Protocol for generation and drug treatment of individualized patient-derived organoids

SUMMARY

Tumor organoids serve as valuable tools for cancer research, but current models suffer from several drawbacks that curtail their clinical utility. Here, we describe the detailed protocol of the recently published IPTO model (IPTO: individualized patient tumor organoid)[1], which preserves the ecosystem of human brain cancers. This protocol outlines techniques for establishing IPTO and evaluating drug responses in IPTO, which can be seamlessly integrated into precision clinical trials for cancer treatment.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological samples		
Patient Derived Brain Tumor Tissue	University hospital Heidelberg, DE	N/A
Patient Derived Brain Tumor Tissue	Huashan Hospital, Shanghai, CN	N/A
Patient Derived Brain Tumor Tissue	Medical Faculty Mannheim, Heidelberg University, DE	N/A
Primary lung cancer samples	DKFZ-Hector Cancer Institute at the University Medical Center Mannheim	N/A
Chemicals, peptides, and recombinant proteins		
Puromycin	Sigma-Aldrich	Cat# P8833; CAS# 58-58-2
mTeSR Plus	Stemcell Technologies	Cat# 100-0276
ReleSR	Stemcell Technologies	Cat# 05872
Accutase	Sigma-Aldrich	Cat# A6964
DMEM/F-12	Gibco	Cat# 11330032
Neurobasal medium	Gibco	Cat# 21103049
hES-quality FBS	Gibco	Cat# 10270106
Knockout serum replacement (KOSR)	Gibco	Cat# 10828-028
Rock Inhibitor	Stemcell Technologies	Cat# 72304; CAS# 129830-38-2
bFGF-2	PeproTech	Cat# 100-18B
Heparin	Sigma-Aldrich	Cat# H3149; CAS# 9041-08-1
GlutaMAX	Gibco	Cat# 35050038
MEM-NEAA	Sigma-Aldrich	Cat# 11140050
CHIR99021	Tocris Bioscience	Cat# 4423; CAS# 252917-06-9
N2	Gibco	Cat# 17502048
B27 – vitamin A	Gibco	Cat# 12587010
B27	Gibco	Cat# 17504044
Insulin Solution	Sigma-Aldrich	Cat# I9278
L-Ascorbic Acid	Sigma-Aldrich	Cat# A4544; CAS# 50-81-7
Matrigel® hES qualified	Corning	Cat# 354277
Matrigel® Basement Membrane Matrix	Corning	Cat# 354234
Antibiotic-Antimycotic	Gibco	Cat# 15240096
Penicillin-Streptomycin	Gibco	Cat# 15140122
Fetal Bovine Serum (FBS)	ATCC	Cat# 30-2020
2-Mercaptoethanol	Sigma-Aldrich	Cat# M3148-25ML; CAS# 60-24-2
Neurobasal-A Medium	Gibco	Cat# 10888-022
human recombinant bFGF	PeproTech	Cat# 100-18B; N/A

human recombinant EGF	Life/Invitrogen	Cat# PHG0311; N/A
Heparin sodium	Sigma	Cat# H3149-500KU-9; N/A
CloneR	Stemcell Technologies	Cat# 5889
DMEM	Gibco	Cat# 31966021
Trypsin-EDTA	Gibco	Cat# 15400054
OptiMEM	Gibco	Cat# 31985062
Panobinostat (LBH589, NVP-LBH589)	Hölzel Diagnostika	Cat# S1030; CAS# 404950-80-7
Temozolomide	Sigma-Aldrich	Cat# T2577; CAS# 85622-93-1
Cell Recovery Solution	Corning	Cat# 354253
D-luciferin	BioVision	Cat# 7903; CAS# 115144-35-9
T7 Endonuclease I	New England Biolabs	Cat# M0302L
Polyethylenimine, MW 25,000, linear	VWR International	Cat# 43896.01; CAS# 9002-98-6
Lenti-X™ Concentrator	Takara	Cat# 631232
Polybrene	Merck Millipore	Cat# TR-1003-50UL
Sodium chloride (NaCl)	neoFroxx GmBH	Cat# LC-5932.1
D- (+)-Glucose	Sigma-Aldrich	Cat# G8270-1KG
Magnesium chloride hexahydrate (MgCl2.6H2O)	neoFroxx GmBH	Cat# LC-5041.4
Calcium chloride dihydrate (CaCl2.2H2O)	Honeywell	Cat# 31307-1KG
Potassium chloride (KCl)	Honeywell	Cat# 31248-1KG
Sodium phosphate monobasic monohydrate (NaH2PO4.H2O)	Sigma-Aldrich	Cat# 71504-1KG-M
Sodium hydrogen carbonate (NaHCO3)	neoFroxx GmBH	Cat# LC-5937.1
Sucrose	Sigma-Aldrich	Cat# S0389-500G
Critical commercial assays		
Bioanalyzer high sensitivity DNA analysis	Agilent	Cat# 5067-4626
Click-iT™ EdU Cell Proliferation Kit for Imaging, Alexa Fluor™ 647 dye	Thermo Fisher Scientific	Cat# C10340
Qubit dsDNA HS Assay Kit	Thermo Fisher Scientific	Cat# Q33231
Monarch Genomic DNA Purification Kit	New England Biolabs	Cat# T3010S
Experimental models: Cell lines		
Human GFP+ induced pluripotent stem cells	Coriell Institute	Cat# AICS-0036-006; RRID: CVCL_JM19
HEK293T	ATCC	Cat# CRL-3216; RRID: CVCL_0063
Recombinant DNA		
pWPI-EF1a-Luc2 (Gibson)	HedgehogBio Science and Technology Ltd	N/A
pHHLVX-EF1α-Luc2-puro	HedgehogBio Science and Technology Ltd	N/A

pMD2.G	Addgene	Cat# 12259
psPAX2	Addgene	Cat# 12260
Software and algorithms		
ImageJ	NIH	https://imagej.nih.gov/ij/; RRID: SCR_003070
Living imaging v4.7.3	PerkinElmer	https://www.perkinelmer.com/lab-products-and-services/resources/software-downloads.html; RRID: SCR_014247
GraphPad Prism 8	GraphPad	https://www.graphpad.com/; RRID: SCR_002798
Adobe Illustrator 2021	Adobe	https://www.adobe.com/products/illustrator.html; RRID: SCR_010279
Other		
Ultra-low attachment 96-well plates	Corning	Cat# 7007
Ultra-low attachment 6-well plates	Corning	Cat# 3473
Pellet Pestle	Fisher Scientific	Cat# 13236679
Motor For Pellet Pestle	Sigma-Aldrich	Cat# Z359971
NanoDrop™ One	ThermoFisher	Cat# Q33238
The Qubit 2.0 Fluorometer	Life Technologies (Invitrogen)	Cat# Q32866

MATERIALS AND EQUIPMENT

Alternative: This protocol utilizes human induced pluripotent stem cells (iPSCs) that are labeled with EGFP to induce host cerebral organoids. Alternatively, human embryonic stem cells (ESCs) expressing EGFP or RFP may also be employed.

Cell culture media

Table 1 Cerebral organoid induction and culture media

Components	Volume
Low-bFGF hESC media	**500 mL**
DMEM/F12	400 mL
KOSR	100 mL
ES-quality FBS	15 mL
GlutaMAX	5 mL
MEM-NEAA	5 mL
2-Mercaptoethanol	3.5 µL
bFGF*#	final conc. 4 ng/mL
ROCK inhibitor *#	final conc. 50 µM
Neural induction media	**50 mL**
DMEM/F12	50 mL
N2 supplement	0.5 mL
Glutamax supplement	0.5 mL
MEM-NEAA	0.5 mL

Heparin solution	50 µL
Improved NeuroDMEM -A	**250 mL**
DMEM/F12	125 mL
Neurobasal	125 mL
B27- vitamin A supplement	5 mL
Anti-anti	2.5 mL
Glutamax supplement	2.5 mL
MEM-NEAA	1.25 mL
N2 supplement	1.25 mL
insulin	62.5 µL
2-Mercaptoethanol	87.5 µL
Improved NeuroDMEM +A (RA+ medium)	**500 mL**
DMEM/F12	250 mL
Neurobasal	250 mL
B27+ vitamin A supplement	10 mL
Glutamax supplement	5 mL
Anti-anti	5 mL
Vitamin C solution (40mM stock)	5 mL
N2 supplement	2.5 mL
MEM-NEAA	2.5 mL
2-ME solution	175 µL
Insulin	125 µL
Cryopreservation Medium (CM)	**10 mL**
RA+ medium	9 mL
DMSO	1 mL
ROCK inhibitor*	final conc. 20 µM
* Add this component before use # Used only once at day 0 as indicated in Lancaster's protocol	

Equipment

Table 2 Equipment

Equipment	Company	Catalog number
Incubator	IncuSafe	N/A
Cellometer Mini Automated Cell Counter	Nexcelom Bioscience	N/A
Orbital shaker	Fisher Scientific	16454310
IVIS Lumina II	Perkin Elmer	NA
BD FACSCanto™ II System	BD Biosciences	NA
LSM780	Carl Zeiss AG	N/A
Axio Vert 7	Carl Zeiss AG	N/A

STEP-BY-STEP METHOD DETAILS

Cerebral organoid induction

Timing: 1 month

Note: The following steps are adapted from Lancaster's protocol [2,3]. Both human induced pluripotent stem cells (iPSCs) and human embryonic stem cells (ESCs) are eligible for cerebral organoid induction. To distinguish host cerebral organoids and inserted tumor explants, these pluripotent stem cells should fluorescent protein-expressing. In this protocol, iPSC with EGFP is used for illustration.

Making EBs

Timing: 5 d (day 0-5)

1. Wash 70~80% confluent iPSC cells in a 6-well plate with 1 mL DPBS.
    - The initial iPSC colony should be characterized by tightly packed colonies with defined borders, where iPSC cells show uniform morphology with a high nucleus-to-cytoplasm ratio, iPSC colonies should have no evidence of differentiation and should display optimal features of pluripotency.
2. Incubate iPSCs with 1 mL EDTA for 4 min at 37°C.
3. Remove EDTA gently and add 1 mL of Accutase. Incubate for another 4 min at 37 °C.
4. Detach the colonies with 1 mL mTeSR and transfer the iPSCs to a 15 mL falcon tube.
5. Pipette up and down to generate single cells and count the cell concentration.
6. Add another 3 mL mTeSR, and resuspend the cells.
7. Centrifuge the cells at 1000 rpm (~ 193 g) for 5 min.
8. Remove supernatant and resuspend the cell pellet with 1 mL low-bFGF hESC medium containing ROCK inhibitor and bFGF, with a final concentration 50 µM and 4 ng/ml, respectively. Take 20 µL cells solution and an equal volume of trypan blue to count live cells by replicates.
9. Plate 150 µL low-bFGF hESC medium supplemented with ROCK inhibitor and bFGF containing 9,000 cells in a 96-well low-attachment U-bottom plate.
10. Put the plate back in the incubator and avoid movement for at least 24 hours.
11. Aspirate the 110 µL medium gently two days later. Add 150-µL low-bFGF hESC medium.

Induction of primitive neuroepithelia

Timing: 6 d (day 5-11)

12. Transfer EB into a low-attachment 24-well plate using a wide bore 200 µL tip. Feed EB with 500 µL neural induction medium.
13. Change the medium every other day.

Embedding neuroepithelial tissues with Matrigel

Timing: 2 h (day 11)

14. Wash the Embedding Sheets with DPBS in 10-cm dishes, and dry them before use.
    - Embedding Sheets are reusable and can be stored in 75% Ethanol in 50-mL Falcon Tubes.
    - Embedding Sheets should be immersed in DPBS to wash off the ethanol thoroughly.
15. Transfer neuroepithelial tissues onto the Embedding Sheet with 200-µL cut tips.
    - Set P200 pipette to 40µL.
    - The diameter of the opening should be at least 0.5 mm larger than the CO.
16. Carefully remove excessive medium with 200-µL uncut tips.
    - The tip opening should be positioned behind the organoid to avoid potential damage.
    - Leave ~5 µL medium with tissues is critical to avoid drying, as later steps take some time to handle.
17. Add 30 µL Matrigel using pre-cooled 200-µL tips.
18. Position the aggregates centrally. Incubate at 37°C for 20min.
    - We usually handle 18 aggregates each time at RT. But with the assistance of a 4°C cooling rack, which slows down the solidification speed of Matrigel, more aggregates could be handled each time.
19. Hold the embedding sheet with sterilized forceps, and gently rinse down the droplet with neural induction medium into an ultra-low attachment 6-well plate.
    - Six droplets in each well are recommended.

Stationary culture of cerebral organoids

Timing: 7 d (day 11-18)

20. Stationary culture of the tissues. Feed the tissues with Improved NeuroDMEM-A with CHIR for three days, followed by Improved NeuroDMEM-A only for another four days.

Cerebral organoids growth

Timing: 7 d (day 18-)

21. Agitate the plates on the orbital shaker at 75 revolutions per minute (rpm). Feed the organoids with Improved NeuroDMEM +A (RA+ medium) from day 20.
22. The medium was changed every 2-3 days throughout the culture period.

Lentivirus production

Timing: 4 d

HEK293T cells seeding

Timing: 20 min (day 1)

Note: HEK293T cells < 10th generation are used to produce second-generation lentivirus.

23. Split confluent HEK193T cells using 0.25% Trypsin from 10-cm petri dishes.

24. Transfer 5 x 10^6 cells to a 10-cm petri dish pre-coated with Laminin. Feed the cells with DMEM+ 10% FBS.

25. Swirl the dishes thoroughly to ensure even distribution of HEK193T cells suspension on the surface of the dish.

Transfection with plasmid mixture

Timing: 1 h (day 2)

26. Exchange DMEM with 10%FBS medium 2 hours prior to transfection.

Note: The following steps should be conducted in an S2 lab.

27. Prepare a plasmid mixture in Opti-MEM in a 1.5 Eppendorf Tumor according to Table 1.

28. Add three times the volume of 1 μg/μL PEI to the total mass of DNA.

Table 3 A plasmid mixture for HEK293T cultured in each 10-cm petri dish (500 μL)

Components	Amount
Opti-MEM	460 μL
pMD2.G #12259 vsvg envelope	2 μg
psPAX2 #12260 gag/pol/rev/tat, 2nd	2 μg
pWPI-EF1a-Luc2 (Gibson)	4 μg
Sum mass of DNA	8 μg
PEI (1 μg/μL)	24 μL

29. Mix the tube well immediately but gently by tapping. Incubate 15 min at RT.

30. Add the transfection mixture onto each plate, swirl the plate carefully to distribute the DNA mix, and incubate it overnight.

31. After 12 hours, change the medium with 5 mL fresh growth medium.

Collecting and concentrating lentivirus

Timing: 12 h (day 3~4)

- After 48 hours, lentivirus is ready to be harvested.

32. Carefully collect media from dishes containing HEK293T cells.

- Pay attention to not disturbing the HEK293T monolayer of cells.

33. Centrifuge the supernatant media at 1000 rpm and 4°C for 5 min.

34. Filter the media using the 0.22 μm syringe into 15 mL tubes.

35. Add Lenti-X Concentrator, gently mix it, and leave it at 4°C overnight.
36. Centrifuge the tubes with a Lenti-X Concentrator at maximum speed for 1 hour at 4°C.
37. Carefully decant the supernatant into the waste bottle.
38. Resuspend the pellet and aliquot the virus using DPBS.

    Note: To determine the optimal amount of virus solution, 2-mm cerebral organoids were directly infected using aliquot virus solution with several dilutions, followed by downstream staining for luciferase and apoptosis markers (e.g., cleaved caspase 3).
39. Single-use aliquots can be stored at-80°C.

Process tumor specimens

Timing: 1 h

Note: Various brain tumors could be cultured with this system, including primary/recurrent GBM, metastatic lymphoma, and metastatic melanoma. Ideal tumor tissues are close to the tumor border without significant necrosis. After tumor resection, transfer them to the lab on ice in appropriate medium (e.g., RA+ medium). Proceed the specimens as soon as possible.

40. Transfer tumors into a 10-cm dish.
41. Cut the tumor sample into small explants with scalpels, size ~1 mm in diameter.
    - Before cutting the tumor tissues, remove the excessive medium.
    - Always keep the tumor tissues wet; otherwise, they will be fragile and easy to shred.
    - Redundant tumor explants can be cryopreserved to acquire fresh frozen (FF) tumor tissues. Cryopreserving and recovering fresh tissue are similar to IPTO, described in *Steps 72-78* and *Steps 79-85*).
42. Wash small tumor explants 3x with 3 mL RA+ medium, and carefully discard the RA+ medium after the tumor explants sink to the bottom of the Falcon Tube/plate.
43. Transfer the tumor explants to a 12-well plate with RA+ medium containing 10 μM ROCK inhibitor using 200-μl cut tips.

Tumor infection with luciferase-carrying virus

Timing: 2 h or overnight

Note: Lentivirus or adeno-associated virus which expressing luciferase can be used to infect tumor tissue.

44. Add appropriate amount of concentrated lentivirus expressing firefly luciferase, with the addition of 8 μg/ml Polybrene. Incubate the mixture at 37°C, 5% CO2 for 6 h.
    - Incubate samples overnight should work.

45. When an adeno-associated virus is used to infect tissue, transfer the tumor pieces to Embedding Sheet, inject 1μL AAV-luc to each tumor piece. Hold the embedding sheet with sterilized forceps, and gently rinse down the droplet with RA+ medium containing 10 μM ROCK inhibitor. Incubate the tumor tissue at 37°C, 5% CO2 for 2 h.

46. Wash samples with RA+ medium 3 times, followed by a co-culture process.

Prepare cerebral organoid

Timing: 0.5 h

47. Generate host organoids following Lancaster's protocol [2,3].

48. Transfer desired host organoids into a 10 cm dish.

- Qualified organoids should age from 4 weeks to 20 weeks in the absence of visible cavity structure.
- The recommended size of the organoid is 2-3 mm in diameter.

49. Make an incision in the middle of the organoids with a scalpel; the incision depth should be 1/2 ~ 2/3 of diameter.

- This step is to increase the tumor-host interaction interface.
- Over-superficial incision leads to insufficient tumor-host interaction, yet over-deep incision may break up the host organoid.
- Always keep the organoids wet; otherwise, they will be fragile and easy to shred.

50. Transfer the host organoids to a 12-well plate using 1-mL cut tips.

51. Wash them tree times with RA+ medium and fed with RA+ medium containing 10 μM ROCK inhibitor.

52. Place the plate in the incubator for later use.

Co-culture of tumor tissues and cerebral organoids

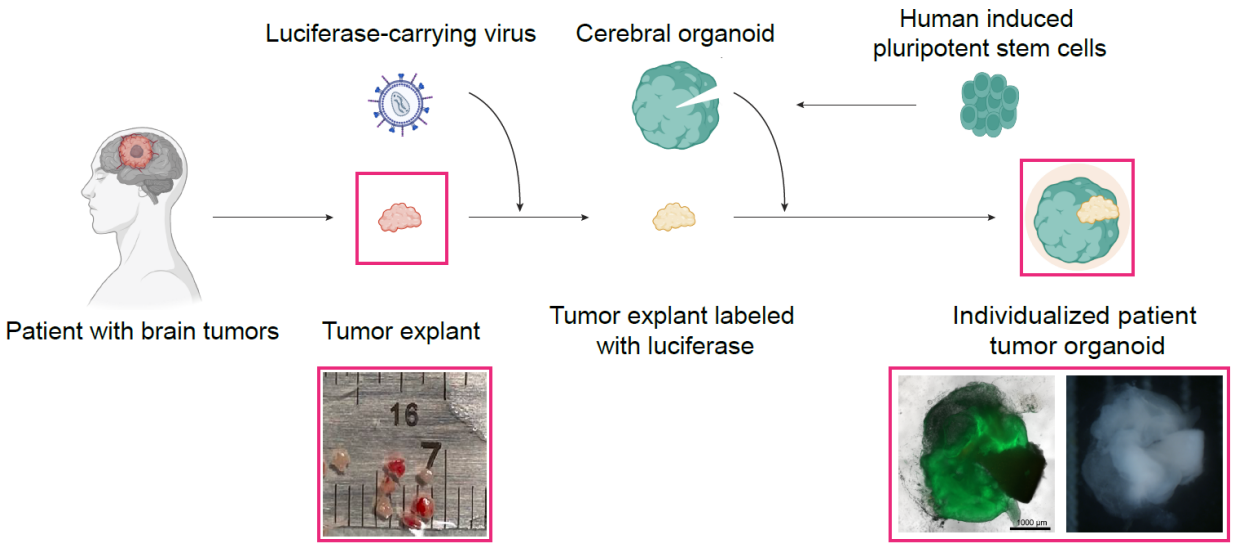

Figure 1. Experimental scheme showing a procedure for generating individualized patient-derived tumor organoids (IPTO).

Timing: 1 h

53. Wash the Embedding Sheets with DPBS in 10-cm dishes, and dry them before use.
    - Embedding Sheets are reusable and can be stored in 75% Ethanol in 50-mL Falcon Tubes.
    - The Embedding Sheet should be immersed in DPBS to wash off the ethanol thoroughly.
54. Transfer CO onto the Embedding Sheet with 1-mL cut tips (prepared in *steps 40-45*).
    - Set P1000 pipette to 40µL.
55. Carefully remove excessive medium with 200-µL uncut tips.
    - The tip opening should be positioned behind the organoid to avoid potential damage.
56. Transfer the tumor explants on the Embedding sheet with 200-µL cut tips
    - The diameter of the opening should be 1.5-2 mm.
57. Carefully remove excessive medium with 200-µL uncut tips.
    - The position of the tip opening should be behind the organoid to avoid potential damage.
    - Leave ~5 µL medium is critical to avoid drying, as later steps take some time to handle.
58. Position the tumor tissue inside the host organoid using autoclaved metal toothpicks or 10-µL tips.
    - 10-µL tips could work, but tumor explants tend to stick to the tips. We prefer to use autoclaved metal toothpicks, as they are smoother.
    - Try to insert the tumor inside host organoids as much as possible to increase the tumor-host interaction interface (**Figure 1**).

59. Carefully remove excessive medium with 200-μL uncut tips.
    - The position of the tip opening should be behind the organoid to avoid potential damage.
60. Add 30 μL Matrigel using pre-cooled 200-μL tips.
61. Position the tumor-organoid mixtures centrally.
    - If the relative position of tumor and host organoid changes, it is important to re-position tumor tissue as described in *Step 18*.
    - We usually handle 9 mixtures each time at room temperature. But with the assistance of a 4°C cooling rack, which slows down the solidification of Matrigel, up to 18 mixtures (the upper limit of each embedding sheet) could be handled each time.
62. Incubate the mixtures at 37°C for 20min.
63. Add 3 mL of RA+ medium containing 10 μM ROCK inhibitor to each well of the 6-well plate.
64. Hold the embedding sheet with sterilized forceps and gently rinse down the droplet with RA+ medium.
    - Six droplets in each well are recommended.
65. Stationary culture the IPTO overnight.
66. Transfer mixtures onto orbital shaker, 75 rpm.
67. Change the medium with RA+ medium 2 days later, and every 2-3 days thereafter.
    - CAUTION: Do not damage the Matrigel while changing the medium

Passage individualized patient-derived tumor organoids

Timing: 2 h

Mature IPTO can be passaged to avoid necrosis. We define "mature IPTO" (Figure 2A and 2B) as compact cell density and: (1) IPTO size (diameter) larger than 3-4 mm and/or (2) the predominance of tumor cells in IPTO. Compact cell density is considered when the edge of IPTO turns dark under the light microscope. The predominance of tumor cells could be judged by a weak GFP signal, which indicates the necessity of a new host organoid. The time for passaging could range from 4 weeks to 16 weeks, depending on growth speed of the tumor cells.

68. Transfer mature IPTOs into a 10-cm dish using 1-mL cut tips.
69. Cut the IPTOs into small pieces with a scalpel, size ~1 mm.
    - Before cutting IPTO, remove excessive medium with 200-μL tips.
    - Always keep the tumor tissues wet; otherwise, they will be fragile and easy to shred.
70. Transfer IPTO pieces to a 15-ml Falcon Tube and wash with 3x with 3 mL RA+ media.
71. Transfer IPTO pieces to a 96-well plate using 1-mL cut tips with RA+ medium containing 10 μM ROCK inhibitor to selected qualified daughter IPTOs.
    - A Stereotype Immunofluorescent Microscopy and IVIS imaging is highly recommended for proportional passage of mature IPTOs.

- Daughter IPTOs should contain enough tumor cells (GFP-) and BLI signals, determined by microscope and IVIS imaging, respectively (**Figure 2C**).

72. Place the plate in the incubator for later use.

73. The preparation of host organoids and co-culturing mature IPTO pieces and host organoids in this step, is the same as described in former sections (*Steps 40-45*).

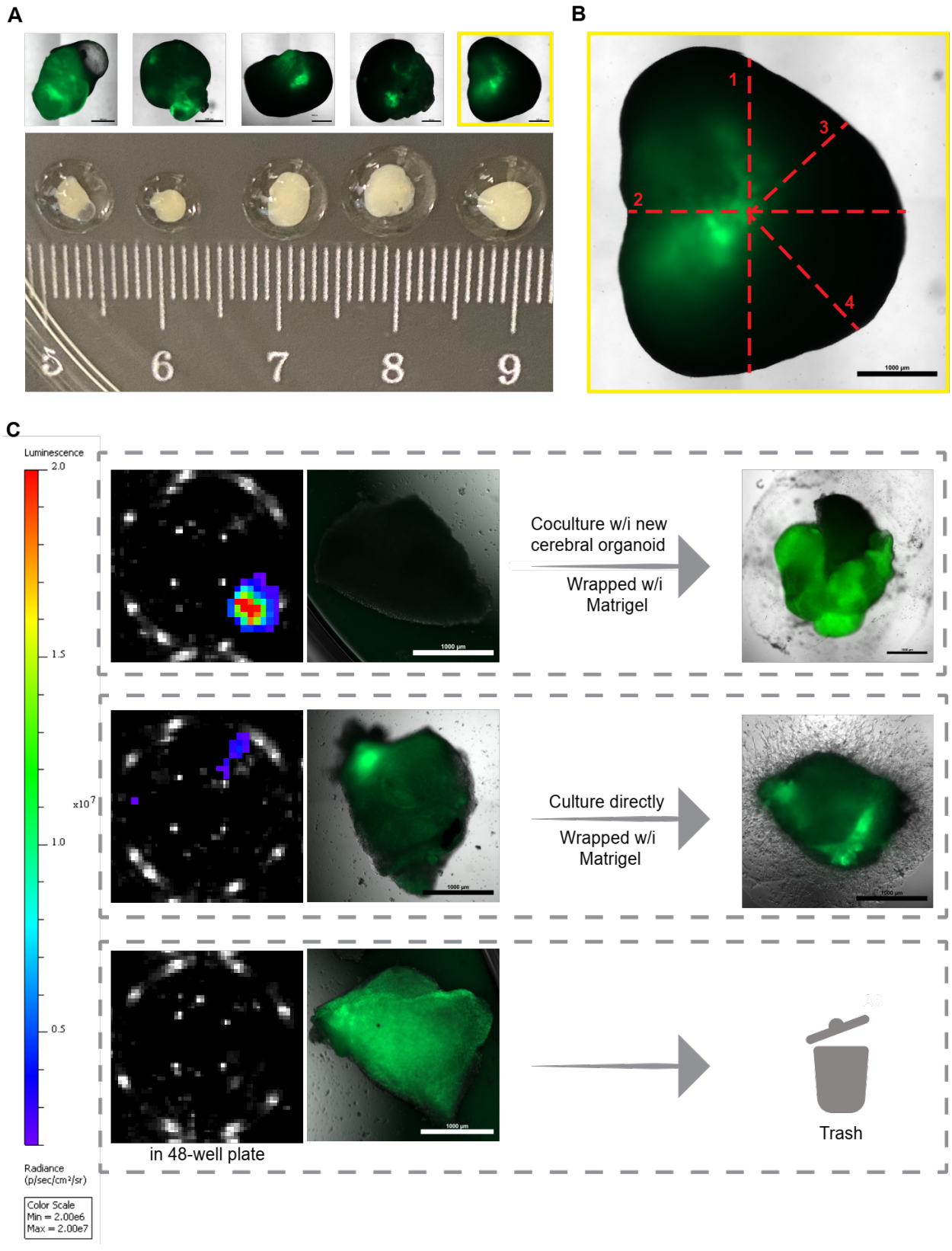

Figure 2. Illustrative figures showing the IPTOs passage process.

(A). Images acquired from fluorescent microscope and stereomicroscope demonstrating IPTOs ready to be passaged. The yellow frame defined the IPTO for subsequent demonstration.
(B). Images showing the order to cut an IPTO.
(C). Images deciphering the processed strategies of cut IPTOs based on their bioluminescence signal and presence of cerebral organoid components (GFP+).

Cryopreserve individualized patient-derived tumor organoids

Timing: 2 h

Note: Mature IPTOs could also be cryopreserved.

74. Process mature IPTOs as described in *Steps 66-69.*
75. Incubate qualified IPTO pieces on an orbital shaker (75 rpm, 37°C, 5% CO_2) with RA+ medium containing 10 μM ROCK inhibitor) for 1 hour.
76. Discard the RA+ medium (containing 10 μM ROCK inhibitor and resuspend IPTO pieces with CM.
77. Treat IPTO pieces at room temperature for another 10 min.
    - The diameter of IPTO pieces should be ~ 1 mm to allow the permeation of CM.
    - This step allows CM to permeate the IPTO pieces. Longer than 10 min is not recommended as DMSO may damage cells.
78. Discard CM and resuspend IPTO pieces with fresh CM.
79. Transfer the IPTO pieces with 1 mL CM in cryotubes (~30 pieces in each tube).
80. Store cryotubes in a CoolCell freezing container at-80°C overnight.
    - Note: Place the samples in liquid nitrogen for long-term storage.

Recover individualized patient-derived tumor organoids

Timing: 2 h

81. Thaw cryovials in a 37°C water bath until only a small chunk of ice remains.
    - Gently swirl the tubes to ensure even thawing.
82. Vials should be thawed as soon as possible.
83. Prepare a 6-well plate with 2 mL RA+ containing 10 μM ROCK inhibitor to dilute the DMSO in the medium later.
84. Gently transferred samples into the 6-well plate with a P1000 pipette with a cut tip.
85. Discard the supernatant and add 3 mL of RA+ medium with 10 μM ROCK inhibitor.
86. Place the plate in the incubator for later use.
87. Follow the procedures for the preparation of host organoids (*Steps 40-45*) and co-culturing (*Steps 51-65*).

Bioluminescence imaging

Bioluminescence imaging

Timing: 20 min

Note: Bioluminescence signal can be detected three days after virus infection.

88. Initialize "Living Image" software (~10 minutes).
89. Discard the old medium and add fresh medium containing 150 μg/mL D-luciferin. Incubate the samples on an orbital shaker for 10 min at 37°C and 5% CO2.
90. Transfer the samples to the IVIS chamber.
91. Set exposure time as 60s and click "Acquire Sequence."
92. Feed the samples with fresh medium after imaging.

Data extraction (Living Image Software, PerkinElmer)

Timing: 10 min

Note: Bioluminescence signal data are retrieved from Living Image Software.

93. In the "Type" dropdown menu, choose "Average Bkg ROI". Click on "Circle", set appropriate diameter, and drag it to an area that no sample is inside.
    - Note: The size of "Average Bkg ROI" should be consistent and the same as "Measurement ROI".
94. Go back to the ROI tools and change "Type" dropdown menu to "Measurement ROI". Click on "Circle", set appropriate diameter, and drag it totally cover your sample.
    - Note: Keep the size of "Measurement ROI" consistent and same as "Average Bkg ROI".
95. Right click on the circle and choose "Set Bkg ROI to BKG1".
96. Then Right click on the circle again and choose "Duplicate ROI in Sequence" for the remaining ROIs.
97. Set the units as "Radiance (Photons)" and export the data to your path.
    - Note: use "Total Flux [p/s]" to represent the bioluminescence signal for downstream analysis.

Evaluation of temozolomide responses in IPTOs

98. Tumor tissues were labeled with firefly luciferase as described earlier and were cultured for two weeks before drug treatments.
    - IPTOs should be cultured in 24-well ultra-low attachment plates, with one IPTO per well, for better monitoring BLI signals for individual IPTO.
99. The treatment groups were exposed to 50 μM temozolomide (Sigma-Aldrich), while the control group was treated with DMSO vehicle.

- Each treatment group was evaluated in quadruplicate.

100. The IPTOs were maintained on an orbital shaker rotating at 75 rpm within a sterile incubator set at 37°C, 5% CO2, and 90% humidity.

101. Media containing fresh drugs or vehicles was replenished every 48 hours after BLI signal monitoring.

102. The BLI signal was monitored every 48 hours and the medium was changed.

- The fold change of BLI signal was utilized as readouts to determine the difference between the control group and treatment group. The BLI fold change referred to the ratio of the relative BLI value in the treatment group to that in the control group, where the relative BLI value was obtained by dividing the signal obtained at the end of the experiment by the signal obtained on the day the treatment was initiated. The BLI fold change lower than 0.85 was considered sensitive when evaluating drug response in IPTOs. For selected experiments, the counts of DAPI and KI67 or EdU were quantified based on immunohistology.

Histological analysis and immunofluorescence

Fixation of organoid tissues

Timing: 12 h

103. Transfer tissues to a 12-well plate using a 1-ml cut tip.

104. Wash with 2 mL of DPBS three times and add 2 mL of 4% (wt/vol) PFA. Leave the samples at 4 °C overnight.

105. Gently aspirate the PFA and wash samples with 2 mL of DPBS on an orbital shaker (150 rpm) 3 times for 10 min each, and aspirate it.

106. Replace the final DPBS wash with 2 mL of 30% (wt/vol) sucrose solution and place it at 4 °C overnight to allow tissues to be totally immersed in the sucrose solution.

Note: Samples could be safely stored for three months at this time.

Embedding tissues for cryosection

Timing: 2 h

Table 4 Embedding solution for cryosection

Components (100 mL)	Amount
Sucrose (g)	10 g
Gelatin (g)	7.5 g
DPBS	To 100 mL

107. Add 2 mL pre-warmed embedding solution into a cryosection mold (Sigma, cat.no. E6032-1CS) to cover the bottom. Place it at 4 °C to allow it to polymerize.

108. Discard the 30% sucrose solution, and add 2 mL of pre-warmed embedding solution. Place samples at 37°C for 15 minutes to equilibrate the tissues (assembled as **Figure 3A**).

109. Carefully transfer the organoids from the 12-well plate to the mold with a polymerized gelatin/sucrose layer (**Figure 3B**). Position the organoids as close to each other as possible (**Figure 3C**).

110. Allow the small amount of embedding solution that was transferred with the organoids to solidify at room temperature for 5 min (**Figure 3D**).

111. Add another 2 mL warm embedding solution in the weighing mold to cover the tissues completely. Place it at 4 °C and allow it to polymerize (**Figure 3E**).

112. Prepare the freezing bath filled with isopentane (2-methylbutane) and surrounded by dry ice (assembled as **Figure 3F**). Wait for the temperature to lower between −50 ~ and −30 °C.

113. Transfer the mold containing polymerized embedding materials to the freezing bath.

114. Carefully take the mold out of the bath using a forcep and store it at −80 °C until ready to section.

Cryosection

Timing: 0.5~1 h for each block

115. Cut the sections using a standard cryostat and collect sections on Ultra Plus slides (Thermo Scientific Menzel, 10417002).

116. 10~20 µm sections are appropriate for downstream staining.

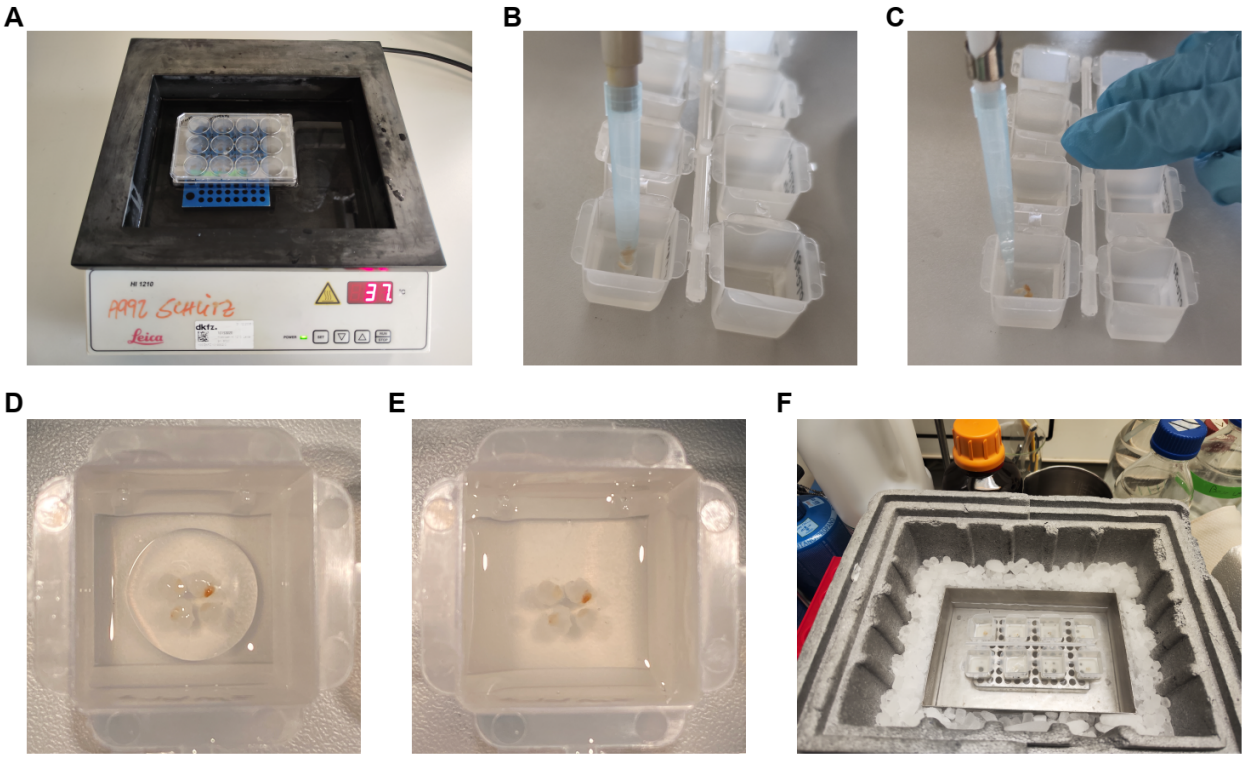

Figure 3. Embedding IPTOs.
(A). The assembly of 37°C water bath.
(B). Organoid tissues are transferred to the mold with a polymerized gelatin/sucrose layer.

(C). Position the organoids as close to each other as possible. **D**. Organoid tissues in a small amount of polymerized gelatin/sucrose mixture.
(E). Organoids tissues in the full amount of polymerized gelatin/sucrose mixture.
(F). The assembly of a freezing bath.

Immunostaining

Timing: 2 d

The following steps describe the immunostaining in tumors.

117. Retrieve cryosection slides and dry them at room temperature for 20 min.

118. Wash the slides with 0.2% triton-100 in PBS 3 times for 5 min each.

119. Treat the samples with Citra-based solution at 80°C for 20 minutes.

120. Wash slides with distilled water three times for 5 min each and with 0.2% triton-100 in PBS three times for 5 min each.

121. Draw a circle around samples using a hydrophobic PAP pen.

122. Block the samples with PBS containing 5% BSA and 0.2% triton-100 in a humidified chamber at room temperature for 1 hour.

123. Add diluted antibodies in PBS containing 5% BSA and 0.2% triton-100. Incubate slides in a humidified chamber at 4°C overnight.

124. Wash slides with 0.2% triton-100 in PBS 3 times for 10 min each.

125. Add diluted secondary antibodies and DAPI in PBS containing 5% BSA and 0.2% triton-100. Incubate slides in a humidified and dark chamber at room temperature for 1 h.

126. Wash slides with 0.2% triton-100 in PBS 3 times for 10 min each.

127. Add a drop of mounting medium and mount the coverslip.

EXPECTED OUTCOMES

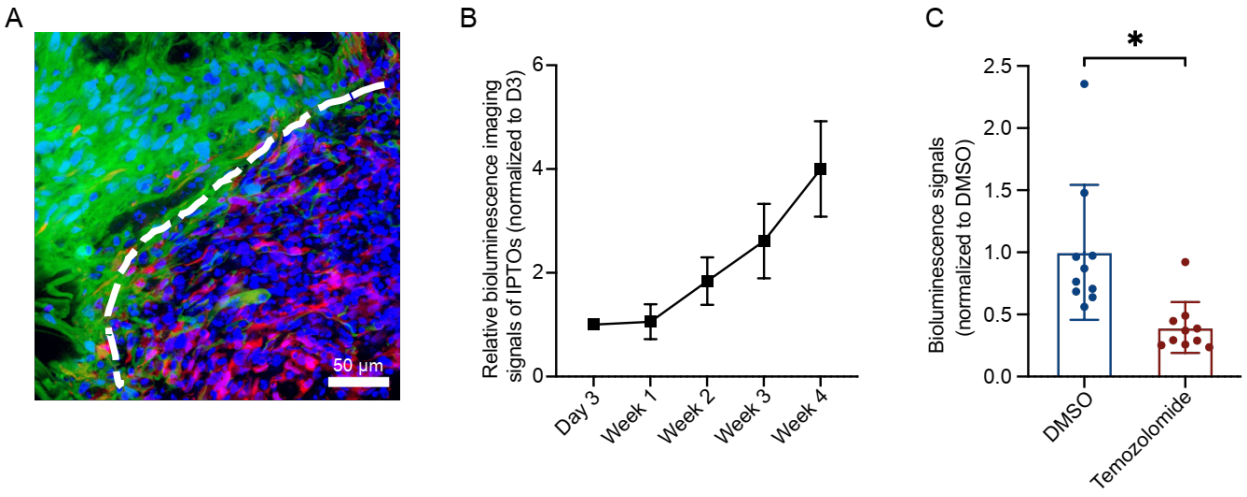

Figure 4. Representative results.

(A). A representative immunostaining images of a 10-week IPTO showing mutual invasion between tumor cells (mCherry) and cells from host cerebral organoids (GFP+). Tumor explants were infected with mCherry rAAV [4] prior to coculture.
(B). A representative plot exhibiting a gradual increase of bioluminescence imaging signal of IPTOs from a patient (n =6).
(C). A representative bar chart indicating IPTOs from one patient significantly responded to 50 μM temozolomide treatment.

This protocol details methods to quickly establish IPTOs and evaluate drug efficacy in IPTOs. Anticipated mutual invasion between tumor cells and cells from host cerebral organoids are showed in **Figure 4A**, respectively. The IPTOs are well characterized in our previous data, which recapitulate the histologic features, epigenetic profiles, genetic alterations, and cell components of their original tumors. This protocol can culture a wide spectrum of brain tumors using bioluminescence imaging within three weeks (**Figure 4B**), which can be confirmed by immunofluorescent staining. More importantly, TMZ responses can be quickly assessed in IPTOs (**Figure 4C**), which closely predict TMZ response in clinic, superior to MGMT promoter methylation. For some highly proliferative tumors, tumor samples can be passaged and expanded.

QUANTIFICATION AND STATISTICAL ANALYSIS

Each experiment was conducted at least in triplicate, as the corresponding figure legend indicated. The results were presented as mean ± SEM. P-values were calculated using a two-tailed Student's t-test or ANOVA in the R package. Statistical significance was determined by P-values of 0.05 (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$). Additional information on sequence analysis is available in the respective STAR Methods sections.

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Haikun Liu (L.Haikun@Dkfz-Heidelberg.de).

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