculated by dividing the number of wells seeded by the number of wells harvested. The cumulative fold expansion was calculated as the product of the passage ratio and the cumulative fold expansion of the previous passages. Dashed lines indicate the day when samples for genetic validation were collected. Shaded intervals indicate the recommended time frame for validation sample collection.

The degree of confluence at the time of the first passage can also differ between cultures. Ideally, cultures are passaged when fully confluent (Figure 4). However, not all samples become confluent before the first passage, even if seeded at high cell density. Notably, these were initiated at high seeding densities, and differences in apparent confluence primarily reflect intrinsic variation in growth dynamics. In other cases, only sparse, large organoid-like structures surrounded by single cells, smaller clusters, or cellular debris are often observed instead (Figure 5). Despite the cultures not being confluent, they still require passage after prolonged periods (over 30–40 days) to fragment the large organoid-like structures into smaller clusters, induce culture regrowth, provide a fresh BME hydrogel scaffold for the cells, and eliminate other tumor-associated cell types.

Moreover, the time that it takes for a newly embedded sample to achieve confluency before the first passage is not indicative of the potential success in developing an organoid culture (Table 1).

Additionally, initial formation of 3D clusters at a high density and, similarly, early 3D structure formation and growth (Figure 6) are not predictive of the ultimate successful development of a stable long-term organoid culture.

Determining the passage ratio

Determining the passage ratio for an organoid culture is an empirical process that requires assessing the culture density and morphology of the organoid-like structures. In Figure 7, we demonstrate the most common healthy organoid morphologies we observed. In newly started cultures and cultures in early development, the passage ratio must be determined for each passage. This is important because the cultures are more unstable, and organoid-like structures can form or collapse within the time course of a passage. Consolidated cultures expand stably, so the passage ratio and frequency can usually be maintained.

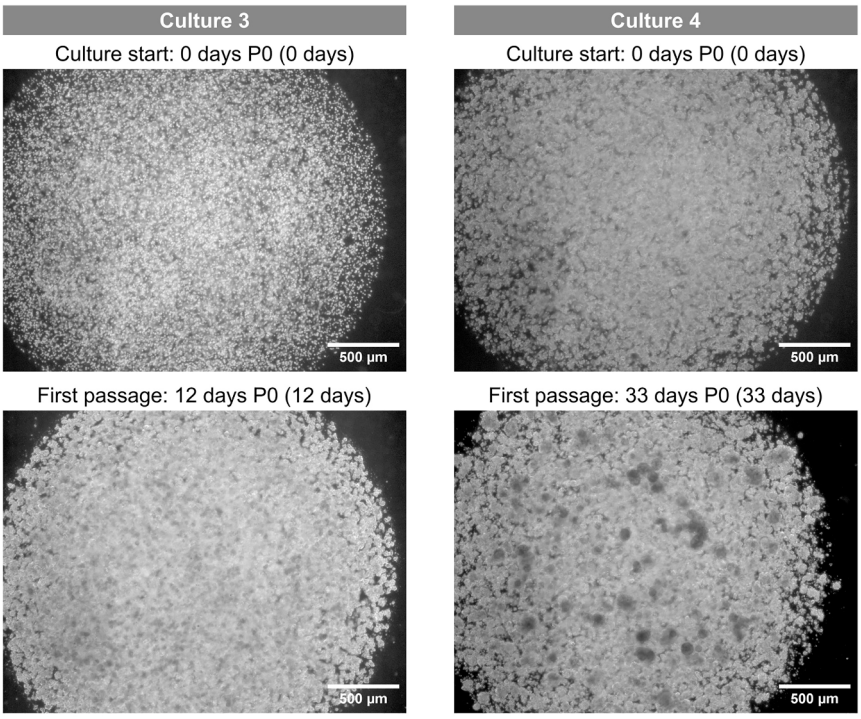

Figure 4. First passage of two confluent HGSC in M1

(A) Culture 3 was passaged 12 days after the culture was started, indicated as 0 days in passage 0 (P0).

(B) Culture 4 was passaged 33 days after the culture was started. Stable organoid cultures were developed from both samples in M1.

The total number of days in culture is indicated in brackets. Images acquired with phase-contrast microscopy at 2.5× magnification. Scale bar: 500 µm.

Passaging a newly started culture for the first time

In this section, we will focus on the time between initial sample seeding and the first passage and on determining the optimal first passaging ratio.

For newly started cultures that become confluent during P0, we recommend expanding them 1:2, independently of the initial number of domes seeded. As shown in Table 2, Culture 3 was expanded from 0.5 wells of a 6-well plate to 1 well, whereas Culture 4 was expanded from 1 well to 2 wells (Figure 4).

For cultures with sparse organoid-like structures, we recommend refraining from culture expansion and keeping a passing ratio of 1:1 (Table 2; Figure 5).

If the density of organoid-like structures is remarkably low and 2 wells are available in each medium, they can be pooled to increase the cell density (ratio 2:1).

In our experience, in early culture development, concentrating the organoid-like structures rather than trying to expand them typically has a positive impact on regrowth, and the cultures often can be expanded in later passages. Thus, when in doubt, we recommend prioritizing high cell densities rather than expanding the culture.

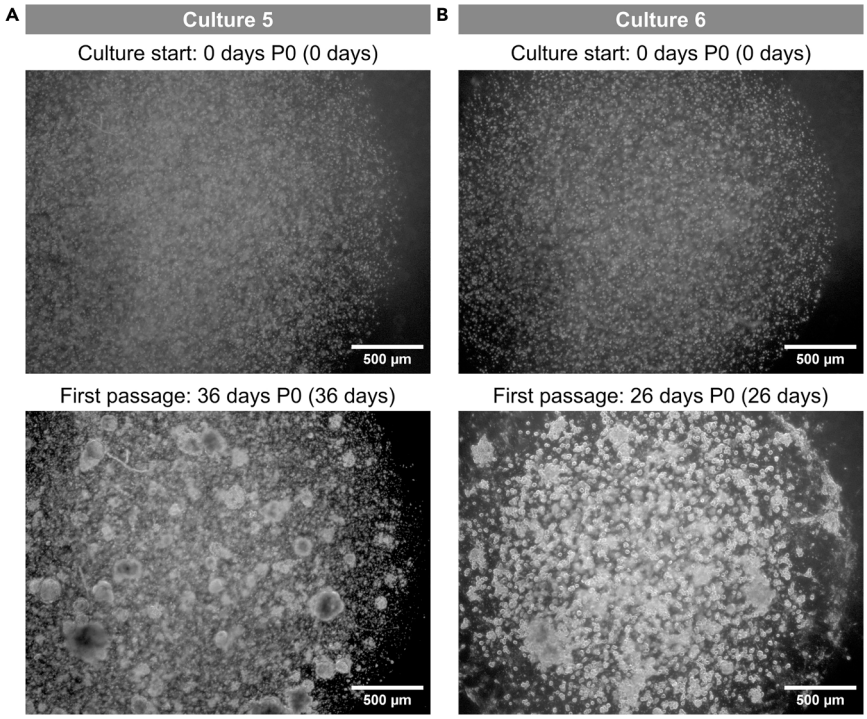

Figure 5. First passage of two sparse HGSC samples in M1

(A) Culture 5 was passaged 36 days after the culture was started, indicated as 0 days in passage 0 (P0).

(B) Culture 6 was passaged 26 days after the culture was started (day 0). Stable organoid cultures were successfully developed from culture 5 in M1.

Culture 6 failed to grow both in M1 and M2. The total number of days in culture is indicated in brackets. Images acquired with phase-contrast microscopy at 2.5× magnification. Scale bar: 500 µm.

Passaging organoid cultures in early development

After the first passage, early organoids can grow and form organoid-like structures at different rates and densities. In this section, we will focus on how to adjust the passage ratio of organoids in early development until the culture becomes stable.

Cultures that form fast-growing organoid-like structures from the beginning can be expanded at a 1:2 or 1:3 ratio in early passages. As shown in Table 2, organoid-like structures in Culture 3 re-grew consistently after 7–8 days following a passage ratio of 1:3. As the regrowth was relatively quick and the culture appeared very dense, we increased the passage ratio to 1:4 and later to 1:5.

Cultures that initially only form slow-growing organoid-like structures typically benefit from little to no expansion, such as Culture 5 (Table 3). In such cases, we only recommend expanding the material

Table 1. Passage frequency and ratios for four organoid cultures

	Culture 3	Culture 4	Culture 5	Culture 6
Medium	M1	M1	M1	M1
Days from P0 to P1	12	33	36	26
Wells seeded in P0	0.5	1.0	0.5	0.5
Wells seeded in P1	1.0	2.0	0.5	0.5
P1 passage ratio	1:2	1:2	1:1	1:1
Successful organoids	Yes	Yes	Yes	No

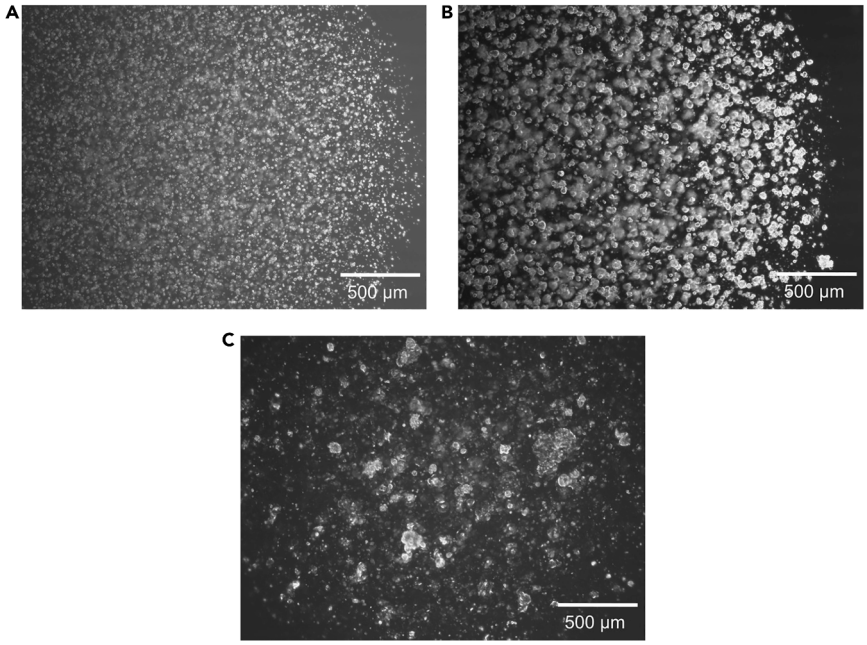

Figure 6. Unsuccessful organoid culture with an initial high density of organoid-like clusters

Culture 7 illustratates a gradual growth decline after the first passage, despite the initial presence of multiple organoid-like clusters.

(A) Culture start in M1 after sample embedding in 3D.

(B) First passage in M1 after 18 days of culture.

(C) End of culture after 6 passages in M1 and 182 days of culture.

Images acquired with phase-contract microscopy at 2.5× magnification. Scale bar: 500 µm.

once the culture becomes denser and the time between passages shortens, indicating exponential culture growth. Earlier expansion attempts could result in slower or no regrowth due to a low density of the organoid-like structures.

As shown in Figure 8, the cell density of Culture 3 was high from the beginning, and fast-growing organoid-like structures formed over just a few days after initial seeding. In addition, the regrowth capacity and the cell density were stable over time. In Culture 5, however, as the organoid density was relatively low in the first four passages, the passage ratio was limited (1:1) to maintain a high cell density. The stabilization of the culture occurred only at passage 5, as depicted by the organoid density. At this point, the culture could start to be expanded.

Cultures in early development can be comprised of sparse large organoid-like structures rather than a lot of smaller ones (Figure 8). Despite confluence not being achieved, cultures must nevertheless be passaged when the organoid-like clusters reach their maximum size. Passaging such cultures is important to fragment the large aggregates and allow regrowth from smaller ones.

Passaging stable organoid cultures

Stable organoid cultures are passaged following a fixed passage ratio and frequency, determined during culture development. The time and number of passages required to achieve a stable organoid culture can differ between patient samples. In our experience, it can take between 2 to 10 passages, regardless of the time between passages.

In our organoid collection, the passage ratios and frequency of stable HGSC organoid cultures vary greatly. Passage ratios can range from 1:2 to 1:6, and the frequencies from 7 to 16 days.

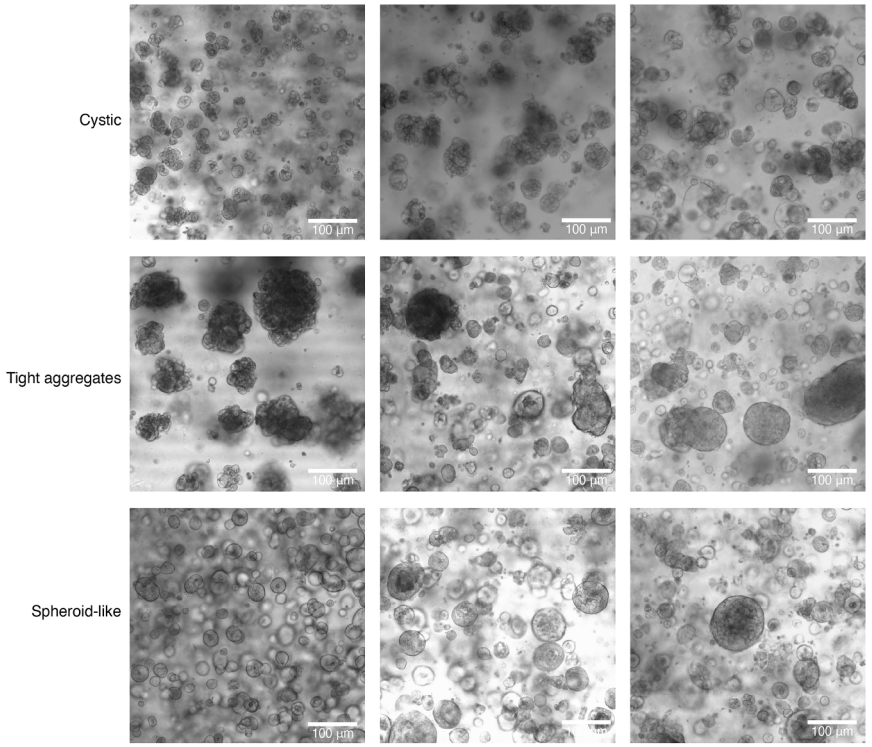

Figure 7. Most common HGSC organoid morphologies

Microscopy images of nine different HGSC cultures in their stable growth phase, ready for passaging categorized based on their morphology as cystic, tight aggregates or spheroid-like. Images acquired with phase-contrast miscroscopy at 10× magnification. Scale bar: 100 µm.

Criteria for terminating an early organoid culture under development

In our experience, 40-50% of early cultures slow down their growth and ultimately collapse, which warrants further research into optimization of additional media formulations that would increase HGSC organoid generation success rates. Growth arrest can occur at any point during organoid development, and currently, we do not have a method to predict whether or when this will occur during organoid development.

Although growth arrest typically becomes apparent after a few passages, it can also occur in later stages, even after initial material expansion. As shown in Figure 9, the formation of organoid-like structures from a patient sample occurred in both media. However, in M2 the growth declined after material expansion in three consecutive passages. By contrast, in M1 the density of organoid-like structures increased gradually over time.

This highlights the importance of culturing tumor samples for several passages in both media, as samples that initially form fast-growing organoid-like structures can gradually lose their long-term growth capacity (Figure 9 – Culture 8 in M2) or it can take multiple passages to start observing their formation (as in Culture 5 in M1 in Figure 8 or in Culture 8 in M1 in Figure 9).

The lack of long-term growth potential often becomes apparent later than passages 2 to 3. As shown in Figure 10, the lack of growth capacity in M1 and the need to terminate the culture were clear already after one passage. However, in M2, it was not until passage 5 that the culture was discarded. Despite the cell density being high in M2, compared to M1, we observed growth arrest in the sample at passage 5.

Table 2. Passage ratios and frequency for Culture 3

Passage number	1	2	3	4	5	6	7	8	9	10	11	*
Passage ratio	1:2	1:3	1:3	1:3	1:3	1:3	1:3	1:4	1:4	1:4	1:5	*
Passage frequency (days)	12	7	7	10	8	7	7	7	5[i]	12[ii]	7[iii]	10
Cryopreservation (wells)	0	0	2	2	2	1	5**	2	3	3	3	1

Overall, we recommend terminating a culture under development when there is a sustained decline in the regrowth capacity for a few consecutive passages, leading to a complete apparent lack of growth. Again, we emphasize the importance of maintaining the cultures, even if minimal growth is observed after a single passage or when the cellular density is low.

Growth in both media

Exceptionally, organoids can sometimes be developed from the same HGSC sample in both M1 and M2. In such a case, we recommend using only one of the cultures for further downstream applications. The selection of the ultimate culture to use can be based on the molecular similarity to the original tissue sample, culture stability and robustness, or the ease of use, among others.

When to cryopreserve organoid cultures under development

Cryopreservation is necessary to reduce the maintenance cost of the organoid culture and to generate a biobanked culture stock, so that early passages of stable organoid cultures can be used later.

We recommend cryopreserving organoids under development when the cultures reach the exponential growth phase, that is, when the organoids in the culture can be expanded. At this point, the passage ratio and frequency can still vary, as determining them requires multiple passages. In our experience, when thawed and resuscitated, all the stable organoid cultures eventually resume their normal growth after cryopreservation (Senkowski et al. 2023).

Success of an organoid culture

The ultimate success of developing a stable organoid culture from viably cryopreserved primary HGSC samples relies on several factors. One is the content of cancer cells in the patient sample, and therefore, we recommend prioritizing samples with high cancer cell purity, if prior sample assessment is possible. Another factor is the anatomical location of the sample. For instance, we noticed a tendency for ascites samples to be easier to develop organoids from than solid tissue samples (e.g., from ovary, peritoneum, or omentum), presumably because they require milder pre-processing and no enzymatic digestion. However, when viable cancer cells are present, it is ultimately the provision of relevant niche signaling that determines the long-term growth capacity of the culture. Thus, the right choice of the growth medium is of utmost importance.

Cross-validation of organoid cultures with the samples of origin

Before any application of the newly derived organoid models in research, it is crucial to confirm their fidelity with the samples of origin and to ensure the lack of cross-contamination from other cultures.

Table 3. Passage ratios and frequency for Culture 5

Passage number	1	2	3	4	5	6	7	8	9	10	*
Passage ratio	1:1	1:2	1:1	1:1	1:1	1:2	1:1.5	1:2	1:2	1:2	*
Passage frequency (days)	36	29	20	29	14	10	14	9	9	10	11
Cryopreservation (wells)	0	0	0	0	0	0	0	0	4**	2	1

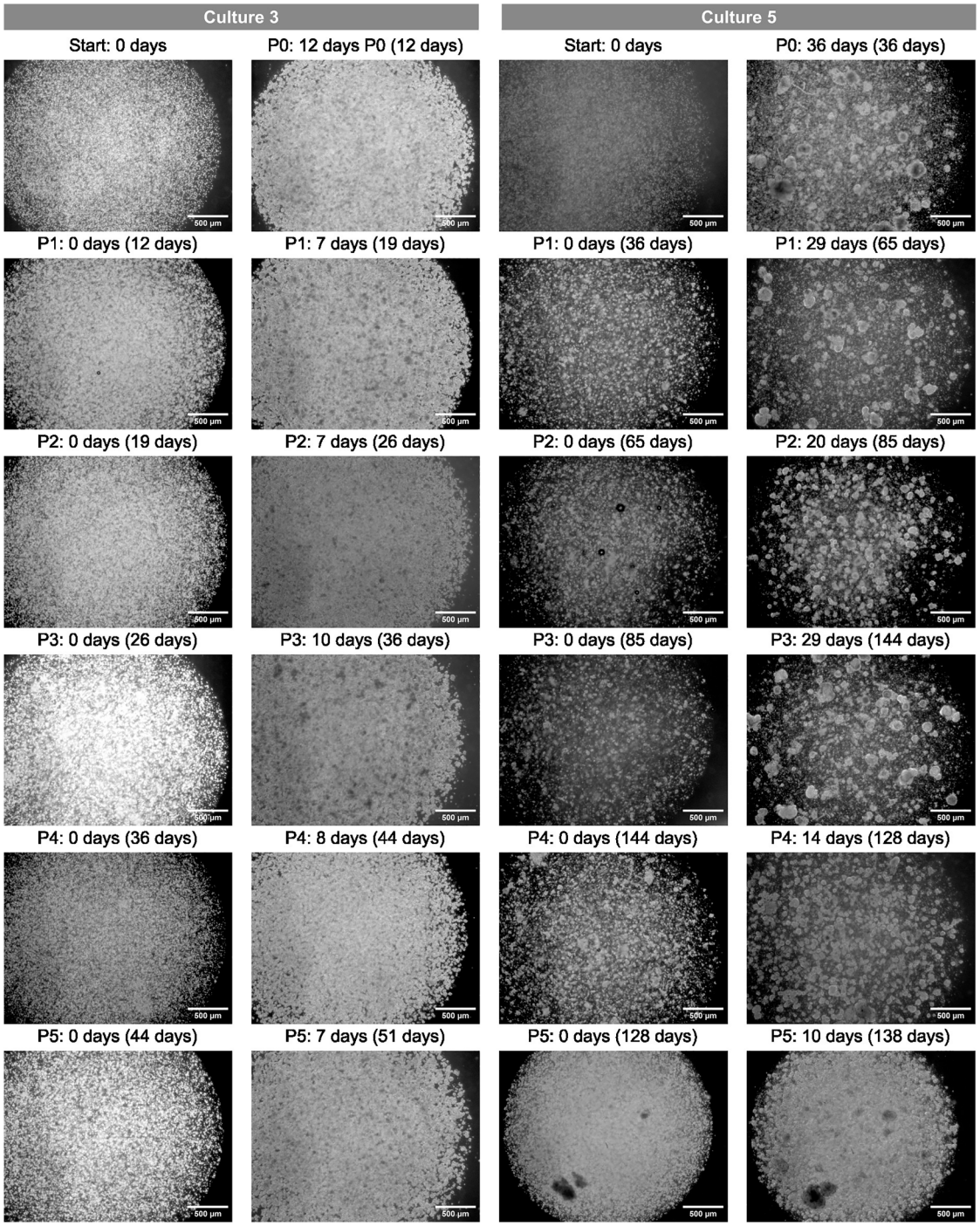

Figure 8. Differences in organoid development of two patient samples cultured in M1

Pictures of Cultures 3 and 5 development in M1. For each culture, the left column shows pictures taken right after seeding/passage (designated by "P"). The right column shows pictures taken right before the passage, demonstrating the evolution of the culture between passages. The total number of days in culture is indicated in brackets. Culture 3 demonstrates a high density from the beginning, whereas in Culture 5 the density progressively increases over several passages. Images acquired with phase-contrast microscopy at 2.5× magnification. Scale bar: 500 µm.

Validation methods can include whole-genome sequencing (WGS), for an in-depth comparison of the genomic copy-number landscape of the organoids and original samples. Nevertheless, *TP53* genotyping can be sufficient to confirm culture identity and tumor purity (at variant allele frequency,

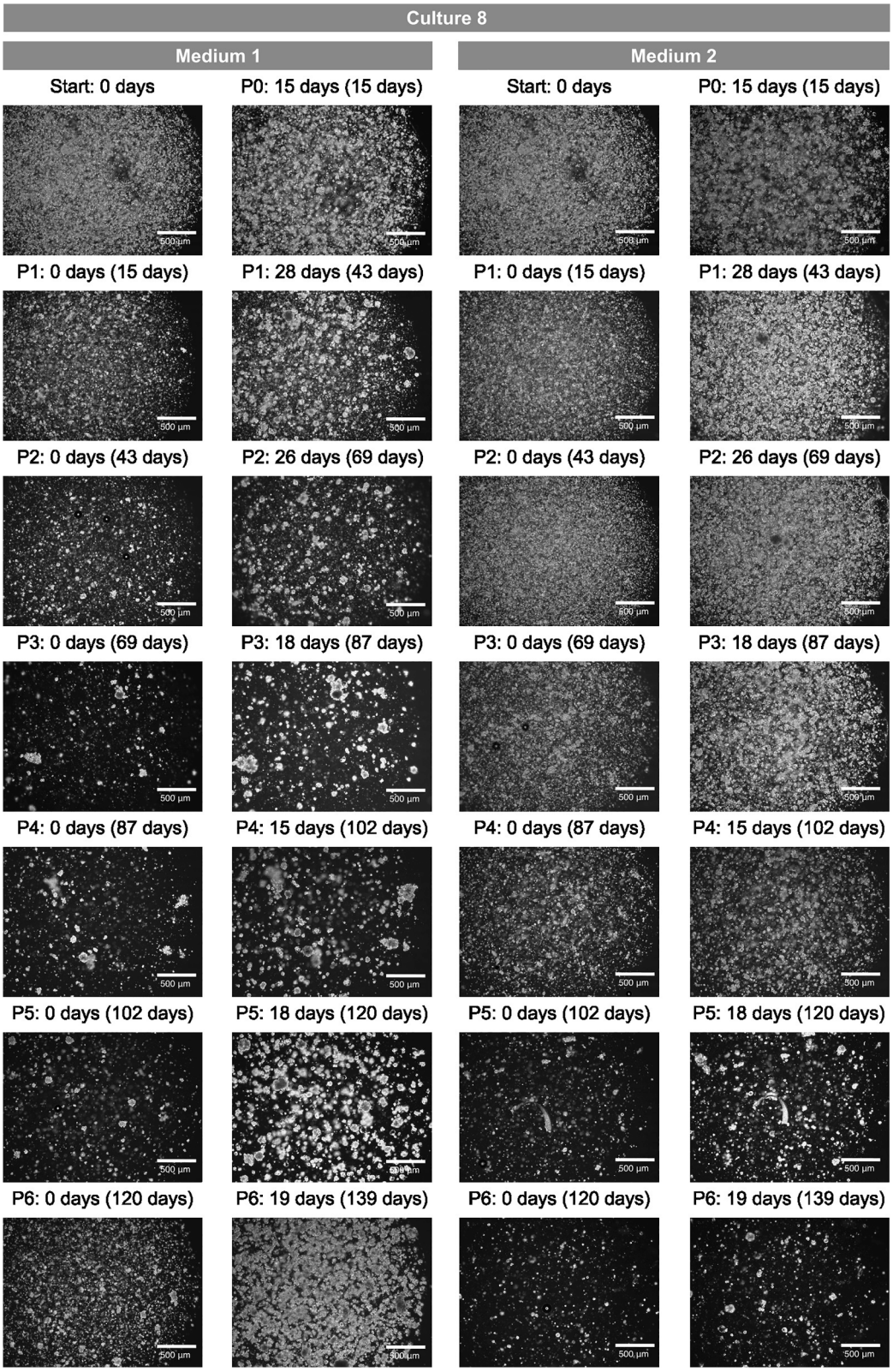
Culture 8
Medium 1
Medium 2
Start: 0 days
P0: 15 days (15 days)
Start: 0 days
P0: 15 days (15 days)
P1: 0 days (15 days)
P1: 28 days (43 days)
P1: 0 days (15 days)
P1: 28 days (43 days)
P2: 0 days (43 days)
P2: 26 days (69 days)
P2: 0 days (43 days)
P2: 26 days (69 days)
P3: 0 days (69 days)
P3: 18 days (87 days)
P3: 0 days (69 days)
P3: 18 days (87 days)
P4: 0 days (87 days)
P4: 15 days (102 days)
P4: 0 days (87 days)
P4: 15 days (102 days)
P5: 0 days (102 days)
P5: 18 days (120 days)
P5: 0 days (102 days)
P5: 18 days (120 days)
P6: 0 days (120 days)
P6: 19 days (139 days)
P6: 0 days (120 days)
P6: 19 days (139 days)

Figure 9. Gradual decline of regrowth capacity of a sample cultured in M1 and M2 in parallel

Pictures of Culture 8 development in M1 and M2. For each medium, the left column shows pictures taken right after seeding/passage (designated by "P"). The right column shows pictures taken right before the passage, demonstrating the evolution of the culture between passages. The sample was cultured in M1 and M2 for the same number of days until Culture 8 consolidated in M1 and stopped proliferating in M2, after a promising expansion for the first three passages. Images acquired with phase-contrast microscopy at 2.5× magnification. Scale bar: 500 μm.

VAF=1). Further validation methods to consider could be immunohistochemical staining for HGSC markers and histological comparison with the original tumor pathology slides, or RNA sequencing, including bulk or single-cell analyses (Senkowski et al., 2023).

For cultures intended to serve as research models over considerable periods of time, and undergoing extensive passaging and expansion, we recommend genomic validation of organoids at both early and late stages of the culture. Every newly developed organoid culture should be validated at the beginning of the exponential growth phase, when the stable expansion rate has been established (usually between passage 4 and 10). We suggest re-validating the culture about 10–15 passages after the first validation, that is, after 4–6 months in culture, post-establishment (Figure 3). Excessively early validation, such as passage 2, are typically not recommended, as the cultures usually do not present a stable expansion rate, or contain cell types other than cancer cells. For validation with WGS, we typically collect 2 pellets (one for the actual validation and one as a backup) that we snap-freeze. Each pellet is harvested from a confluent well with 10 domes of BME-hydrogel, to ensure enough DNA for whole-genome sequencing.

One of the characteristics of HGSC cells is their high chromosomal instability. Thus, we recommend carefully assessing the morphology and growth rate of a culture over time and terminating the culture if there are major changes. In our research, we typically use the cultures for downstream applications up to passage 25, and, if more analysis is needed, we re-establish the culture from the low-passage aliquot.

LIMITATIONS

The development of HGSC organoid cultures is an empirical process. Currently, we lack tools to reliably predict which samples are more likely to generate stable organoids when cultured in 3D and which medium will ultimately result in the generation of a successful culture. Additionally, although our method has the highest reported success rate of developing stable, long-term HGSC organoids, further optimization of additional media formulations or sample pre-processing protocols could further increase the success rate of organoid development. Finally, as this protocol is mostly focused on technical aspects and takes advantage of the media formulations reported by us previously (Senkowski et al. 2023), it does not take into consideration other physicochemical factors that could highly influence the culture of HGSC organoids, such as the oxygen and carbon dioxide pressure, BME hydrogel stiffness and others.

TROUBLESHOOTING

Problem 1

Incomplete pellet formation during organoid passage. Related to the passage of organoid cultures – step 47.

Potential solution

- Many dead cells in the sample (and hence, a large amount of cellular debris) or incomplete BME hydrogel digestion might influence the successful pellet formation.
- Spin down the organoid cell pellet at a higher centrifugal force (350 to 400 × *g*).
- If this does not work, pre-wet a P1000 pipette tip in TrypLE Express and gently resuspend the pellet before centrifuging it at a higher centrifugal force (350 to 400 × *g*).

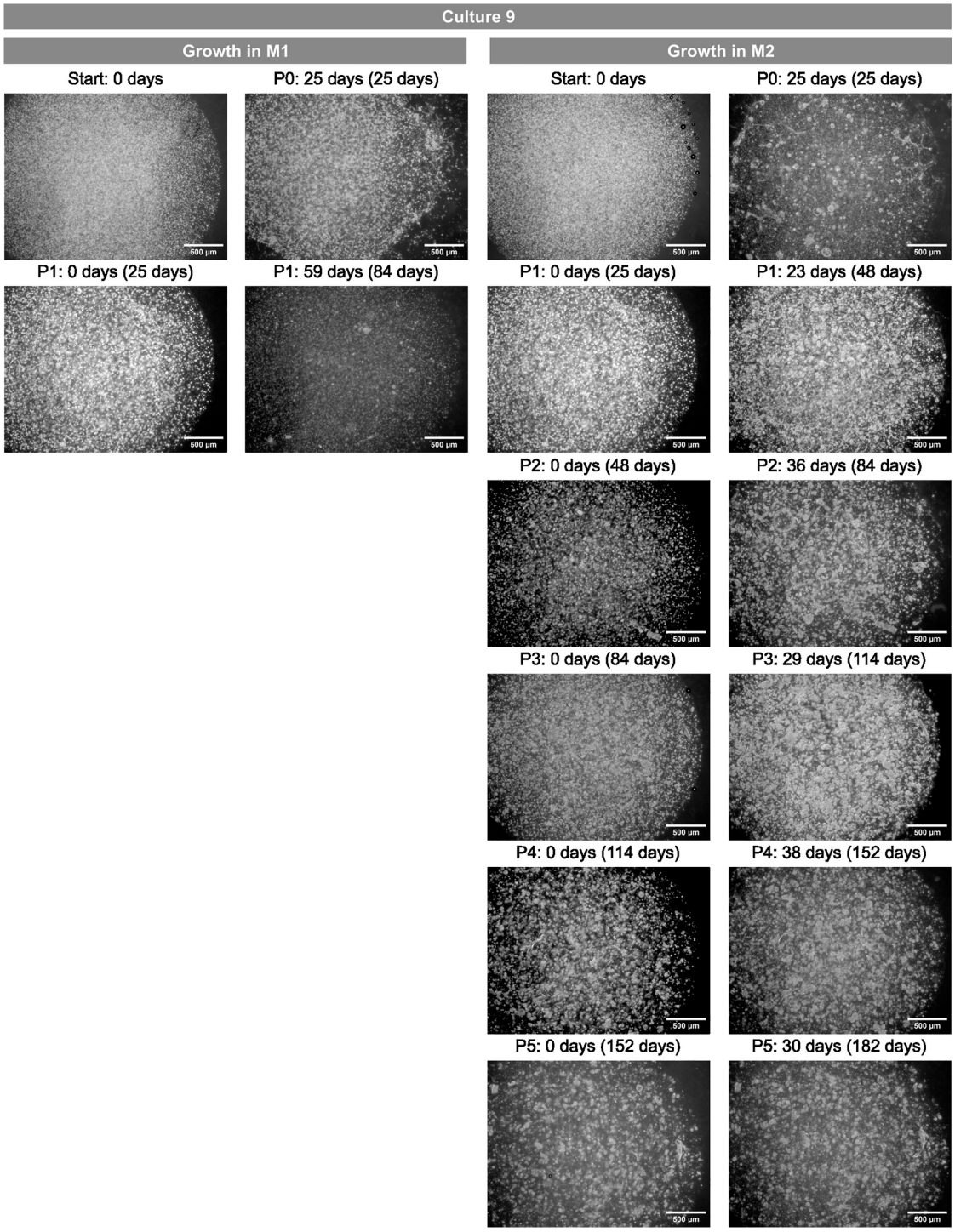

Figure 10. Delayed growth arrest of a sample in M2

Pictures of Culture 9 development in M1 and M2. For each medium, the left column shows pictures taken right after seeding/passage (designated by "P"). The right column shows pictures taken right before the passage, demonstrating the evolution of the culture between passages. The sample was cultured for 84 days in M1 and for 181 days in M2. The lack of regrowth capacity of the dissociated cells to form larger organoid-like structures becomes apparent after 1 passage in M1 and 5 passages in M2. Images acquired with phase-contrast microscopy at 2.5× magnification. Scale bar: 500 µm.

Problem 2

BME hydrogel domes merge after seeding. Related to the establishment of organoid cultures from patient samples – step 16; passage of organoid cultures – step 51.

Potential solution

- Ensure the plates used for seeding organoids are hydrophilic (e.g., cell culture-treated).
- Pre-warm the plate before seeding to ensure solidification of the BME hydrogel domes from the bottom.
- Avoid over-diluting the BME hydrogel with excessive leftover supernatant in the pellet, as it makes domes spread more on the plate.
- Avoid seeding the BME hydrogel domes too close to each other.
- Transport the plates carefully to the cell incubator while the BME hydrogel is still in a liquid state, as abrupt movements can cause the BME hydrogel domes to merge.

Problem 3

BME hydrogel domes collapse during medium addition and/or medium change. Related to the establishment of organoid cultures from patient samples – step 19; change of medium – steps 27–29; passage of organoid cultures – step 54.

Potential solution

- After digesting the culture and aspirating the supernatant around the cell pellet using a vacuum aspiration system, gently aspirate the remaining supernatant using a mechanical pipette, taking care not to disturb the cell pellet. Having a high volume of supernatant left can over-dilute the BME hydrogel and impair dome stability. Sometimes, the pellets are loose and get mixed with the supernatant. In these cases, use a higher concentration or undiluted BME hydrogel.
- Use the correct BME hydrogel concentration. Avoid over-diluting it with PBS, as this can affect dome stability.
- Avoid the formation of air bubbles when seeding the organoids.
- Add medium gently using a low flow mode on the electronic pipette controller.
- Passage the culture with collapsing domes, to avoid losing more material, and identify which one of the above-mentioned factors led to the collapse of the BME hydrogel domes.
- Usage of pure gel in cultures with a lot of dead cells. Digestion of the pellet with an extra 15′ with TrypLE.

Problem 4

Organoid-like structures are present and growing, but the culture is overgrown with stromal cells present in the tumor sample (Figure 11). Related to the establishment of organoid cultures from patient samples.

Potential solution

- Stromal cells from the tumor have a limited lifespan in M1 and M2, which are specifically formulated to promote the growth of the cancer cells, and they usually die after around 2 months of culture. Thus, we recommend proceeding with the organoid culture as normal, but not cryopreserving any material while the stromal cells are present.
- If the stromal cell overgrowth is severe and directly influences the growth of the organoid-like structures (they disappear, attach to neighboring cells or plastic) or destabilizes the BME hydrogel domes, we recommend passaging the culture, even when the organoid-like structures do not seem large enough. Passaging will destabilize the stromal cell network, decrease their viability, and facilitate the outgrowth of organoid-like structures.

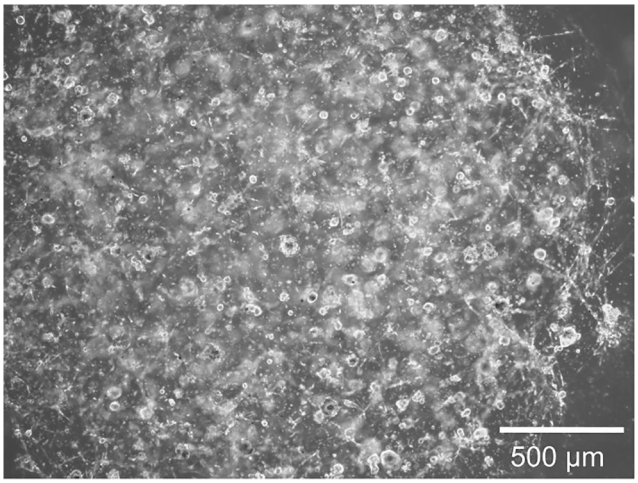

Figure 11. Presence of stromal cells during organoid development

Sample was maintained for 20 days in M2. Visible organoid-like structures and major fibroblast growth. A successful organoid culture was ultimately developed from the sample. Image acquired with phase-contrast microscopy at 2.5× magnification. Scale bar: 500 μm.

Problem 5

The culture is dominated by single cells of small size, which, after a few days of culture, die and are found floating in the culture medium (related to the whole process of establishing an organoid culture) (Figure 12). Related to the establishment of organoid cultures from patient samples.

Potential solution

- Most cells in the sample are likely immune cells. These cells have a short lifespan in M1 and M2 and will die during the first 2 to 3 weeks of culture.
- We recommend proceeding with the culture as normal. If large numbers of dead cells escape the BME hydrogel domes and destabilize them, we recommend passaging the culture to preserve the cancer cells within the BME hydrogel domes. After the first passage, most single cells should disappear.

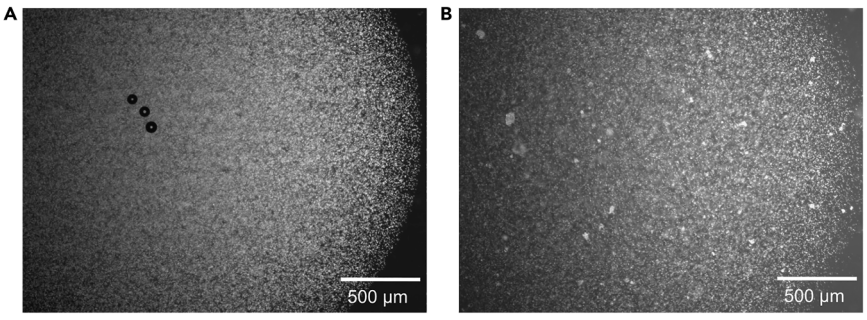

Figure 12. Organoid culture dominated by immune cells

(A) Cell suspension embedded in the BME hydrogel on Day 0, with single cells dominating the hydrogel dome.
(B) The same BME hydrogel dome after 35 days of culture in M1, without passaging. Single cell density has decreased, and scattered organoid-like cell clusters are present.
A successful organoid culture was ultimately developed from this sample in M1. Images acquired with phase-contrast microscopy at 2.5× magnification. Scale bar: 500 μm.

Problem 6

There is an apparent fungal or bacterial contamination in the culture. This can occur during the establishment of organoid cultures, due to contaminated patient samples, or during the culture of stable organoid cultures, as fungal contamination can arise due to environmental reasons. Related to the establishment of organoid cultures from patient samples.

Potential solution

- In case of contamination in newly started organoid cultures, if there is some spare material from the original sample, we recommend terminating the contaminated culture and starting a new one from the spare material.
- If starting a new organoid culture is not a possibility, and the contamination is only visible in one of multiple wells of the plate, we recommend removing the contaminated medium from that well and adding 5 mL of 70% ethanol for 5 min. Then, remove and discard the ethanol and BME hydrogel domes from the well and rinse it with PBS. Culture the other wells as normal, but keep the plate in a separate cell incubator, away from other cultures, for at least 30 days, and monitor the culture daily. This procedure can be used in case of fungal contamination during the culture of stable organoid cultures.
- If all wells are contaminated with fungi and there is no spare primary material available from that sample, amphotericin B or Fungin could be used, according to the manufacturer's instructions, to eradicate the infection. If so, keep the plate in a separate cell incubator, away from other cultures, for at least 30 days and monitor the culture daily.

Problem 7

There are only a few viable cells in the initial processed tumor sample or very few organoid-like structures re-growing in between passages of a newly started culture (Figure 13). Related to the establishment of organoid cultures from patient samples.

Potential solution

- Even when it is not possible to seed or passage the cells into a dense culture, we do not recommend seeding the cells in less than 5 domes (100 μL of BME hydrogel) per culture. Resuspending cell pellets in lower gel volumes might result in accidental loss of cell material, as low cell numbers usually will not result in easily observable pellets after centrifugation. Furthermore, the remaining supernatant might dilute the BME hydrogel, which could result in unstable domes prone to fragmentation.
- In cases of extremely low cell numbers, we recommend prioritizing the successful transfer of the cellular material during passaging and seeding rather than increasing the density of the culture, even if the cells are over-diluted with an excessive amount of the BME hydrogel. Viable cancer cells in a suitable medium should nonetheless resume growth, even though often at a slow rate.

Problem 8

During passaging, organoids have been over-dissociated into a mostly single-cell suspension. After seeding, there are mostly single and dead cells present. Related to the establishment of organoid cultures from patient samples.

Potential solution

- Many established organoid lines tolerate over-dissociation well and re-grow from single-cell suspensions without delay. However, some can be sensitive to over-pipetting and show increased cell death. For such cultures, avoid prolonged (>25 min) TrypLE Express incubation and over-pipetting the cell pellet post-digestion. Resuspending the organoid pellet in the BME hydrogel should

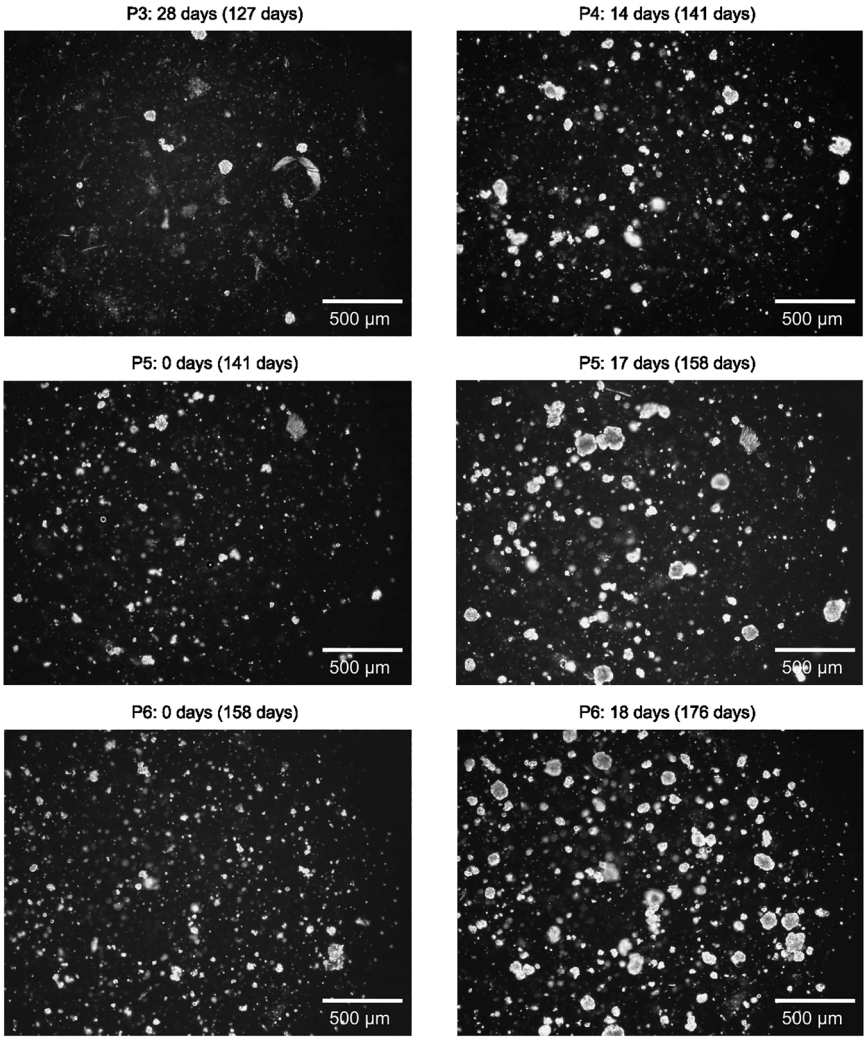

Figure 13. Organoid culture with a few visible organoid-like clusters

Pictures show the development of an organoid culture in M1. Notice that the size and number of organoid-like structures increase during the passages. A successful organoid culture was ultimately established in M1. Images acquired with phase-contrast microscopy at 2.5× magnification. Scale bar: 500 µm.

be stopped immediately once the suspension appears homogeneous to the naked eye. If unsure, a sample of the suspension could be inspected under a microscope to determine whether additional pipetting is needed.

- In the case of increased cell death and delayed organoid regrowth, we recommend allowing extra time, beyond the regular passaging interval, before the next passage. Oftentimes, the passaging ratio should also be reduced, to re-establish the previous passaging ratio and interval at the following passages.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Wojciech Senkowski (wojciech.senkowski@bric.ku.dk).

Technical contact

Technical questions on executing this protocol should be directed to and will be answered by the technical contact, Wojciech Senkowski (wojciech.senkowski@bric.ku.dk).

Materials availability

Patient-derived HGSC organoid cultures are available at the OvaCure Collection (www.ovacurecollection.com) through the Auria Biobank (Turku, Finland).

Data and code availability

Additional data supporting the figures can be provided upon request to the lead/technical contact, Wojciech Senkowski (wojciech.senkowski@bric.ku.dk).

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AUTHOR CONTRIBUTIONS

L.G.-M. conceptualized, drafted, and edited the manuscript, with writing contributions from W.S. The protocol for organoid establishment was initially generated by W.S. and included methodology and investigation from L.G.-M. W.S., L.G.-M., and L.M.-G. performed the experimental work. L.M.-G., E.V., J.H., K.W., and W.S. reviewed and edited the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

DECLARATION OF GENERATIVE AI AND AI-ASSISTED TECHNOLOGIES IN THE WRITING PROCESS

During the preparation of this work, the author(s) used Grammarly to correct the grammar and spelling and improve readability. The tool was only used in the first draft of the manuscript. After using this tool or service, the author(s) reviewed and edited the content as needed and take(s) full responsibility for the content of the publication.

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