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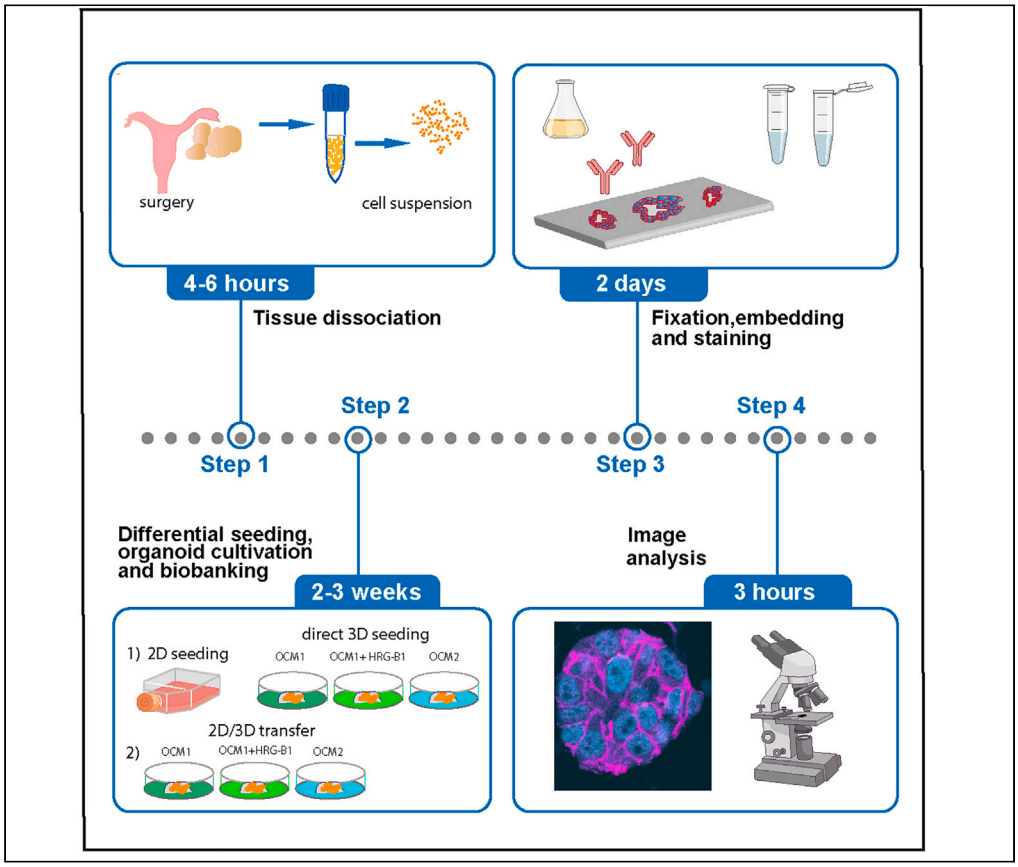
Protocol to optimize the biobanking of ovarian cancer organoids by accommodating patient-specific differences in stemness potential



We present a protocol for effective biobanking of epithelial ovarian cancer organoids, considering the heterogeneous clinical presentation and high recurrence rates. Our protocol involves parallel testing of three media to identify patient-specific optimal conditions. We describe steps for tissue dissociation, differential seeding, organoid cultivation, and biobanking. We outline procedures for fixation, embedding, and staining for confocal imaging. Furthermore, we demonstrate that brief cultivation of isolates in 2D on plastic enhances organoid-forming potential in selected lines, expanding their application scope.

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

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Highlights

Biobanking of ovarian cancer organoids based on patient-specific medium requirements

The systematic approach accounts for variability in samples and improves yield

Enables robust long-term expansion, which is necessary for downstream applications

Embedding and staining procedure of organoids to obtain high-resolution confocal images

Trillsch et al., STAR Protocols 4, 102484
September 15, 2023
https://doi.org/10.1016/j.xpro.2023.102484

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Protocol to optimize the biobanking of ovarian cancer organoids by accommodating patient-specific differences in stemness potential

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https://doi.org/10.1016/j.xpro.2023.102484

SUMMARY

We present a protocol for effective biobanking of epithelial ovarian cancer organoids, considering the heterogeneous clinical presentation and high recurrence rates. Our protocol involves parallel testing of three media to identify patient-specific optimal conditions. We describe steps for tissue dissociation, differential seeding, organoid cultivation, and biobanking. We outline procedures for fixation, embedding, and staining for confocal imaging. Furthermore, we demonstrate that brief cultivation of isolates in 2D on plastic enhances organoid-forming potential in selected lines, expanding their application scope.
For complete details on the use and execution of this protocol, please refer to Hoffmann et al.[1]

BEFORE YOU BEGIN

This protocol describes in detail an approach to generate stable long-term organoid culture from solid tumor deposits of epithelial ovarian cancer. Based on the principal findings previously published,[1] we show the requirement to test different media compositions and modes of seeding on the primary isolates to identify adequate exogenous support for the maintenance of the stemness *in vitro*, which shows patient-specific variability in this malignancy. While most organoids show optimal growth in low WNT, and high BMP environments (OCM1 medium), some lines do benefit from supplementation of RSPO1 and FGF10 (OCM2). Also, we demonstrate the positive effect by adding Heregulinß-1, to the minimal organoid OCM1 medium in individual cases. Our experimental matrix, designed to accommodate observed differences in progenitor potential also includes brief cultivation of the isolates in 2D on plastic, where we also observed improved organoid forming potential in some of our lines. If the optimal *in vitro* cultivation conditions have been achieved, ovarian cancer organoids have unlimited expansion potential and are suitable for a broad range of applications, including multiwell drug testing, imaging analysis as well as genomic editing.

A complete least of reagents and equipment is listed in the key resources table.

Preparation for progenitors isolation

The Lab where tissue processing and cell isolation are performed must have Biosafety Level 2 safety permission, and Laminar Flow cabinets. As primary surgery material, in the absence of comprehensive testing of all relevant pathogens (HCV, HBV, HIV, etc), could potentially be infectious strict

compliance with institutional bio-safety regulations regarding the usage of personal protective equipment (e.g., Lab coats, gloves), decontamination of surfaces and waste disposal is necessary.

1. Thaw an appropriate amount of Cultrex RGF BME Type 2 on ice for a minimum of 2 h before use. Overnight slow thawing is also a possibility.
   a. Cultrex RGF BME2 in original vials is stored at −80°C.
   b. Smaller aliquots (1 mL) from large vials (5 or 10 mL) always prepare following the slow overnight thawing on ice.
   c. These 1 mL aliquots for immediate use (within 1 month) can be stored at −20°C.
2. Prepare one-time scalpels and sterilized dissection tools (scissors and tweezers), and filter inserts for falcons (400 μm).
3. Water bath pre-warmed at 37°C.
4. Medium preparation.
   a. Tissue Collection medium (can be stored for four weeks at 4°C) −20 mL per 50 mL Falcon.
   b. Organoid growth medium OCM1, OCM1+ HRG1-B1, OCM2 (prepared fresh one time per week).
   c. Enzymatic digestion solution (prepared fresh immediately before use).
5. Liquid nitrogen container (small amount of liquid nitrogen for snap freezing).
6. Transport box with cool packs.

Production of RSPO-1 conditioned medium

7. Take out 1 vial of 293T HA RSPO1 Fc cell line, stored at −80°C.
8. Defreeze cells quickly at 37°C and wash with 10 mL ADF++ with 10% FCS.
9. Resuspended Cell pellet in 12 mL ADF+++ and seeded in a T75 flask.
10. When the cells reach confluence split 1:6, with TrypLE (4 min, 37°C) and seed in a new T75 flask.
11. The next day add Zeocin (1,25 μL/mL) to the medium.
12. Upon confluency split 1:20 into T175 flasks (the number of the flask is variable depending on the desired production volume) in 30 mL ADF++10% FCS supplemented with Zeocin.
13. When cells reach ∼50% confluence start conditioned Media production: change medium to 40 mL ADF++ with 5% FCS without Zeocin.
14. After 3 days collect 1 st supernatant.
15. Centrifuge 10 min at 1200 × *g* to get rid of the debris and store supernatant in the sterile bottle at 4°C.
16. Replenish cells with a new medium (ADF++ 5% FCS).
17. Collect the second supernatant after additional 3–4 days.
18. Centrifuge for 10 min at 1200 × *g* and pool with the first supernatant.
19. Filter the conditioned medium through a 0.2 μm Bottle Top Filter.
20. Aliquot to 15 mL falcon tubes and store for long-term storage (up to half a year) at −20. Thawed aliquots are stable in the fridge for 1 week.

Institutional permissions

Collection of patient material from ovarian cancer surgeries and generation of organoids has been performed with the approval of the Ethics Committee of LMU University (17-471), following all relevant EU, national, and local regulations. Written consent has been obtained from every patient.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
E-Cadherin (Cdh1) antibody	BD	610181
SOX17 antibody	R&D Science	AF1924
DAPI	Thermo Fisher	62248
Cy™5 AffiniPure Donkey Anti-Mouse IgG	Jackson Immuno	715-175-151

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Donkey anti-Goat IgG Alexa Fluor Plus 488	Thermo Fisher	A32814
Biological samples		
Ovarian cancer tumor tissue	N/A	N/A
Chemicals, peptides, and recombinant proteins		
Human recombinant BMP2	Gibco	PHC7146
Human recombinant EGF	Gibco	PHG0311L
Human FGF10	PeproTech	100-26
Human recombinant Heregulinβ-1	PeproTech	100–03
A-83-01 (TGF-β RI Kinase inhibitor IV)	Merck	616454
N2 (100×)	Gibco	17502–048
B27 50×	Gibco	17504–044
Nicotinamide	Sigma	N0636
N-acetyl-L-Cystein	Sigma	A9165
Y-27632	Tocris	1254
Advanced F12	Gibco	12634028
GlutaMAX	Thermo Fisher	35050038
HEPES (1 M)	Gibco	156630080
FBS	Gibco	10270106
Cryo SFM	PromoCell	C-29912
PBS	Gibco	14190–094
Collagenase I	Thermo Fisher	17100–017
RBC buffer	Sigma	11814389001
TrypLE Express	Gibco	12604–013
Penicillin-Streptomycin	Sigma-Aldrich	P4333-100
Primocin	InvivoGen	ant-pm-05
Zeocin	Invitrogen	R25001
Pen Strep	Gibco	15140–122
100 Sterican 26 G	Braun	4657683
100 Sterican 27 G	Braun	4657705
Omnifix 1 mL	Braun	3570519
PFA	Morphisto	11762.01000
Formalin	Morphisto	10192.05000
Roticlear	Roth	A538.5
Histogel	Thermo Fisher	Epredia™ HG-4000-012
DAKO citrate buffer, pH 6.0, 10× antigen retriever	Sigma-Aldrich	C9999-1000ML
Leica Paraplast Standard	Leica Biosystems	*39602012*
Trypan blue solution	Sigma-Aldrich	T8154
Tween-20	AppliChem	A4974-0100
Triton X-100	Sigma	T8787
Cultrex Reduced Growth Factor Basement Membrane Extract, Type 2, Pathclear, Alternative: Matrigel, Growth Factor Reduced Basement membrane matrix	Bio-Techne,R&D Systems Corning	3533-005-02 356231
Experimental models: Cell lines		
293T HA Rspo1-Fc	R&D	3710-001-01
Alternative: R-Spondin1 expressing Cell line	Sigma-Aldrich	SC111
Software and algorithms		
LAS X core	Leica Microsystems	https://webshare.leica-microsystems.com/latest/core/widefield/
Other		
Costar® 48-well Clear TC-treated	Corning	3548
Falcon® 24-well Polystyrene	Corning	351447
CELLSTAR cell culture flask, 75 cm^2	Greiner Bio-one	658175
CELLSTAR cell culture flask, 25 cm^2	Greiner Bio-one	690160

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
CELLSTAR cell culture flask, 175 cm^2	Greiner Bio-one	661175
Nunc Cryovials	Thermo Fisher	375418PK
Surgipath Paraplast	Leica	39602012
PluriStrainer 400 µm	pluriSelect	43-50400-01
Feather scalpel	Pfm Medical	200130010
Bottle-top vacuum filter 0,2 µm	Corning	430049
Parafilm	Omnilab	5170002
Leica TCS SP8 X White Light Laser Confocal	Leica Microsystems	

MATERIALS AND EQUIPMENT

Prepare stock solutions of growth factor reagents and store them at −20°C, except for A83-01 and Nicotinamide (storage at +4°C). Thaw stock solutions components immediately before pipetting the "ready to use" 3D or 2D medium. Avoid the negative effects of repeated freezing and thawing cycles by aliquoting the stock solutions.

Ovarian cancer medium 1 -OCM1

Reagent	Final concentration	Amount
BMP2 (10 µg/mL)	10 ng/mL	20 µL
EGF (10 µg/mL)	10 ng/mL	20 µL
A83-01 (0.5 mM)	0.5 µM	20 µL
Y-27632 (3 mM)	9 µM	67 µL
N2 100×	1×	200 µL
B27 50×	1×	400 µL
NAC 500 mM	1.25 mM	50 µL
NIC 1 M	5 mM	100 µL
Primocin	1×	40 µL
ADF++		19.063 mL
Total		20 mL

Herβ−1 (HRG1-B1) is added to OCM1 at a final concentration of 50 ng/mL.

Ovarian cancer medium 2-OCM 2

Reagent	Final concentration	Amount
RSPO1 conditioned medium	10%	2 mL
EGF (10 µg/mL)	10 ng/mL	20 µL
FGF10 (100 µg/mL)	100 ng/mL	20 µL
A-83-01 (0.5 mM)	0,5µM	20 µl
Y-27632 (3 mM)	9 µM	67 µL
N2 100×	1×	200 µL
B27 50×	1×	400 µL
NAC 500 mM	1.25 mM	50 µL
NIC (1 M)	5 mM	100 µL
Primocin	1×	40 µL
ADF++		17.084 mL
Total		20 mL

3D media containing growth factors should be prepared once per week and kept at 4°C

2D medium

For 12 mL medium (T75 flask): 11.2 mL ADF++, 600 µL FCS (5% final concentration), 12 µL EGF (10/ng/mL final concentration), and 61 µL ROCK inhibitor+120 µL pen/strep 1% (final concentration).

ADF ++:490 mL Advanced F12 supplemented with 5 mL Glutamax (100×) + 5 mL (1 M HEPES)-can be kept for longer periods (1 month) at 4°C.

Tissue Collection medium

ADF++.

Dilution medium for IF staining

1× PBS 0.01 M, 1% BSA, 0.05% Tween-20.

STEP-BY-STEP METHOD DETAILS

Surgical sampling, tissue processing, and initiation of organoids culture

Timing: 4–6 h

To initiate organoid culture from solid tumor deposits of EOC, perform tissue dissociation and cell isolation on fresh material to maximize the preservation of progenitors' capacity *in vitro*. This section outlines a step-by-step approach to seed primary isolates in different media and 2D to 3D vs. direct 3D seeding format, to identify optimal organoid-forming conditions for each patient.

1. Transfer the tissue to a Petri dish, within a cell culture Laminar flow.
   a. Wash extensively in PBS without Ca^{++}, Mg^{++}.
   b. Homogenize with scissors.

   Note: Samples are transported directly to the lab where cell isolation protocol is immediately implemented. However, it is possible to store tissue samples within a collection medium for up to 20 h at 4°C without negatively affecting organoid forming efficiency or longevity of the culture.

2. Collect small fragments (3–5 mm) into Cryo tubes and perform snap freezing in liquid nitrogen.
3. Further, put 1–2 fragments into formalin for fixation (24 h) and embed them in paraffin for comparative staining with organoids.
4. Mince further the remaining tissue with a scalpel to maximize mechanical dissociation before enzymatic digestion. It is recommended that during homogenization tissue is soaked in PBS, to avoid a drying-out effect.
5. Digest tissue in PBS (w/o Ca^{++} and Mg^{++}) + collagenase I (stock solution 5 U/μL, final con 1U/μL) + Y-27632 3 μM final concentration.

   Note: Define the volume of the digestion mixture based on the amount of tissue sample. For optimal results, 2 cm^3 of the tumor should be dissociated in a total of 15 mL in a 50 mL Falcon tube. If tissue material is limited (e.g., biopsies) downscale the volume of the digestion accordingly. Biopsy samples require only 2–3 mL of enzymatic solution to achieve adequate dissociation.

6. Incubate 1.5 h in a 37°C water bath with sporadic vigorous vortexing (every 20 min for 10 s) to enhance dissociation.
7. Inactivate and wash with 15 mL ADF++. Centrifuge 5 min at 300 g.
8. Discard the supernatant, add 5 mL new ADF++, and filtrate through the cell strainer 400 μm into the fresh 50-mL-Falcon tube.
9. Centrifuge 5 min at 300 g.
10. Discard the supernatant, add 5 mL RBC Buffer, and incubate in the water bath for 5 min.
11. Stop lysis with 5 mL ADF++ and centrifuge for 5 min at 300 g.
12. Resuspend pellet in 3 mL ADF++.
13. Cell counting Neubauer Chamber or automatic cell counter.
    a. For manual counting, dilute the cell suspension 1:2 with Trypan blue (for example 10 μL of the sample+ 10 Trypan blue), to determine the number of viable cells.

b. Fill the sample in the Neubauer counting chamber and evaluate the number of cells in 4 big squares.
c. Calculate the cell yield per mL, by averaging the number of cells per square, factoring in dilution, and multiplying by 10^4.

14. For direct 3D seeding (Graphical abstract) Seed 30 000 cells per 25 μL Cultrex in 48-well format according to the scheme.
    a. Calculate the total number of cells needed for all Cultrex wells and separate accordingly ADF++ cells suspension to the new tube. Spin down the cells for 5 min at 300 g and remove the supernatant. Add cold Cultrex at an appropriate amount (250 μL for 10 wells in 48-well format).
    b. Distribute isolates in Cultrex droplets (25 μL) to the pre-warmed empty 48-well plate.
    c. Make sure to thoroughly mix the cells in the Culturex without creating bubbles.
    d. Incubate the plate for a minimum of 30 min at 37°C so that Cultrex solidifies.
    e. Add appropriate growth medium, 250 μL per well.

Note: This is an optional format. 24 well format with 50 μL of Cultrex and 50 000 seeded cells per well can also be chosen.

15. Remaining cells 1 × 10^6 seed to T75 Flask in the 2D medium.
    a. If fewer cells are available consider seeding in the T25 flask.
16. Allow cells in the flask to adhere to the 3–5 days. Do not change the medium first 72 h.

2D to 3D transfer and evaluation of organoid growth

Timing: 2–3 weeks

This section describes how primary isolates isolated and expanded in 2D are transferred to 3D cell culture, and how to perform a comparative assessment of organoid growth under different conditions.

17. Detach cells, by washing the monolayer 2× with 5 mL PBS followed by 1 mL of TrypLE.-Incubate 10 min at 37°C.
18. Determine cell number as in 13) and seed as in 14).
19. Image the cultures (directly seeded in 3D or organoids derived after 2D/3D transfer) at least 1× per week to document long-term growth.
20. Select the best medium condition for the long-term expansion at 14–21 days post-isolation.
21. Wait at least 14 days before classifying the sample as "no growth" as the speed of organoid formation varies substantially between donors.
22. If organoid growth is observed in several media expand all conditions, differences in growth pattern are in some cases apparent only during extended passaging.

⚠ CRITICAL: Do not propagate primary isolates in 2D culture, by splitting. Perform a transfer to Cultrex BME 2 in passage 0 as soon as cells reach confluency.

⚠ CRITICAL: Tissue fragments are being selected and retrieved by an experienced gynecologic oncologist during multi-visceral surgery for ovarian cancer (primary debulking, interval debulking following neoadjuvant chemotherapy, or surgery for recurrent disease), diagnostic laparoscopy, or by CT-directed biopsy. If possible, preferred localizations with viable tumor load include the peritoneum of the abdominal wall, pelvis, paracolic gutter, and diaphragm, as well as adnexa and greater omentum (obviously necrotic areas in large tumor nodules should be disregarded, e.g., omental cake). Place tissue fragments immediately after surgery in the falcon with collection medium and prepared for transport at 4°C (transport box with cool packs).

Passaging of organoids, long-term cultivation, and biobanking

Timing: 2–3 weeks

This section describes how to perform the long-term expansion of organoid lines by enzymatic digestion and mechanical dissociation. For optimal biobanking, preserve new lines when organoids have been sufficiently expanded to enable the generation of several stock vials. The availability of multiple stocks from each donor line safeguards long-term experimental planning. As freezing and thawing are associated with a certain degree of cell loss, pool 2–3 wells per 1 stock vial.

23. Add 250 µL of cold ADF++ medium (in 48-well format) or 500 µL for 24 well and thoroughly scrape the bottom of the plate with a pipette tip with intermittent pipetting up and down, to ensure that Cultrex is disrupted. Transfer cell suspension to 15 mL Falcon, add an additional 1 mL of cold ADF++, mix, and keep on ice.
    a. You can pool technical replicate wells 2–3 together to ensure even expansion.
    b. Spin down the organoids for 5 min at 300 g.
    c. Discard the supernatant and if the Cultrex layer is still visible on top of the pellet repeat the washing step.
24. Add 1 mL of TrypLE (stored at 20°C–25°C).
25. Incubate in a water bath for 7–10 min. Periodic vortexing for 10 s is recommended to improve the efficiency of dissociation. If large cell aggregates are still present (visible against the light in the falcon tube) you can passage the suspension 3–5 times through a 1 mL cannula with a 27G syringe.

Note: Passing through the syringe is optional, and can be performed with needles in the range of 23 gauge to 27 gauge depending on the requirement for the dissociation efficacy. Dissociation to smaller clumps and single cells does improve the uniformity of the suspension and leads to lower variability between the wells.

26. Add 1 mL of cold ADF++ to stop the reaction.
27. Centrifuge 5 min at 300 g.
28. Remove supernatant.
29. Add an appropriate volume of Cultrex. For example for the 50 µL of parental passage (1 × 24 well) resuspend in 150 µL of fresh Cultrex to achieve a 1:3 split.
30. Pipet up and down carefully and distribute to the prewarmed plate.
31. After incubation of min 30 min at 37°C add medium, 500 µL per well.
32. Medium change 2× per week.
33. Make pictures in regular intervals of the cultures to allow for systematic comparisons between different conditions.
34. If organoid formation efficiency is similar in different media, consider long-term passaging of multiple conditions. In many cases, clear differences in expansion potential are obvious only after >1 month of cultivation.

⚠ CRITICAL: Make sure that ADF++ is properly cooled before harvesting the organoids and that mixing steps are thoroughly performed. The efficiency of removal of the extracellular matrix which dissolves below 4°C is critical for optimal dissociation of the cells in the subsequent steps.

⚠ CRITICAL: When removing the medium in step 28 try to leave as little as possible residual liquid as it dilutes the Cultrex BME2 and could increase the fragility of the droplets.

Cryopreservation of organoids

Timing: 30 min

This section describes the procedure for freezing organoid lines. As freezing and thawing are associated with a certain degree of cell loss, 2–3 wells should be pooled per 1 stock vial.

35. Identical to step 23.
36. Take 1.8 mL Nunc cryovials, label them (name, passage, kind of medium, date of freezing), and put them on ice. Precool also the freezing medium (Cryo SFM) on ice.
37. Add 1 mL of CryoSFM to the organoid pellet and transfer to cryo tubes.
38. Store the tubes in precooled Mr. Frosty and place them at −80°C overnight.
39. Transfer the stock tubes to regular −80°C storage.
40. For long-term stable storage (>2 months) transfer the stocks to liquid nitrogen.

Note: We recommend the freezing of the organoids in the first week after passaging within the proliferative stage while fixing the organoids, and imaging analysis should be performed 10–14 days after the split when organoids are differentiated and reach optimal size.

Thawing of the organoids

Timing: 1 h

This section describes how to bring organoids into the culture after long-term storage.

41. Label and prepare a 15 mL falcon with prewarmed 9 mL ADF++.
42. Take the sample out of the −80°C freezer or liquid nitrogen and transfer it small table container prefilled with liquid nitrogen.
43. Transfer Organoid-stock carefully to a 37°C warm water bath while shaking softly.
44. Transfer organoid suspension drop by drop to the prepared falcon with 9 mL ADF++, and mix carefully.
45. Spin down for 5 min at 300 g.
46. Identical to steps 28–31.

Fixation of organoids and embedding in HistoGel

Timing: 2 h

This section describes the procedure of organoid fixation and preparation for embedding in paraffin, by using HistoGel.

47. Collect organoids like in step 23.
48. Add 3 mL of 4% PFA and incubate for 1 h at 20°C–25°C.
49. Wash two times with 5 mL PBS with spinning down the organoids for 3 min at 300 g.
50. Resuspend in 4 mL fresh PBS and store at 4°C until embedding.

Pause point: Fixed organoids are stable, and can be kept in the fridge for 1 month before embedding.

51. Prewarm HistoGel (stored at −20°C) in the heat block at 65°C.
52. Fixed organoids are usually sedimented at the bottom of the falcon and are visible so supernatant can be easily removed (Figure 1). If the pellet is disrupted during handling, centrifuge briefly (3 min at 300 g) and then remove PBS.
53. Add 100 µL of warm Histogel pipet up and down and transfer to the piece of Parafilm, leaving it to solidify at 20°C–25°C. around 15 min.
54. Transfer the firm droplet to the tissue Paraffin cassette.
55. Proceed according to standard tissue embedding protocols.

Figure 1. Preparation of the fixed organoids for the paraffin embedding

56. Cut 5–10 μm thick slices with a microtome, and mount them on slides.
57. Dry slides for 1 h at 65°C.
58. Store them until stained in a dry place.

⚠ CRITICAL: Embedding in HistoGel simplifies the embedding procedure and enables identical downstream workflow as for tissue fragments (Figure 1, Methods video S1).

Immunofluorescence staining of the organoid sections

Timing: 2 days

This section describes the protocol for Immunofluorescence staining of the organoid sections, for phenotypic characterization by confocal microscopy.

59. Move microscope slides through a series of glass trays filled with solutions:
    a. 2 × 15 min Roticlear.
    b. 15 min 100% Ethanol.
    c. 1 min 100% Ethanol.
    d. 10 min 96% Ethanol.
    e. 5 min 70% Ethanol.
    f. 5 min 50% Ethanol.
60. Wash 3 × 5 min in PBS on the shaker 100 r/min.
61. Place slides in thermostable chambers (plastic) and add antigen retrieval solution (TRIS-EDTA pH 9.0 or Citrate pH 6.0).
62. Place chamber with slides in Steamer for 30 min at 100°C, and leave them to cool down at 20°C–25°C.
63. Same as step 60.
64. Permeabilize for 15 min in 1% Triton X (in PBS) solution.
65. Same as step 60.
66. Mark the area on the slides where organoids/ tissue are located with a PAP pen liquid blocker to help the maintenance of the droplet in subsequent steps. Block with 100 μL per slide of 10% of donkey serum* in a Dilution medium.

Note: The type of serum depends on the selection of the host species for the secondary antibodies.

67. Wash one time with PBS.
68. Add primary antibodies in a dilution medium in 100 μL final volume.

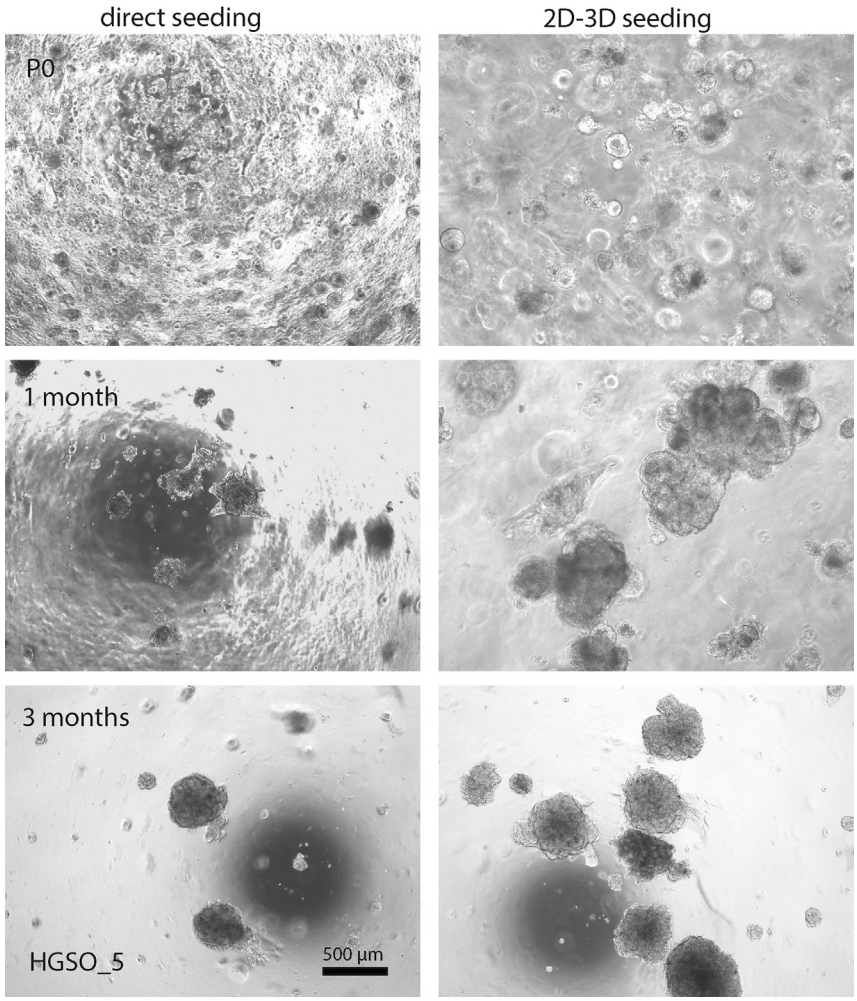

Figure 2. Example of High-grade serous (HGS) ovarian cancer line, the most frequent histological EOC type, where the brief expansion of epithelial progenitors in 2D positively influenced organoid growth in subsequent 3D culture. Scale bar=500 μm.

69. Incubate >16 h at 4°C in a moist-filled incubation tray.
70. Same as step 60.
71. Add secondary antibodies in a dilution medium in 100 μL droplets, and incubate for 2 h at 20°C–25°C.
72. Same as step 60.
73. Add DAPI diluted in Dilution medium 1:1000 and incubate for 10 min at 20°C–25°C.
74. Same as step 60.
75. Mount with Mowiol and place the coverslip. After drying overnight seal the slides with nail polish.
76. Image with Confocal microscope.

EXPECTED OUTCOMES

With this protocol, we outline the experimental procedure which, if fully implemented, enables the generation of stable organoid lines from ~50% of solid tumor deposits of EOC irrespective of the stage of disease (primary debulking, recurrent disease, and post-neoadjuvant surgical samples). In parallel, seeding and comparative analysis ensures the identification of optimal growth conditions supporting the maintenance of the regeneration potential *in vitro*, as illustrated by examples of

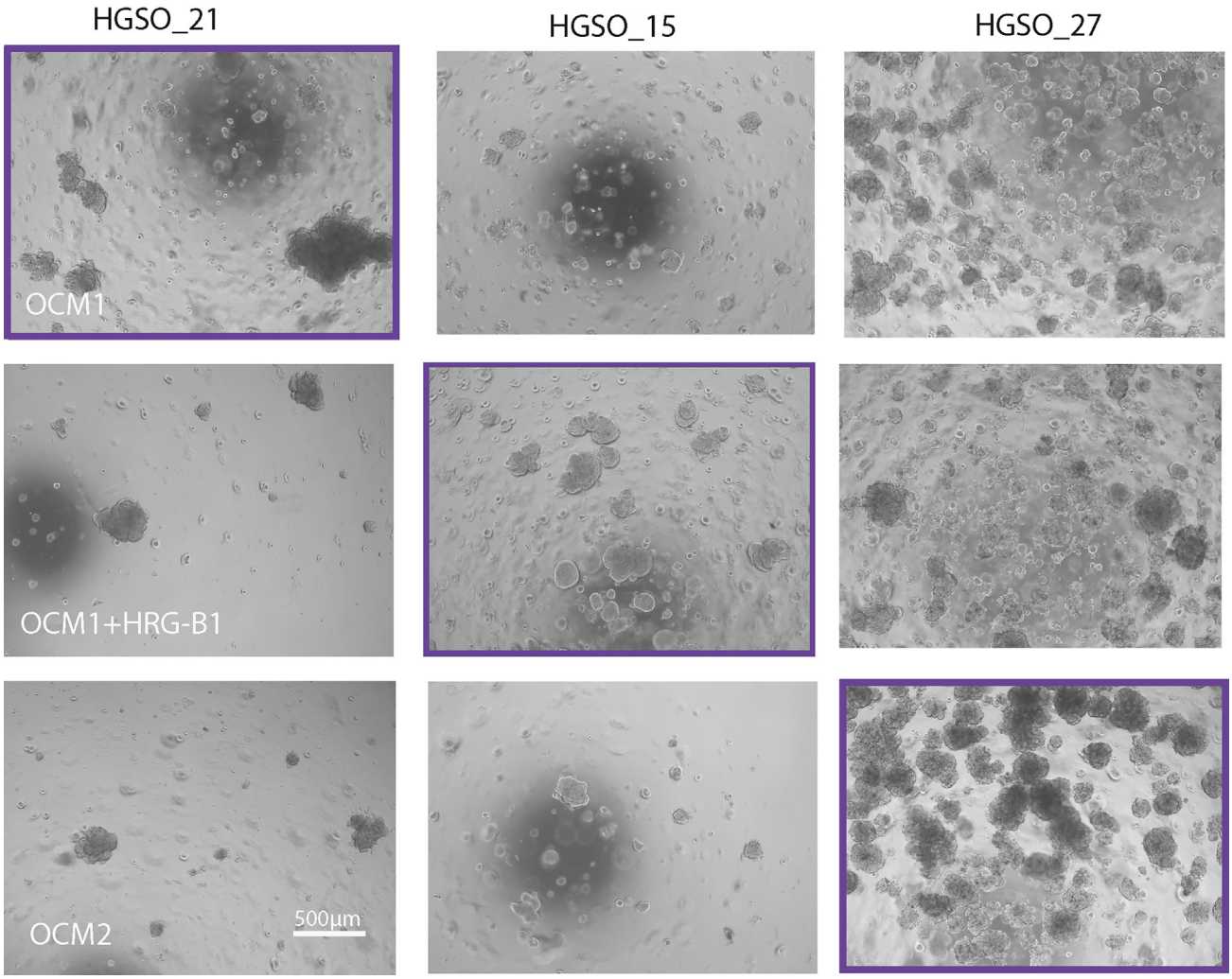

Figure 3. Examples of 3 donor tissues with diverging outcomes in different media, with the best result observed in OCM1, OCM1+HRG-B1 and OCM2 from left to right respectively. Scale bar= 500µm.

different donor samples (Figures 2 and 3). Ovarian cancer tissue from individual patients contains inherent variability in the regulation of stemness potential which at this stage cannot be prospectively predicted based on clinical criteria; thus, a parallel approach is required to identify the best medium for each line. If adequate conditions are provided, EOC organoids do have the capacity for unlimited long-term expansion (Figure 4). Examples of growth arrest of organoids or limited expansions, as previously reported and observed by us in some cases, appear to reflect the deficiency in medium composition supporting stemness maintenance of individual tumors *in vitro* rather than a reduced stemness capacity per se. These fully expandable organoid lines are suitable for a broad range of applications including drug testing studies of individual drug response and are amenable to gene editing (e.g., generation of CRISPR clones).

If cancer samples are obtained by the experienced surgeon based on routine assessment and identification of solid tumor deposits, the resulting organoid cultures have complete purity as routinely confirmed in comparison to parental tumor tissue which fatefully recapitulates the phenotype of the epithelial compartment (Figure 5) including adhesion/polarity as evident by CDH1 staining pattern as well as expression of homeobox master regulator transcription

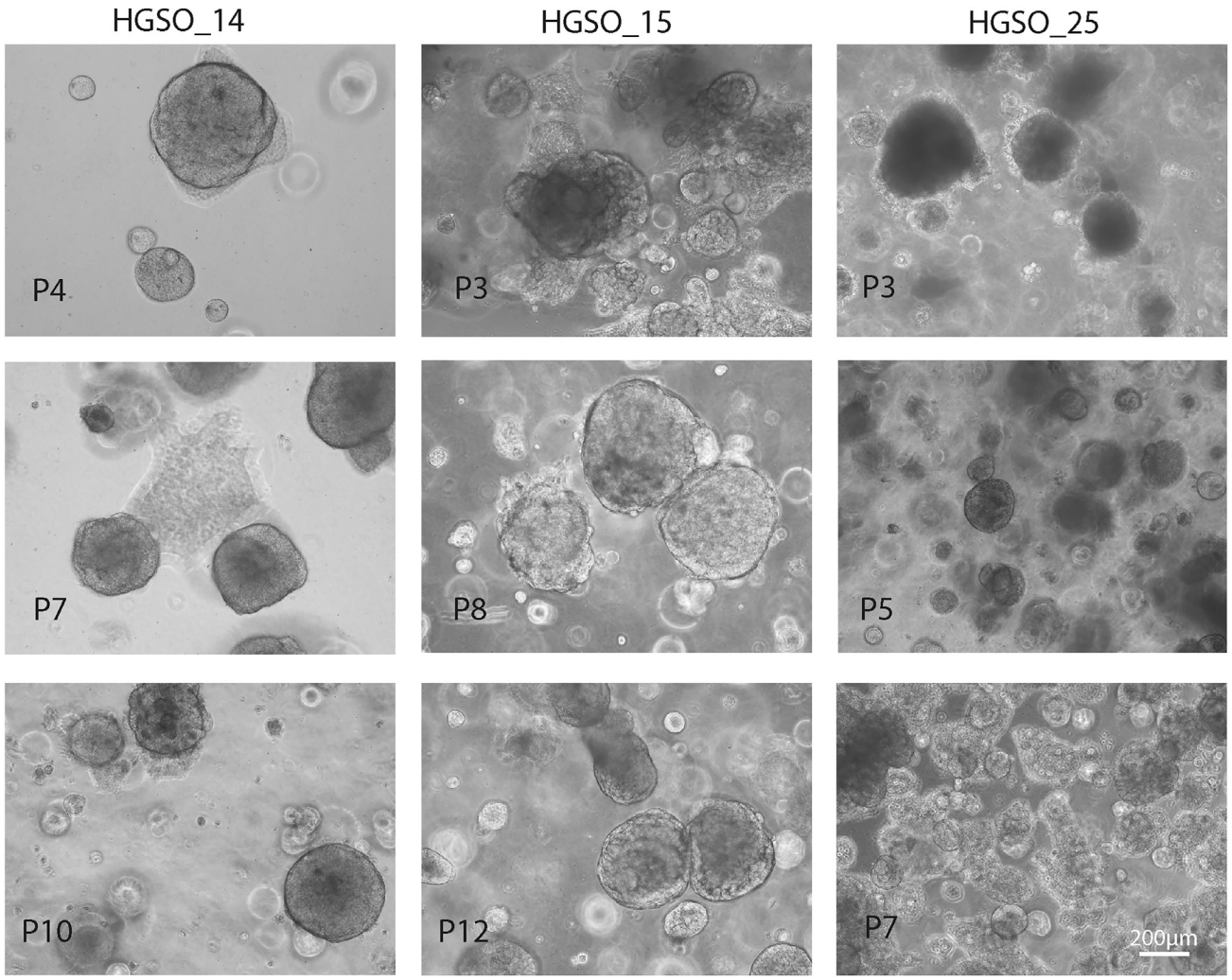

Figure 4. Illustration of the sustained long-term growth potential of the EOC organoid lines. Scale bar=200µm.

factors (SOX17). Here, we also describe in detail the procedure for embedding and immunofluorescence staining of organoid sections that yields high-resolution confocal images on the subcellular level.

LIMITATIONS

Previous studies, though in principle successful, already indicated inherent difficulties in performing bio-banking of ovarian cancer PDOs, concerning both efficacy of organoid formation as well as long-term passaging potential.[2–4]

Although our protocol greatly improves the efficacy and durability of biobanking of EOC, organoids by using specific media composition, there is still room for improvement. The protocol needs to be further adapted presumably by modifying the activity of key paracrine signaling pathways (e.g., NOTCH, FGF, EGF), to identify the media that are required for organoid growth from all EOC tumor deposits. Focusing on samples in which we did not achieve full expansion, we did observe organoid formation in the majority of cases, but some cultures were characterized by slow growth and complete arrest by passage P3. Overall, with this protocol, some degree of organoid formation is

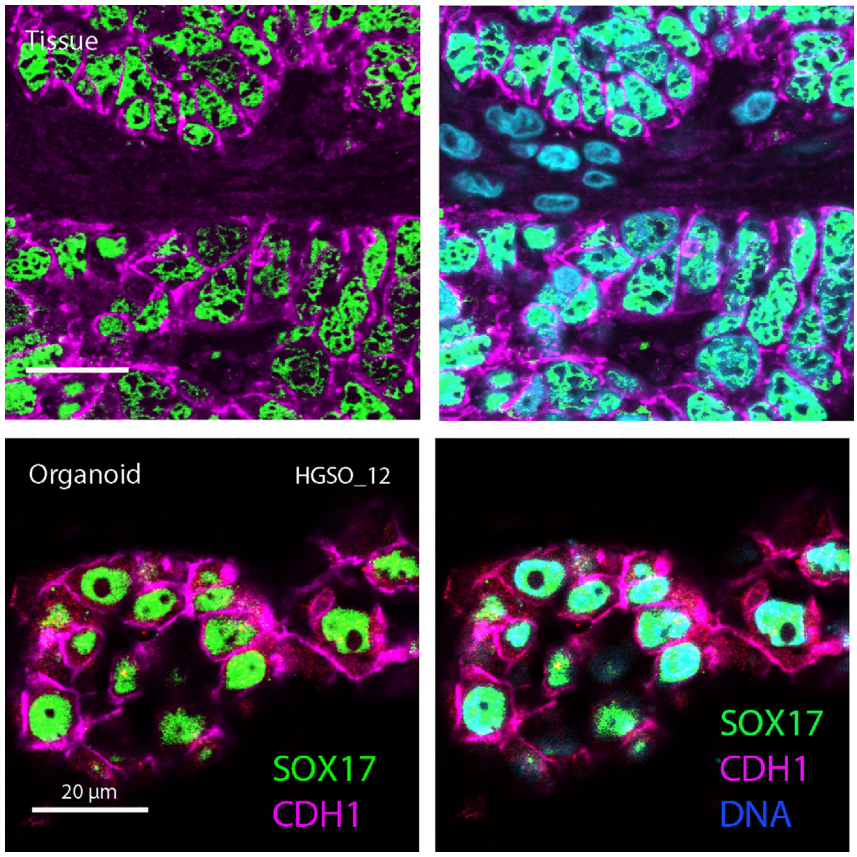

Figure 5. Confocal images of parental tumor tissue and organoid line, showing ubiquitous expression of SOX17 in tumor compartment and intact epithelial architecture (CDH1). Scale bar=20 µm

achieved in approximately 80% of samples. In the remaining 20% of cases, we did not observe organoid formation at all, despite the confirmed presence of cancer tissue in representative fragments of parental tissue. Thus, we conclude that further adaptations of the medium are necessary to fully cover the spectrum of variability in stemness regulation in ovarian cancer. As EOC organoids mimic tissue architecture and maintain *in vitro* main characteristics of epithelial phenotype, the presence of abundant adhesion and junctional complexes represents a challenge for effective enzymatic dissociation. Here we describe a combination of mechanical and enzymatic dissociation to improve the rate of cell dissociation in cases where large clumps remain after TrypLE. Using the needle with 23-27G (0,4–0,6 mm) and 1 mL syringe enables standardization of the mechanical dissociation approaches. In contrast, the procedure initially performed with pulled glass Pasteur pipette requiring specific manual skills is prone to large experimental variation (opening 0,5 mm–1 mm).[5] Further refinement of the enzymatic conditions and for cell dissociation, could also be tested such as including DNAse I treatment as previously described by[6] as the general protocol for the expansion of cancer PDOs.

TROUBLESHOOTING

Problem 1

Organoids form but only a very small number and are characterized by slow growth.

Potential solution

Carefully follow the progress of the culture and wait as long as you see evidence of continuous growth (increase in diameter) while evaluating the phenotypic appearance of the organoids: the

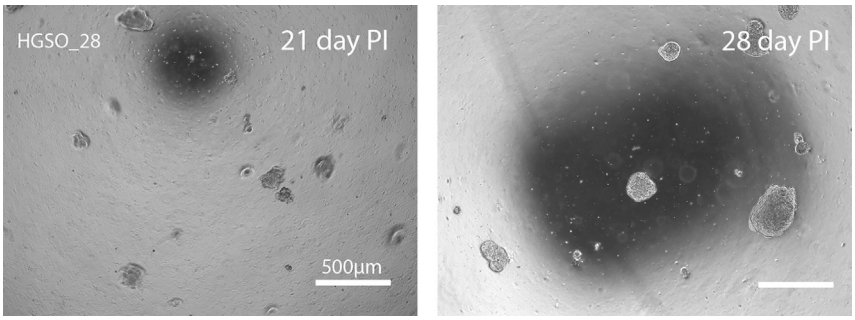

Figure 6. Example of slow-growing organoid line, showing vital growth pattern for 1 month after seeding. Scale bar= 500µm

presence of intact edges, the color and darkness (evidence of terminal differentiation), and the presence or absence of stress vacuoles (Figure 6). If continuous growth is noted, healthy organoids can be kept in one passage for up to 1 month.

Consider pooling the culture after splitting, and re-seeding at higher density.

Problem 2

No organoids form from fresh material.

Potential solution

Double-check media composition, and if all components have been properly stored. We also recommend that the preparation of media and stock solutions is performed by trained staff in a strictly controlled manner to minimize the risk of pipetting mistakes.

Check tumor purity of paraffin samples by IHC and, if needed, optimize the sampling procedure. While the presence of healthy epithelial progenitors as a potentially "contaminating" fraction is not of relevance in EOC due to its early peritoneal dissemination pattern and large tumor volume being removed in debulking surgery, a proper selection of samples used for organoid generation is an important contributing factor.

Check the conditions of the transportation of the tissue. In a collection medium, kept at 4°C vital progenitors can be isolated up to 20 h post-surgery. Longer delay is likely to negatively influence not only organoid forming efficiency but also the capacity for long-term expansion.

Problem 3

Organoid growth is at high density.

Potential solution

Consider reseeding organoids, by retrieving them (step 23) and proceeding to step 29. Do not perform dissociation procedures (enzymatic or mechanic) in intervals shorter than 10 days.

Problem 4

High well-to-well variability in organoid size and density.

Potential solution

While passaging several wells, organoids should be pooled (step 23a) and dissociated within one tube. In addition, improving dissociation efficiency should be considered (step 25). When seeding multiple new wells from one pool sample continuous slow pipetting of Cultrex suspension, up and down (3–4 times) should be applied between each well to avoid sedimentation of the cells during the process.

Problem 5

Cultrex droplet integrity is disrupted.

Potential solution

The frequency of this problem can be greatly reduced by careful handling of the medium change procedure, avoiding direct pipetting on the top of the matrix, and applying medium to the side along the walls of the wells. If the integrity of the droplet is sufficient to perform medium change organoids can be left within the culture as their growth is not affected. If fragments of the Cultrex completely lose contact with the bottom of the plate, consider reseeding the organoids (step 23) followed by step 29 or perform full passaging of the culture.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Mirjana Kessler (Mirjana.Kessler@med.uni-muenchen.de).

Materials availability

Organoid lines generated in this study, following guidelines and with approval of the Ethical Commission, which defines conditions for wider distribution and use for research purposes. This can be considered on individual bases upon reasonable request and is dependent on institutional approval.

Data and code availability

The protocol describes all datasets generated within this study.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2023.102484.

ACKNOWLEDGMENTS

The study is funded by the German Cancer Research Center (DKFZ) Heidelberg, German Cancer Consortium, DKTK, Partner site Munich. Paraffin embedding of tissue and organoids has been performed at the Core facility of the Institute of Anatomy, Faculty of Medicine, LMU Munich, Munich. Confocal imaging has been performed at the Core facility Bioimaging at Biomedical Center (BMC), LMU Munich. The authors want to thank Simone Hofmann, Gaby Weimer, Cornelia Herbst, Sabine Fink, and Martina Rahmeh for technical help.

AUTHOR CONTRIBUTIONS

M.K. designed and supervised the study, planned technical implementation, and performed data acquisition and analysis. F.T., A.B., B.C., and S.M. performed surgical tumor sampling and provided conceptual advice in the refining of the experimental steps. F.K. participated in the experimental implementation of the protocol. M.K. and F.T. wrote the manuscript. All authors critically read and critically approved the manuscript.

DECLARATION OF INTERESTS

M.K. is listed as an inventor on a patent related to a medium for ovarian cancer organoids. F.T. received research funding, honoraria, and travel expenses from and is a member of the advisory board of AstraZeneca, Clovis, Eisai, ImmunoGen, Medac, MSD, PharmaMar, Roche, and Tesaro/GSK. S.M. received research funding, honorary, or travel expenses from and is a member of the advisory board of AbbVie, AstraZeneca, Clovis, Eisai, GlaxoSmithKline, Hubro, Medac, MSD, Novartis, Nykode, Olympus, PharmaMar, Pfizer, Roche, Sensor Kinesis, Teva, and Tesaro.

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