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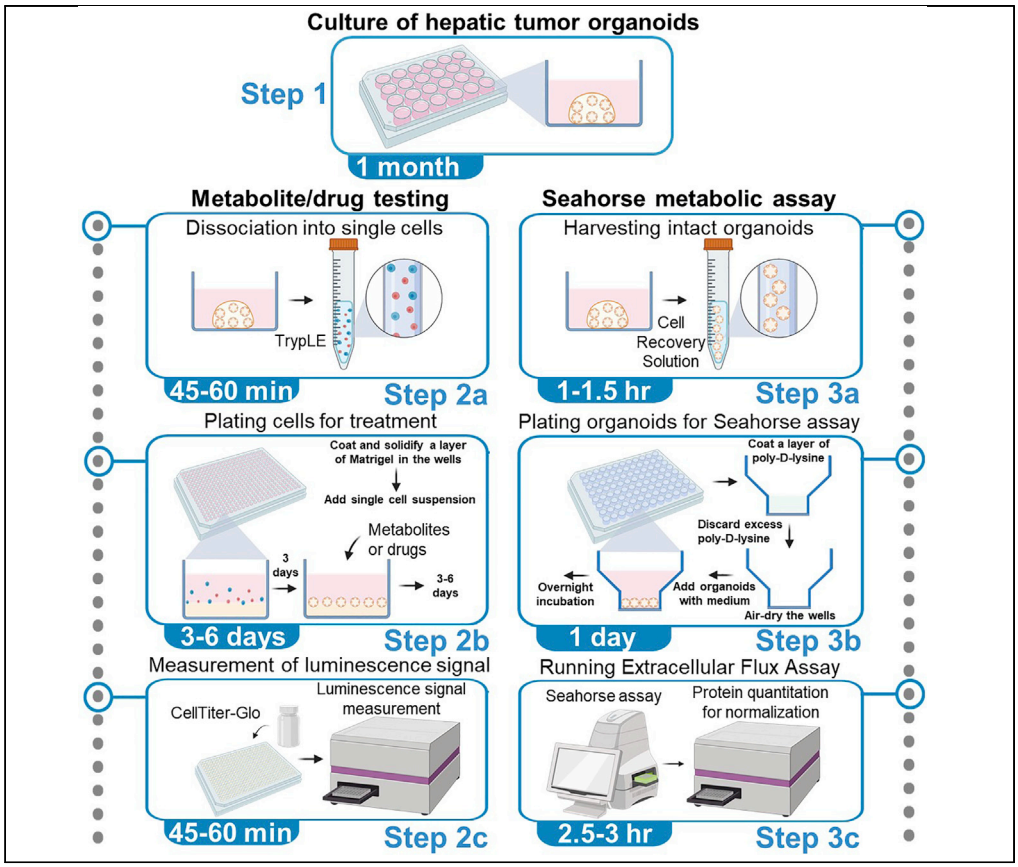
Protocols to culture and harvest hepatic tumor organoids for metabolic assays



Three-dimensional organoids, which resemble the pathophysiology and structural architecture of the original tissues, are a preferable *in vitro* model system for assessing metabolic activities in response to various environmental or nutritional changes. Here, we describe step-by-step protocols to establish and culture mouse and human hepatic tumor organoids. We also describe two straightforward and efficient approaches to harvest tumor organoids for investigating the effects of metabolites/drugs on viability and metabolic functions of tumor organoids.

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

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Highlights

Protocol to culture mouse and human hepatic tumor organoids in metabolic assays

Two optimized approaches to harvest organoids for downstream functional assays

Straightforward and efficient testing of candidate metabolites/drugs in organoids

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Protocols to culture and harvest hepatic tumor organoids for metabolic assays

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SUMMARY

Three-dimensional organoids, which resemble the pathophysiology and structural architecture of the original tissues, are a preferable *in vitro* model system for assessing metabolic activities in response to various environmental or nutritional changes. Here, we describe step-by-step protocols to establish and culture mouse and human hepatic tumor organoids. We also describe two straightforward and efficient approaches to harvest tumor organoids for investigating the effects of metabolites/drugs on viability and metabolic functions of tumor organoids.

For complete details on the use and execution of this protocol, please refer to Tong et al. (2018), Leung et al. (2020), Tong et al. (2021), and Xu et al. (2021).

BEFORE YOU BEGIN

The experimental procedures described in this protocol include the steps necessary to establish, culture and harvest hepatic tumor organoids to assess their viability and metabolic functions in response to nutrient availability and metabolic drug treatment. We successfully applied this protocol to both human and murine hepatic tumor organoids. This protocol can be broadly applied to any metabolite/drug and Seahorse metabolic assay, and it is not limited to the examples provided below.

This protocol is specifically designed for hepatic tumor organoids. We have not tested it using organoids established from other tissues. Users are advised to determine the optimal conditions in terms of establishment, culturing, harvesting and plating organoids according to their standard organoid culture protocol.

Before implementing this protocol, users need to ensure that the use of any human clinical specimens and any animal experiments comply with their institutional guidelines.

Institutional permissions

Hepatocellular carcinoma (HCC) tissues used for organoid establishment were obtained from patients with HCC undergoing hepatectomy or liver transplantation at Queen Mary Hospital, Hong Kong, with informed consent obtained from all patients and a protocol approved by the University of Hong Kong/Hospital Authority Hong Kong West Cluster Institutional Review Board. Five organoid lines were derived from tissues from five HCC male patients, all of Chinese origin, with age ranging from 56–66 years old. The animal study protocol was approved by and performed in accordance with

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the Committee of the Use of Live Animals in Teaching and Research at The University of Hong Kong and the Animals (Control of Experiments) Ordinance of Hong Kong.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological samples		
Human HCC patient-derived organoids	Tong et al. (2021)	N/A
Murine HCC organoids	Zhou et al. (2021)	N/A
Chemicals, peptides, and recombinant proteins		
Poly-D-lysine hydrobromide (Poly-D-lysine)	Sigma-Aldrich	Cat#P7280
Phosphate-buffered saline (PBS)	Sigma-Aldrich	Cat#P4417
Matrigel, growth factor reduced, phenol red-free (Matrigel)	Corning	Cat#356231
Advanced DMEM/F-12	Gibco	Cat#12634028
HEPES, filtered with 0.22 μm filter	Sigma-Aldrich	Cat#H3375
HEPES (100×)	Gibco	Cat#15630080
GlutaMAX™ Supplement	Gibco	Cat#35050061
Penicillin–Streptomycin (P/S) (5,000 U/mL)	Gibco	Cat#15070063
N-2 Supplement (100×)	Gibco	Cat#17502048
B-27™ Supplement (50×), minus vitamin A	Gibco	Cat#12587001
N-Acetyl-L-cysteine	Sigma-Aldrich	Cat#A9165
[Leu15]-Gastrin I human (Gastrin)	Sigma-Aldrich	Cat#G9145
Human EGF Recombinant Protein (EGF)	PeproTech	Cat#100-15
Recombinant Human FGF-10 (FGF-10)	PeproTech	Cat#100-26
Recombinant Human HGF (HGF)	PeproTech	Cat#100-39
Nicotinamide	Sigma-Aldrich	Cat#N0636
A83.01	Tocris	Cat#2939
Forskolin (FSK)	Tocris	Cat#1099
CHIR 90021	Tocris	Cat#4423
Recombinant Human KGF (FGF-7)	PeproTech	Cat#100-19
Y-27632 dihydrochloride (ROCK inhibitor)	AbMole	Cat#M1817
RSPO1 conditioned medium	Prof. Hans Clevers, Hu et al. (2018)	N/A
L-Glutamine (200 mM)	Gibco	Cat#25030081
Sodium Pyruvate (100 mM)	Gibco	Cat#11360070
Glucose, powder	Gibco	Cat#15023021
TrypLE™ Express Enzyme (1×), no phenol red (TrypLE)	Gibco	Cat#12604021
Cell Recovery Solution	Corning	Cat#354253
RIPA Buffer (10×)	Cell Signaling Technology	Cat#9806
DMEM medium, high glucose (DMEM)	Gibco	Cat#11965092
Ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA)	Sigma-Aldrich	Cat#E5134
Collagenase IV	Sigma-Aldrich	Cat#5138
DNase I	GoldBio	Cat#D-301-1
Potassium chloride	Sigma-Aldrich	Cat#60130
Calcium chloride	Sigma-Aldrich	Cat#793639
Fetal bovine serum (FBS)	Gibco	Cat#10270106
Critical commercial assays		
Seahorse XF Cell Mito Stress Test Kit	Agilent	Cat#103010-100
Seahorse XFe96 Analyzer or Seahorse XFe24 Analyzer	Agilent	N/A
Seahorse XFe96 FluxPaks or Seahorse XFe24 FluxPaks	Agilent	Cat#102416-100 or Cat#102340-100
Seahorse XF base medium, without phenol red	Agilent	Cat#103335-100
CellTiter-Glo® 2.0 Luminescent Cell Viability Assay (CellTiter-Glo solution)	Promega	Cat#G9241

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Software and algorithms		
Seahorse Wave Desktop Software	Agilent	https://www.agilent.com/en/product/cell-analysis/real-time-cell-metabolic-analysis/xf-software/seahorse-wave-desktop-software-740897
Other		
384-well white plates, optically clear polymer bottom	Thermo Fisher Scientific	Cat#142762
24-well tissue culture plates, tissue culture treated	TPP	Cat#92024
15-mL tubes	SPL Life Sciences	Cat#50015
50-mL tubes	SPL Life Sciences	Cat#50050
Automated cell counter (LUNA-II)	Logos Biosystems	https://logosbio.com/automated-cell-counters/brightfield/luna-ll
Microplate reader (The VICTOR^3 Multilabel Plate Reader)	PerkinElmer	https://resources.perkinelmer.com/lab-solutions/resources/docs/BRO_VICTOR3.pdf

Warning for hazards:

***N*-Acetyl-L-cysteine**: Cause serious eye irritation. Wash hands thoroughly after handling. If in eyes, rinse cautiously with water for several minutes. If eye irritation persists, get medical advice/attention.

Nicotinamide: Cause serious eye irritation. Wash hands thoroughly after handling. If in eyes, rinse cautiously with water for several minutes. If eye irritation persists, get medical advice/attention.

Forskolin: Harmful in contact with skin. Wear protective gloves/protective clothing/eye protein/face protection when handle. Wash with soap and water if in contact with skin.

Ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA): Harmful if inhaled. May cause damage to organs through prolonged or repeated exposure. Do not breathe dust/fume/gas/mist/vapours/spray. Use only outdoors or in a well-ventilated area. If inhaled, remove victim to fresh air and keep at rest in a position comfortable for breathing. Call a poison center or doctor/physician if you feel unwell.

Calcium chloride: Cause serious eye irritation. If in eyes, rinse cautiously with water for several minutes.

MATERIALS AND EQUIPMENT

Perfusion buffer (50 mL)			
Reagent	Stock concentration	Final concentration	Amount
Phosphate-buffered saline (PBS), 1×	N/A	N/A	48.45 mL
HEPES, filtered with 0.22 μm filter	1 M	25 mM	0.5 mL
Ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA)	0.5 M	0.5 mM	50 μL
Perfusion buffer is prepared freshly before the experiment.			

Human liver digestion buffer (50 mL)			
Reagent	Stock concentration	Final concentration	Amount
Phosphate-buffered saline (PBS), 1×	N/A	N/A	49 mL
Collagenase IV	250 mg/mL	2.5 mg/mL	0.5 mL
DNase I	10 mg/mL	0.1 mg/mL	0.5 mL
Human liver digestion buffer is prepared freshly before the experiment.			

Murine liver digestion buffer (50 mL)			
Reagent	Stock concentration	Final concentration	Amount
Base buffer (1 L), pH7.4, filtered with 0.22 μm filter			
Phosphate-buffered saline (PBS), 1×	N/A	N/A	976.5 mL
HEPES, filtered with 0.22 μm filter	1 M	15 mM	15 mL
Potassium chloride	5% (w/v)	0.025%	5 mL
Glucose	1 M	2.5 mM	2.5 mL
Calcium chloride	500 mM	500 μM	1 mL
Murine liver digestion buffer (50 mL)			
Base buffer	N/A	N/A	48.75 mL
Collagenase IV	250 mg/mL	1.25 mg/mL	0.25 mL
DNase I	10 mg/mL	0.1 mg/mL	0.5 mL
HEPES, filtered with 0.22 μm filter	1 M	10 mM	0.5 mL
Base buffer can be stored at 4°C for 1 month. Murine liver digestion buffer is prepared freshly before the experiment.			

Neutralization buffer (50 mL)			
Reagent	Stock concentration	Final concentration	Amount
DMEM medium, high glucose (DMEM)	N/A	N/A	49 mL
Fetal bovine serum (FBS)	N/A	N/A	0.5 mL
HEPES, filtered with 0.22 μm filter	1 M	10 mM	0.5 mL
Neutralization buffer is prepared freshly before the experiment.			

Basal medium (Ad+++ medium) for organoid culture (50 mL)			
Reagent	Stock concentration	Final concentration	Amount
Advanced DMEM/F-12	N/A	N/A	48.5 mL
GlutaMAX	100×	1%	0.5 mL
HEPES (100×)	100×	1%	0.5 mL
Penicillin-Streptomycin (P/S)	100×	1%	0.5 mL
Ad+++ medium can be stored at 4°C for 1–2 months.			

Complete culture medium for human HCC organoids (~50 mL)			
Reagent	Stock concentration	Final concentration	Volume
Ad+++ medium	N/A	N/A	45 mL
B-27™ Supplement (50×), minus vitamin A	50×	2%	1 mL
N-2 Supplement	100×	1%	0.5 mL
Nicotinamide	1 M	10 mM	0.5 mL
N-Acetyl-L-cysteine	625 mM	1.25 mM	0.1 mL
[Leu15]-Gastrin I human (Gastrin)	10 μM	10 nM	50 μL
Recombinant Human FGF-10 (FGF-10)	100 μg/mL	100 ng/mL	50 μL
Human EGF Recombinant Protein (EGF)	100 μg/mL	50 ng/mL	25 μL
Recombinant Human HGF (HGF)	50 μg/mL	25 ng/mL	25 μL
A83.01	12.5 mM	5 μM	20 μL
Forskolin (FSK)	25 mM	10 μM	20 μL
Y-27632 dihydrochloride (ROCK inhibitor)	25 mM	10 μM	20 μL
RSPO1 conditioned medium	N/A	10%	5 mL
It is preferable to prepare fresh complete culture medium with growth factors before use. Excess medium can be stored at 4°C for no more than 1 week.			

Complete culture medium for mouse HCC organoids (~50 mL)			
Reagent	Stock concentration	Final concentration	Volume
Ad+++ medium	N/A	N/A	42.5 mL
B-27™ Supplement (50×), minus vitamin A	50×	2%	1 mL
Nicotinamide	1 M	10 mM	0.5 mL
N-Acetyl-L-cysteine	625 mM	1.25 mM	0.1 mL
[Leu15]-Gastrin I human (Gastrin)	10 μM	10 nM	50 μL
Recombinant Human FGF-10 (FGF-10)	100 μg/mL	100 ng/mL	50 μL
Human EGF Recombinant Protein (EGF)	100 μg/mL	50 ng/mL	25 μL
Recombinant Human HGF (HGF)	50 μg/mL	25 ng/mL	25 μL
A83.01	12.5 mM	1 μM	4 μL
CHIR 90021	15 mM	3 μM	10 μL
Recombinant Human KGF (FGF-7)	50 μg/mL	50 ng/mL	50 μL
Y-27632 dihydrochloride (ROCK inhibitor)	25 mM	10 μM	20 μL
RSPO1 conditioned medium	N/A	15%	7.5 mL

It is preferable to prepare fresh complete culture medium with growth factors before use. Excess medium can be stored at 4°C for no more than 1 week.

Cell Mito Stress Test assay medium (50 mL)			
Reagent	Stock concentration	Final concentration	Volume
Seahorse XF basal medium (pH 7.4)	N/A	N/A	48.5 mL
Sodium pyruvate	100 mM	1 mM	0.5 mL
L-Glutamate	200 mM	2 mM	0.5 mL
Glucose	1 M	10 mM	0.5 mL

It is preferable to prepare fresh assay medium with supplements before use. Excess medium can be stored at 4°C for no more than 1 month.

Cell Mito Stress Test compounds—Stock solutions		
Reagent	Final concentration	Volume of assay medium
Oligomycin	100 μM	630 μL
FCCP	100 μM	720 μL
Rotenone/antimycin A	50 μM	540 μL

It is preferable to prepare stock solutions before use. Excess stock solutions can be stored at −20°C for no more than 1 month.

Cell Mito Stress Test compounds—Working solutions for hepatic tumor organoids				
Reagent	Working concentration	Volume of stock solution (XFe96/XFe24)	Volume of assay medium (XFe96/XFe24)	Total volume (XFe96/XFe24)
Oligomycin	1.5 μM	375 μL/300 μL	2125 μL/1700 μL	2.5 mL/2 mL
FCCP	1 μM	250 μL/200 μL	2250 μL/1800 μL	2.5 mL/2 mL
Rotenone/Antimycin A	0.5 μM	250 μL/200 μL	2250 μL/1800 μL	2.5 mL/2 mL

Working solutions should be freshly prepared before use.

Note: The concentrations of the compounds are optimized for hepatic tumor organoids. The optimal concentrations of the compounds used may vary by cell and assay medium type. Users are recommended to optimize the concentrations for each compound with titrations.

Note: All buffers and solutions should be prepared inside a laminar flow hood, except specified otherwise.

Microplate readers for spectrophotometry and luminescence

Alternatives: Any microplate readers that are equipped to measure colorimetric absorbance (595 nm for the Bradford protein assay) and luminescence signal from 96- or 384-well plates.

Seahorse XF analyzer

Alternatives: Any Seahorse analyzer with the use of compatible sensor cartridges and cell culture microplates.

STEP-BY-STEP METHOD DETAILS

Establishment and culture of hepatic tumor organoids

Timing: 1 month

This section describes the experimental steps required to establish, expand and culture hepatic tumor organoids from human and murine hepatic tumors. All experimental steps should be performed in a sterile environment, e.g., laminar flow hood, and sterile and autoclaved tools should be used when they are in contact with the tumor tissues.

For human HCC tissues

1. Obtain liver tumor biopsies from donors undergoing hepatectomy or liver transplantation. Liver biopsies are kept in at least 25 mL DMEM medium, high glucose (DMEM) in 50 mL centrifuge tube in ice before processing.
2. Inside a laminar flow hood, aspirate the medium without disturbing the tumor tissues and leave around 10 mL DMEM in the tube.

   Note: UV the tissue culture hood for 30 min. Disinfect the tissue culture hood and all the pipettes with 75% ethanol and swipe dry with paper towel.

3. Pour the tumor tissues together with the remaining DMEM into a 10-cm cell culture dish.
4. Cut out 1 cm^3 tumor tissues with sterile blades and transfer to a 6-cm cell culture dish containing 3 mL fresh DMEM.

   Alternatives: Extra tissues can be snap frozen in liquid nitrogen or fixed in 4% paraformaldehyde.

5. Go to step 12.

For murine HCC tissues

6. Anesthetize the mice with CO_2.
7. Open the abdomen of the mice with sterile surgical scissors to expose the liver.
8. Perfuse the liver with perfusion buffer via the inferior vena cava to the portal vein to wash out blood.
9. After perfusion, dissect the tumor nodules and cut out 1 cm^3 with sterile blades.
10. Transfer to a 6-cm cell culture dish containing 3 mL fresh DMEM.

    Alternatives: Extra tissues can be snap frozen in liquid nitrogen or fixed in 4% paraformaldehyde.

11. Go to step 12.

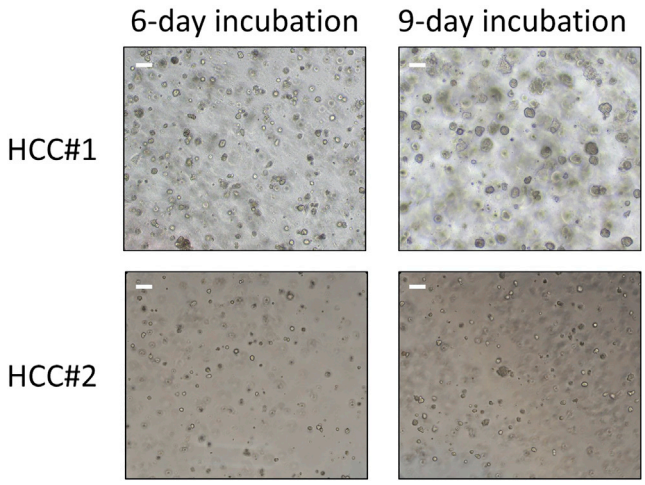

Figure 1. Hepatic tumor organoids
Images of organoids with varying proliferation capacities grown for 6 and 9 days. Scale bar = 100 μm.

Dissociation of human and murine HCC tissues

12. Mince the tumor tissues together with the medium in 6-cm cell culture dish with sterile blades until the tissues becomes less than 1 mm in diameter.
13. Transfer the tissue-medium mixture to 50 mL centrifuge tube with a sterile transfer pipette.
14. Add 10 mL human or murine liver digestion solution to the mixture.
15. Incubate the mixture with digestion solution in 37°C water bath for 30 min with occasional swirling of the tube.

Alternatives: Use a water bath with a shaker set at moderate speed.

16. Stop the digestion process by adding ice-cold neutralization buffer.
17. Filter the mixture through a 70 μm cell strainer.
18. Centrifuge at 180 × *g* for 5 min at 4°C. Discard the supernatant.

Culture of human and murine HCC organoids

19. Resuspend dissociated cells in 5 mL complete organoid culture medium.
20. Count the cell number with an automated cell counter or a hemocytometer.
21. Mix the dissociated single cells with Matrigel, growth factor reduced, phenol red-free (Matrigel) at a 1:4 v/v ratio.
    a. For plating in each 24-well plate, we recommend 15,000 cells (or 30,000 cells depending on the proliferation rate; please refer to Figure 1 below) in 10 μL of medium to be mixed with 40 μL of Matrigel.
    b. If the concentration of the cells is too low for plating, centrifuge the cells at 800 × *g* for 5 min and resuspend in less volume of medium (at least 15,000 cells in 10 μL) according to the concentrations counted in step 20.

⚠ CRITICAL: Thaw and maintain Matrigel on ice. The cell-Matrigel mixture is kept on ice before plating.

22. Dispense 50 μL of the cell-Matrigel mixture into each well of a 24-well plate.

⚠ CRITICAL: Pipette the cell-Matrigel mixture gently. Avoid introducing bubbles into the mixture.

Note: The cell-Matrigel mixture should sit at the center of the well as a dome and should not spread.

23. After dispensing the mixture into the required number of wells, put the plate in a 37°C, 5% CO_2 incubator for at least 15 min.
24. Gently add 500 μL of complete culture medium to each well, with care taken to not disrupt the Matrigel mixture.

Note: We recommend adding the medium against the wall of the wells to avoid touching the Matrigel droplet.

25. Incubate the organoids in a 37°C, 5% CO_2 incubator.
26. Change fresh complete medium every three days, with care taken to not disturb the Matrigel droplet when aspirating or adding medium. Troubleshooting 1.
27. Culture the organoids for 9–14 days until the diameters of the organoids reach approximately 50–75 μm (Figure 1). Troubleshooting 2.

⚠ CRITICAL: The hepatic cells are very sticky and tend to be retained inside the pipette tips. It is critical to wet the inner wall of a new pipette tip each time before pipetting the cell suspension. Dip the pipette tip into organoid medium and pipetted up and down 2–3 times to rinse the tip. Alternatives include using low binding tips or using a sterile solution of PBS.

Note: We do not recommend growing the organoids for more than 14 days in the same well without further passage. Matrigel will be more difficult to digest by TrypLE™ Express Enzyme (1×), no phenol red (TrypLE) or cell recovery solution for prolonged culture of organoids.

Note: All the hepatic organoid lines derived using this protocol are morphologically similar that cells are densely packed and arranged to form compact and spherical organoids. Please refer to Figures 1 and 2 for representative images.

Note: The growth rates of different hepatic organoid lines can vary (Figure 1). For organoid lines that grow slower, the user may plate more cells (2 times the suggested cell number, i.e., 30,000 cells) inside one Matrigel droplet to enhance the growth of the organoids. For human HCC organoid lines, we cannot conclude on the growth rate differences observed among various lines as some patients harbor certain/multiple mutations while some do not. For murine HCC organoid lines derived from various mutations-driven tumors, we did observe that certain mutation could promote proliferation/certain organoid lines grow faster than others while carrying the mutation, i.e., beta-catenin activation.

Note: Organoids grown too large have reduced viability that will result in more dead cells after dissociation. For the hepatic tumor organoid lines that we have tested, the optimal size of the organoids necessary for dissociation to perform functional assays is 50–75 μm in diameter. At this range of sizes, the single cells dissociated can keep up to 90% viability.

Note: For organoid expansion, dissociation and passage are needed. The benchmark or the signals for passage of organoid lines include the following: 1) the medium changes to yellowish color the day after changing fresh medium, 2) organoids and cells are dislodged from the Matrigel droplet and floating in the medium, and 3) cells migrate through the

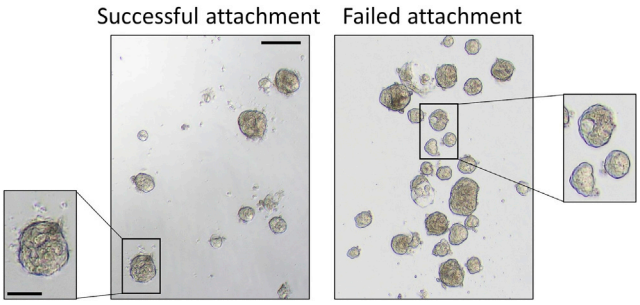

Figure 2. Plating and attachment of hepatic tumor organoids

Left image showing successful plating and attachment of organoids without Matrigel. Organoids do not float in the medium and attach to the bottom of the well. Single cells appear at the peripheral of the organoid to aid with the attachment (inset). Right image showing unsuccessful attachment of organoids after plating. The organoids float in the medium and concentrate at the center of the well. The organoids have more clear and smooth boundaries and there are no single cells attached to the peripheral of the organoid (inset). Scale bar of large image = 100 μm. Scale bar of inset = 50 μm.

edge of the Matrigel droplet and attach to the bottom of the well. The number of culture days before the next passage should be optimized for each organoid line.

Note: There is not a referenced minimum number of times that the organoids should be passaged before performing the assay. We did passage the organoids to at least P2 (from P0) before performing assays, to make sure the cells are indeed expandable and proliferating after passage. We have not experienced organoid lines to stop growing after certain passage numbers. We and others have confirmed that hepatic tumor organoids are genetically stable for at least 3 months.

Note: Regarding expanded details about hepatic organoid growth, maintenance, and passaging, users can also refer to the methods described in Broutier et al. (2017), Hu et al. (2018), Huch et al. (2015) and Nuciforo et al. (2018).

Harvesting and plating dissociated organoids for metabolite/drug testing

Timing: 45–60 min

This section describes the experimental steps required to harvest hepatic tumor organoids and plate the dissociated single cells to measure viability under metabolite/drug treatment. In general, the basic steps necessary for harvesting and dissociation can be applied to any type of organoid.

28. Before harvesting the organoids, coat the 384-well plate with the required number of wells with 8 μL of Matrigel per well in a laminar flow hood.

⚠ CRITICAL: The plate and Matrigel are placed on an ice box to prevent oligomerization and solidification of the Matrigel while dispensing it. Since only 8 μL of Matrigel is added to each well, the surface tension of Matrigel is high, which prevents its spread across the surface of the well. Some cells could attach to the area without the Matrigel coating and grow 2-dimensionally without forming 3D organoids. It is important to tap the plate after adding Matrigel to ensure that Matrigel is evenly distributed and covers the entire surface of the well.

29. Put the plate into a 37°C, 5% CO_2 incubator for at least 15 min to solidify the Matrigel.
30. Take out the cultured organoids from the incubator, and aspirate the medium in each well.

31. Add 500 μL of pre-warmed TrypLE to each well to recover organoids from the Matrigel.
32. Gently pipette TrypLE and Matrigel droplet mixture up and down 2–3 times to dislodge the Matrigel and the organoids from the well.
33. Transfer the mixture into a new sterile 15-mL centrifuge tube with 1000 μL pipette tip.

Note: Each 15-mL centrifuge tube can accommodate organoids from up to 8 wells (i.e., approximately 4–6 mL in volume).

34. Incubate the mixture in a 37°C water bath for 5 min with occasional swirling of the tube to mix TrypLE with organoids.

Alternatives: Use a water bath with a shaker set at moderate speed.

35. Pipette the mixture rigorously for 20–30 times to mechanically disrupt and dissociate the organoids into single cells.
36. Neutralize the TrypLE solution with the same volume of complete organoid medium.
37. Centrifuge the tube at 800 × *g* for 5 min.
38. Aspirate the medium. Troubleshooting 3.

⚠ CRITICAL: During aspiration, avoid touching the cell pellet, which can be dislodged easily.

39. Gently resuspend the dissociated cells are for 10 times in complete culture medium using 200 μL or 1000 μL pipette tips depending on the number of Matrigel drop and the medium volume. For each Matrigel drop, we recommend resuspending in 60 μL complete culture medium.

Alternatives: To test the effect of depleting a metabolite/growth factor from the medium, the dissociated cells are resuspended in the medium without the addition of a particular metabolite/growth factor.

40. Count the cell number with an automated cell counter or a hemocytometer, and calculate the number of 384-well plates and total cell numbers needed.
    a. For each 384-well plate, we suggest plating 1,500 cells in 30 μL of medium per well.
    b. For each experimental group, at least three technical replicates are highly recommended.
41. Dispensed 30 μL of solution containing 1,500 cells into each 384-well coated with Matrigel.

⚠ CRITICAL: Avoid touching the Matrigel while dispensing the cell solution. The cells may migrate through the crack of Matrigel and attach to the bottom of the well. Add the cell suspension against the wall of the wells.

Note: A multichannel pipette is used to dispense the cell suspension to shorten the cell plating time and increase consistency.

42. To avoid edge effects, we recommend not using the peripheral wells of the plates and filling those wells with 100 μL of water.
43. Incubate the organoids in 37°C, 5% CO_2 incubator for 3 days without changing the medium to allow the single cells to grow into small organoids.
44. After a 3-day incubation period, prepare the metabolites/drugs and respective controls to be tested at 2× concentration in 30 μL of medium (testing medium). The plating medium in each well is not changed or discarded. Add the testing medium to the wells so that the final concentration becomes 1×.

Alternatives: To test the effect of depleting a metabolite/growth factor from the medium, go to step 46 after the 3-day incubation. The incubation can be extended beyond 3 days, but we recommend adding 30 μL of the testing medium on Day 3 to avoid drying out of the wells.

45. Incubate for 3–6 days to measure the effects of metabolites/drugs to the organoid growth.

Note: The incubation time can vary for different metabolites/drugs. We recommend testing the effects at multiple time points. The suggested time points for hepatic tumor organoids are 3, 6 and 9 days (Tong et al., 2021).

46. After incubation at specific time points, dispense 15 μL of CellTiter-Glo® 2.0 Luminescent Cell Viability Assay (CellTiter-Glo solution) into each well, and resuspend the mixture.
47. Incubate the plate in the dark at room temperature for 30 min.
48. Measure the luminescence signal with a luminescence plate reader.

⚠ CRITICAL: The hepatic cells are very sticky and tend to be retained inside the pipette tips. It is critical to wet the inner wall of a new pipette tip each time before pipetting the cell suspension. The pipette tip was dipped into organoid medium and pipetted up and down 2–3 times to rinse the tip.

Optional: Leftover cells after plating for metabolite/drug testing can be plated for propagation and expansion in a 24-well plate.

Note: We recommend plating the cell-Matrigel mixture in 24-well rather than 384-well plates because the medium and the nutrients/drugs would not efficiently diffuse through the Matrigel and reach the organoids that are grown away from the surface of the Matrigel.

Note: We do not recommend aspirating and changing the medium of the organoids after plating in 384-well plates, as this may disrupt and remove the organoids from the wells.

Note: We recommend including negative control cells with only the organoid medium added as the background reading for this experiment.

Coating plates, harvesting and plating 3D organoids for Seahorse assays

This section describes the experimental steps to 1) coat Seahorse microplates with poly-D-lysine hydrobromide (poly-D-lysine) to facilitate the attachment of 3D organoids, 2) harvest intact hepatic tumor organoids, and 3) plate the intact 3D organoids for Seahorse metabolic assays. The coating procedures can be applied to any type of tissue culture plate and are not limited to Seahorse microplates. Note that the organoids are not dissociated into single cells in this section.

Coating Seahorse cell culture microplate with poly-D-lysine

Timing: 1–1.5 h

Day before the assay.

49. Prepare working solution of poly-D-lysine at 50 μg/mL with sterile water.
50. Coat the cell culture microplate with poly-D-lysine for 1 h at room temperature.
    a. For the XFe96 cell culture microplate, we recommend adding 20 μL.
    b. For the XFe24 cell culture microplate, we recommend adding 100 μL.
51. Aspirate the poly-D-lysine and wash the wells twice with 0.2 mL (XFe96) or 1 mL (XFe24) of sterile water. Air-dry the plate inside the laminar flow hood.

Alternatives: Prepare the coated plate one day before plating the organoids. The plates are wrapped with parafilm, stored at 4°C in a refrigerator and brought to room temperature before use.

Note: Seahorse XF poly-D-lysine (PDL)-coated cell culture microplates are also available. We recommend using self-coating poly-D-lysine, as the concentrations of poly-D-lysine used for coating can be adjusted and optimized for each organoid line.

Harvesting and plating organoids for the Seahorse assay

Timing: 45–60 min

Day before the assay.

52. Aspirate the medium of the organoids in a 24-well plate.
53. Add 500 μL of ice-cold cell recovery solution to each well to recover organoids from the Matrigel.
54. Gently pipette the cells up and down 2–3 times to dislodge the Matrigel and organoids from the well.

⚠ CRITICAL: Do not pipette aggressively; this will disrupt the organoid structure.

55. Transfer the mixture into a new sterile 15-mL centrifuge tube.

Note: Each 15-mL centrifuge tube can accommodate organoids from up to 8 wells (i.e., approximately 4–6 mL in volume).

56. Place the tube in ice for 30 min and rotate it from end to end manually every 5 min.

⚠ CRITICAL: Do not mix with a vortex mixer to avoid disrupting the organoid structure.

57. Neutralize the cell recovery solution with the same volume of complete organoid medium.
58. Centrifuge the tube at 800 × *g* for 5 min.
59. Aspirate the medium. Troubleshooting 3.

⚠ CRITICAL: During aspiration, avoid touching the organoids, which can be easily dislodged.

60. Gently resuspend the organoids in complete culture medium. For each well of organoids, we recommend resuspending with 200 μL of medium.
61. Pipette 10 μL of organoid suspension into any tissue culture plate. Place the plate under the microscope to count the number of organoids in a 10-μL volume.
62. Calculate the required number of wells for the experiment and adjust the volume of organoid suspension to achieve the required concentration.
    a. For the XFe96 cell culture microplate, we recommend plating 50 organoids in 80 μL of medium per well.
    b. For the XFe24 cell culture microplate, we recommend plating 250 organoids in 200 μL of medium per well.
63. Add the organoids with medium to the coated microplate. Leave the four wells at the corner as a background correction control. Fill those four wells with medium only.
64. Place the microplate at room temperature for an hour to facilitate even distribution of the organoids in the well.
65. Incubate the organoids overnight in 37°C, 5% CO_2 incubator, and check the plating of the organoids the next day (Figure 2).

Note: To preserve the 3D structure of the organoids, it is critical to pipette gently.

Note: Matrigel is not added in this assay. Our testing experiment showed that the organoids embedded inside Matrigel did not respond to the test compounds, as no changes in the oxygen consumption rate (OCR) were observed over time.

Seahorse metabolic assay

Timing: ~180 min

This section describes the procedures used to perform the Seahorse Cell Mito Stress assay. The general procedures can be applied to other Seahorse metabolic assays. The assay medium and test compounds vary for different metabolic assays; please refer to the manufacturer's protocols for each assay.

Day before the assay

66. Turn on the Seahorse analyzer and warm the machine overnight.
67. Aliquot at least 20 mL of Seahorse XF Calibrant into a 50 mL conical tube. Place this XF Calibrant in a non-CO_2 37°C incubator overnight.
68. Place the sensor cartridge upside down next to the utility plate.
69. Fill each well of the utility plate with 200 μL (XFe96) or 1 mL (XFe24) of sterile water.
70. Lower the sensor cartridge onto the utility plate, submerging the sensors in the water.
71. Verify the water level is high enough to keep the sensors submerged.
72. Place assembled sensor cartridge and utility plate in a non-CO_2 37°C incubator overnight. To prevent evaporation of the water, verify that the incubator is properly humidified.

Note: For details, please refer to the manufacturer's protocol.

https://www.agilent.com/cs/library/usermanuals/public/XFe96_DAY_BEFORE_CARTRIDGE_HYDRATION.pdf.

Day of the assay

73. Prepare the Cell Mito Stress Test assay medium for the Cell Mito Stress Test assay.
74. Warm the assay medium to 37°C in a water bath for subsequent use.
75. Remove the microplate with organoids from the 37°C, 5% CO_2 incubator.
76. Check the attachment of the organoids under a microscope. Troubleshooting 4.
77. Carefully remove the organoid medium with a pipette.

Note: We do not recommend using suction, as the organoids could be aspirated from the well.

78. Slowly add 180 μL (XFe96) or 500 μL (XFe24) of Seahorse assay medium per well.

⚠ CRITICAL: The assay medium was added to the wall of the wells to prevent organoids from lifting off. Make sure the organoids are not dislodged after adding the medium under the microscope.

Note: The testing metabolites/drugs and their respective controls can be added to the assay medium at this stage. We recommend at least three replicates for each testing group.

79. Place the microplate in a non-CO_2 incubator at 37°C for an hour.

80. Prepare stock and working solutions of Cell Mito Stress Test compounds.
81. Take out the XF Calibrant and the hydrated sensor cartridge with utility plate from the incubator.
82. Place the sensor cartridge upside down next to the utility plate.
83. Aspirate the water from the utility plate.
84. Fill each well of the utility plate with 200 μL of the pre-warmed XF Calibrant.
85. Lower the sensor cartridge onto the utility plate, submerging the sensors in calibrant.
86. Place assembled sensor cartridge with utility plate in a non-CO_2 37°C incubator for 45–60 min prior to loading the injection ports of the sensor cartridge.
87. Take out the utility plate from the incubator.
88. Load the sensor cartridge with the aid of port loading guides (for XFe96) with working solutions of compounds according to the following table.

Cell Mito Stress Test compounds—Working solutions for hepatic tumor organoids

Port	Reagent	Port concentration	Volume of working solutions added to each port (XFe96)	Volume of working solutions added to each port (XFe24)
A	Oligomycin	10×	20 μL	56 μL
B	FCCP	10×	22 μL	62 μL
C	Rotenone/Antimycin A	10×	25 μL	69 μL

For the location of the ports, please refer to Figure 3.

Note: For loading XFe96 sensor cartridge, we recommend using the A/D and B/C port loading guides provided inside the package of the sensor cartridge.

Note: For details, please refer to the manufacturer's protocol.

https://www.agilent.com/cs/library/usermanuals/public/DAY%20OF%20LOADING%20CARTRIDGE%20XFe96-XF96.pdf.

89. Set up the assay program in the Wave software of Seahorse analyzer. In the assay program, assign the wells to each experimental group. Assign the wells at the four corners as blank.

Note: We use the Cell Mito Stress Test assay template stored in the software for running the program.

90. Save the assay program and click **Start Run** in the program.
91. Place the loaded sensor cartridge with the lid removed in the correct orientation with the triangular notch of the cartridge at the left-bottom corner.
92. Click **I'm ready** in the program and calibrate the sensor cartridge. This step will take approximately 20–30 min.
93. When prompted, replace the calibration plate with a cell culture microplate and remove the lid. Click **I'm ready** in the program. The standard Cell Mito Stress Test assay will take approximately 120 min.
94. Remove the microplate and sensor cartridge after running the program.
95. Save the experimental result file for subsequent analysis. Troubleshooting 5.

Note: For details of the operation of the Seahorse analyzer, please refer to the manufacturer's protocol.

https://www.agilent.com/cs/library/usermanuals/public/usermanual-xfe-analyzer-operating-manual-cell-analysis-S7804-90001-en-us-agilent.PDF.

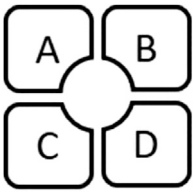

Figure 3. The arrangement of Ports A, B, C, and D in the sensor cartridge

Note: For details of the running of the Wave program, users may refer to the manufacturer's protocol.

https://www.agilent.com/cs/library/usermanuals/public/S7894-10000_Rev_C_Wave_2_6_User_Guide.pdf.

Protein quantification for normalization

Timing: 30–45 min

This section describes the procedures for normalization based on protein quantification.

96. After the assay, carefully remove the medium in the microplate with a pipette.

Note: We do not recommend using suction, as the organoids could be easily dislodged after the assay.

97. Wash each well with 200 μL (XFe96) or 1 mL (XFe24) of ice-cold phosphate-buffered saline (PBS) once, and carefully discard the PBS with a pipette.

⚠ CRITICAL: The washing step was performed gently to prevent the organoids from lifting off.

98. Lyse the organoids for protein extraction by adding 10 μL (XFe96) or 50 μL (XFe24) of 1× RIPA buffer to each well. Pipette up and down for 10 times with 20 μL or 200 μL pipette tips to facilitate protein lysis.
99. Incubate the microplate on ice for 20–30 min. Resuspend the mixture occasionally to facilitate protein lysis.
100. Centrifuge the microplate at 3000 g at 4°C for 15 min.
101. Use the supernatant for measurement of protein concentrations by standard Bradford protein assay.
102. Calculate the protein concentrations of each well and normalize oxygen consumption rates (OCR) from the Seahorse assay result with the protein concentrations in Wave program.
103. Calculate the basal respiration, ATP production and maximal respiration from the normalized OCR from the Seahorse analysis (Figure 4).

Note: For details of normalization of the assay result in the Wave program, users may refer to the manufacturer's protocol.

https://www.agilent.com/cs/library/usermanuals/public/S7894-10000_Rev_C_Wave_2_6_User_Guide.pdf.

Alternatives: Other quantification methods, such as cell numbers and total DNA measurement, can be used for normalization purposes. For details, the user may refer to the manufacturer's protocol.

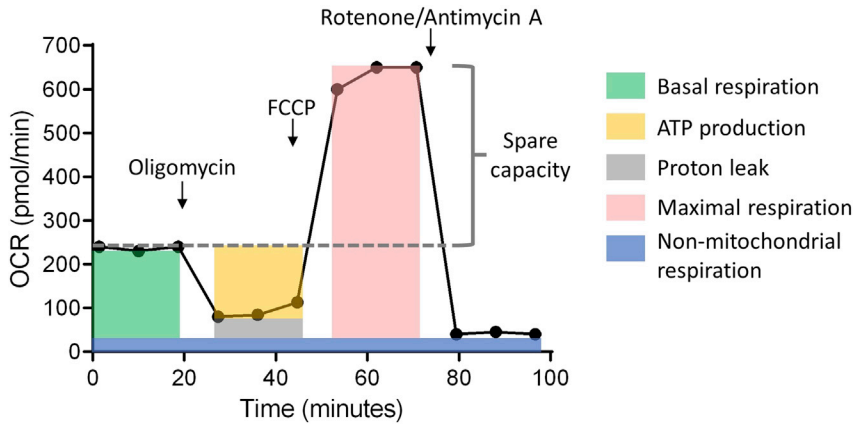

Figure 4. Different metabolic parameters in the Seahorse Cell Mito Stress Test

Schematic showing the metabolic profile and key parameters of mitochondrial functions from the Seahorse Cell Mito Stress Test.

https://www.agilent.com/cs/library/technicaloverviews/public/Methods_and_Strategies_for_Normalizing_Tech_Overview_022118.pdf.

EXPECTED OUTCOMES

This protocol describes two approaches for harvesting and plating hepatic tumor organoids for subsequent functional assays to assess the treatment response to metabolite/drug administration and their metabolic profile using Seahorse assays.

Regarding the assessment of the treatment response toward metabolite/drug treatment, we use the CellTiter-Glo assay to quantify the viability of organoids based on ATP quantitation. In an example of investigating the glutamine dependency of organoids, we cultured the organoids in organoid medium with or without the addition of GlutaMAX for 6 days. The readings of the luminescent signal after treatment are shown below (Table 1). The growth inhibitory effect of glutamine depletion was compared by measuring the fold-change between the two experimental groups.

> *Note:* The signal intensity values can be largely different for different organoid types, the number of viable cells present and the machine used to measure the luminescent signal. We recommend performing at least 3 technical replicates for each experimental group.

Regarding the investigation of mitochondrial functions using the Seahorse Cell Mito Stress Assay, the metabolic profile and metabolic parameters represented by the oxygen consumption rates (OCRs) over time are displayed in Figure 4. Below is an example showing the mitochondrial function perturbation under the treatment of CB-839 (glutaminase inhibitor) and/or S6Ki (LY2584702, p70S6 kinase inhibitor) (Figure 5A). The metabolic parameters, including basal respiration, ATP production and maximal respiration, were calculated and are represented in bar charts as shown below (Figure 5B).

LIMITATIONS

Accumulating evidence revealed from studies that utilize single-cell sequencing technologies indicates that liver cancer displays a high degree of intratumoral heterogeneity and is composed of multiple tumor clones (Heinrich et al., 2021). Tumor organoids are an *in vitro* model that can recapitulate the structural architecture and both the genetic and nongenetic heterogeneities of the original tumor tissues. One limitation of this protocol is that the treatment response and metabolic profile of an individual tumor clone/cell cannot be measured. The degree of metabolic heterogeneity

Table 1. Luminescent signals of organoids cultured in medium with or without glutamine

	+ Glu	- Glu
3 technical replicate wells for each group	243827	113018
	181858	97597
	204232	120490

among different tumor clones in the organoids cannot be compared directly. In addition, the current organoid culture protocol lacks neighboring cells, such as fibroblasts and immune cells, in the tumor microenvironment, which contribute to regulating the metabolic activities of the tumor organoids in response to environmental and nutritional variabilities. Studies and protocols have been established for the co-culture of fibroblasts or immune cells with tumor organoids. Users may modify the culture system described in this protocol to include fibroblasts and immune cells to study the metabolic impact of these nontumor cells.

TROUBLESHOOTING

Problem 1

Due to gravity, dissociated single cells inside the Matrigel droplet tend to sink to the bottom of the well. For some organoid lines, cells grown in 2D are observed within the first few days of incubation.

Potential solution

Plates can be pre-warmed in a 37°C, 5% CO_2 incubator for the first 15 min. After plating the cell-Matrigel mixture into the well, quickly flip the plate upside down and incubate it in a 37°C, 5% CO_2 incubator for the first 15 min. The cells will remain at the periphery of the Matrigel droplet instead of sinking to the bottom.

Problem 2

Cholangiocyte organoids in the shape of a balloon are grown together with hepatocyte organoids which are dense and compact.

Potential solution

Put the plate with organoids embedded in Matrigel under a microscope and remove the lid. Look through the microscope at 10× magnification. Using a 200 μL pipette tip, carefully pick up the organoids that are dense and compact into a 15 mL centrifuge tube containing 10 mL complete organoid culture medium. Avoid picking up the cholangiocyte organoids. Centrifuge the tube at 800 × *g* for 5 min. Proceed with dissociation of organoids into single cells and replate the dissociated cells together with Matrigel in 24-well plate. Repeat this process in the next passages until no more cholangiocyte organoids are observed.

Problem 3

After incubation with TrypLE or cell recovery solution and subsequent centrifugation, there is an additional transparent layer of Matrigel observed on top of the cell/organoid pellet. This is the undissolved Matrigel.

Potential solution

Matrigel becomes harder to digest or dissolve by TrypLE or cell recovery solution when the organoids are cultured in the same well for more than 14 days. As suggested in the protocol under Culture of hepatic tumor organoids, we do not recommend culturing the organoids without passage for more than 14 days. If the organoid line grows too slow, users are advised to seed more cells in the beginning to enhance the growth of the organoids.

If an additional transparent layer of Matrigel is observed after centrifugation, aspirate the medium and add 500 μL of fresh TrypLE or cell recovery solution for another round of trypsinization/organoid

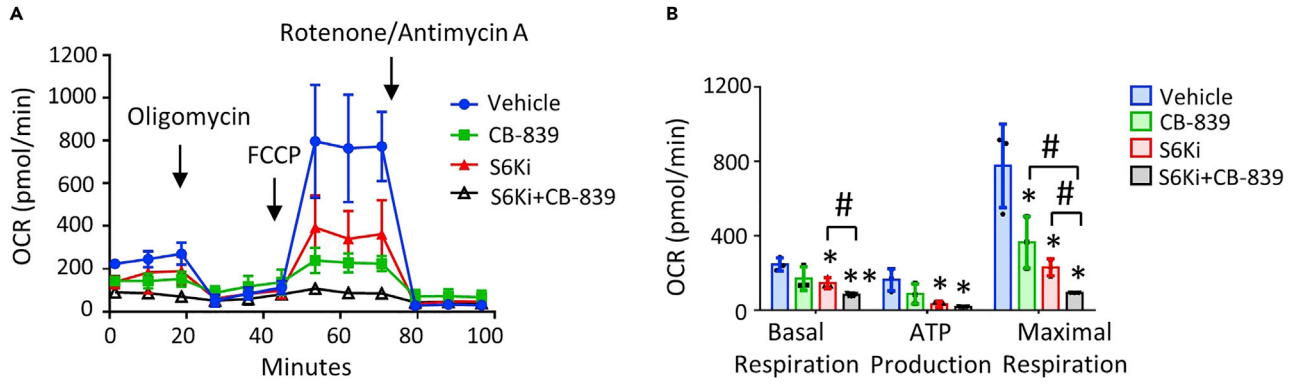

Figure 5. Respiratory profile and metabolic parameters of hepatic tumor organoids under treatment with CB-839 and/or S6Ki

(A) Respiratory profile of hepatic tumor organoids treated with CB-839 and/or S6Ki as measured by oxygen consumption rates (OCRs) over time.

(B) Bar chart showing the mitochondrial function of organoids after treatment as represented by three metabolic parameters (basal respiration, ATP production and maximal respiration). Data are displayed as the mean ± SD. Statistical tests were performed by one-way ANOVA. * and ** represent $p<0.05$ and $p<0.01$, respectively (versus vehicle treatment). # represents $p<0.05$ (single versus combination treatment). For a detailed graphical presentation of the results and the molecular mechanisms involved, please refer to Tong et al. (2021).

recovery. Usually, one additional round can remove all the remaining Matrigel. If the Matrigel layer still persists, we recommend replacing it with a new bottle of TrypLE or cell recovery solution.

Problem 4

Organoids are not attached to the cell culture microplate coated with poly-D-lysine.

Potential solution

The suggested concentration of poly-D-lysine for coating the microplate is 50 μg/mL. If the organoids do not attach to the wells using this concentration of poly-D-lysine, the user can test a titrated concentration from 50 to 100 μg/mL. Note that high concentrations of poly-D-lysine may cause toxicity and, hence, reduce the viability of the organoids.

Problem 5

The oxygen consumption rates (OCRs) are below 0 pmol/min.

Potential solution

In the case where the OCR shows negative values, users can first check whether the organoids are detached from the wells after the assay. Detachment and floating of the organoids in the medium can result in inaccurate measurement of OCR by the Seahorse analyzer.

Another possible reason for the negative OCR is that the number of viable cells in the wells is too low. Users may increase the seeding density of the organoids.

If negative OCR values appear after the addition of the assay compounds, i.e., oligomycin A or rotenone/antimycin A, the concentrations of these two compounds may be too high. Users can reduce the concentrations of these compounds.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Dr. Stephanie Ma (stefma@hku.hk).

Materials availability

Materials associated with this study are available upon request from the lead contact.

Data and code availability

This paper does not report datasets or original code.

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AUTHOR CONTRIBUTIONS

M.T. and S.M. conceived the protocols; M.T. and S.M. wrote the manuscript; M.T. performed and analyzed the experiments; M.T. and S.M. acquired funding for this study; and S.M. supervised the project.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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