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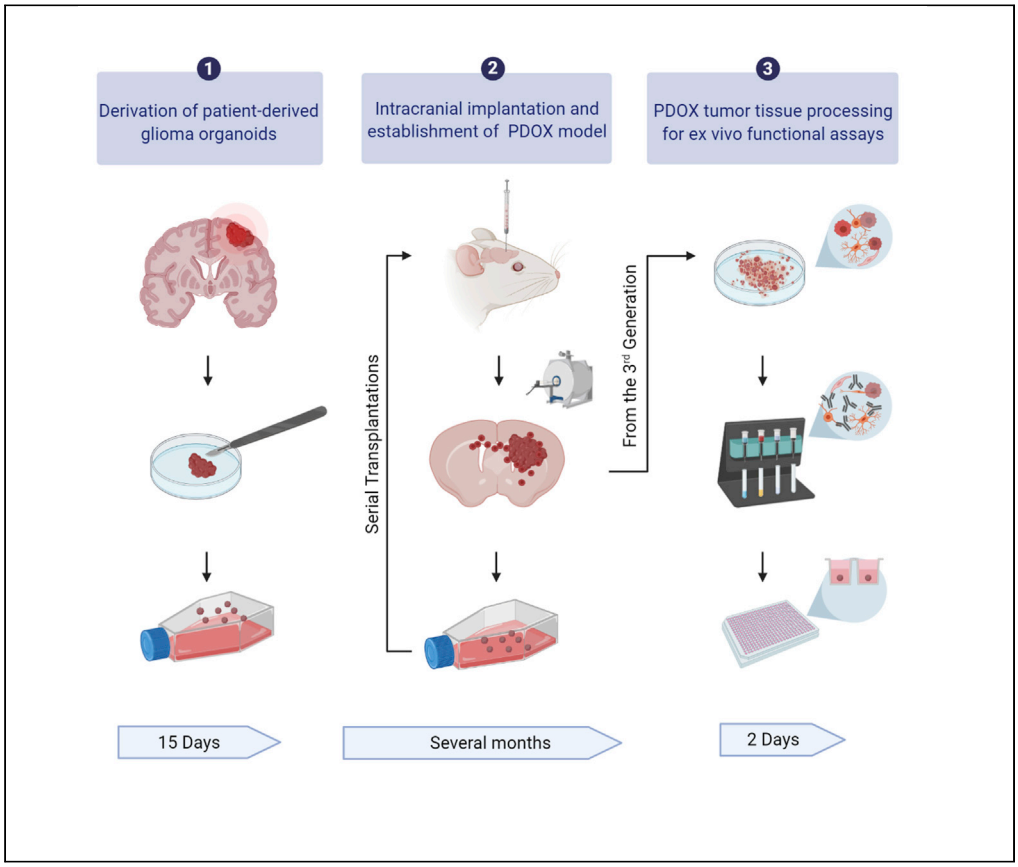
Protocol for derivation of organoids and patient-derived orthotopic xenografts from glioma patient tumors



Tumor organoids and patient-derived orthotopic xenografts (PDOXs) are some of the most valuable pre-clinical tools in cancer research. In this protocol, we describe efficient derivation of organoids and PDOX models from glioma patient tumors. We provide detailed steps for organoid culture, intracranial implantation, and detection of tumors in the brain. We further present technical adjustments for standardized functional assays and drug testing.

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Highlights

Organoids can be generated from diverse glioma patient tumors

High-grade glioma organoids give rise to patient-derived orthotopic xenografts

Serial transplantation in vivo allows for consistent expansion of human tumor cells

The adapted protocol for reconstitution of uniform organoids for functional assays

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Protocol for derivation of organoids and patient-derived orthotopic xenografts from glioma patient tumors

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SUMMARY

Tumor organoids and patient-derived orthotopic xenografts (PDOXs) are some of the most valuable pre-clinical tools in cancer research. In this protocol, we describe efficient derivation of organoids and PDOX models from glioma patient tumors. We provide detailed steps for organoid culture, intracranial implantation, and detection of tumors in the brain. We further present technical adjustments for standardized functional assays and drug testing.

For complete details on the use and execution of this protocol, please refer to Golebiewska et al. (2020).

BEFORE YOU BEGIN

This protocol requires viable tumor tissue material obtained during surgery of glioma patients. Ethical approvals are required prior the collection and patients must give informed consent. Glioma patient tumors used in this protocol were collected at the National Department of Neurosurgery at the Centre Hospitalier de Luxembourg (CHL) after informed consent of the patients, and with the approval from the local research ethics committee (National Committee for Ethics in Research (CNER), Luxembourg; protocol REC-LRNO-20110708). All mouse experiments must be approved by the internal animal welfare structures and the national authorities responsible for animal experiments. Animal experiments described in this protocol were approved following the European Directive 2010/63/EU, under the references: LRNO-2014-01, LUPA2019/93 and LRNO-2016-01. Established PDOX models generated in this study are available at PDX Finder portal (www.pdxfinder.org).

Agar flasks for organoid culture

Timing: 1 h

1. Preheat 150 mL DMEM medium w/o supplements to 42°C.

2. Weigh 1.8 g of Noble Agar and transfer to a sterile glass bottle.
3. Add 60 mL of sterile water and boil in microwave to dissolve, the agar should appear transparent.
4. Cool down to 42°C in the water bath. It is not necessary to autoclave the agar as boiling in the microwave sterilizes the solution. We advise to use a dedicated microwave within the tissue culture facility.
5. Work under the biological safety cabinet II (BSC II). Mix gently with preheated 150 mL of DMEM medium (w/o supplements) to achieve a final Agar concentration 0.85% (w/v).
6. Transfer liquid agar in a sterile culture flask with vent/close cap (15–16 mL per T75, 5–6 mL per T25) and leave to solidify under the BSC II w/o the cap.
7. Store flasks at 4°C with closed caps.

⚠ CRITICAL: Agar solidifies rapidly at temperatures below 42°C, thus it is important to apply DMEM pre-warmed at 42°C. Store agar Flask at 4°C for up to one month.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, buffers, and reagents		
BD Difco™ Dehydrated Culture Media: Noble Agar	Fisher Scientific	11798223
Bone wax surgical specialties 2.5 *g*	Surgical Specialties Corporation	901
Bovine Serum Albumin	Sigma-Aldrich	A9418
BRAUNOL 7.5/100 G 1 FL HDPE 1000	Braun	3864154
Bupivacaine hydrochloride (Marcain 0.25%)	Aspen	N/A
DMEM High Glucose without L-Glutamine	Westburg	12-614F
DMSO, Dimethyl sulfoxide	Sigma	D4540-100ML
Fetal Bovine Serum, qualified, heat inactivated, Brazil	Gibco	10500064
Gadoterate, Dotarem, 0.5 mmol/mL	Guerbet	N/A
Hanks' Balanced Salt solution (HBSS) w/o Ca2+/Mg2+	Sigma	HBSS-H6648
Isoflurane	CP Pharma	G228L19A
Ketamine (Nimatek), 100 mg/mL	Dechra	N/A
Mouse Cell Depletion Kit	Miltenyi Biotec	130-104-694
Myeline Removal Beads II	Miltenyi Biotec	130-096-433
NaCl 0.9% (20 × 10 mL)	BRAUN	235 0748
Neural Tissue Dissociation Kit	Miltenyi Biotec	130-092-628
Non-essential amino acids 10 mM 1000× stock	Westburg	LO BE13-114E
Ocry-gel Ogen Honden/Katten Tube 10 g	TVM	48026T613/3
Penicillin-Streptomycin (Pen-Strep)	Westburg	DE17-602E
Sodium pyruvate solution 100mM	Sigma-Aldrich	S8636-100mL
Buprenorphine (Vetergesic Multidose) 0.3 mg/mL	Ecuphar	N/A
UltraGlutamine I (Alanyl-L-Glutamine) 200 mM (1000×)	Westburg	LO BE17-605E/U1
Xylasine (Rompun) 2%	Bayer	N/A
Experimental models: organisms/strains		
Mouse: NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ (NSG)	Charles River	N/A
Software and algorithms		
ImageJ		N/A
PC-SAM	SA Instruments Inc.	N/A
Preclinical Scan	MR Solutions	N/A
Others		
10 mL Serological pipettes	Greiner	607180
15 mL CELLSTAR® Polypropylene Tube	Greiner	188271
25 mL Serological pipettes	Greiner	760180
50 mL CELLSTAR® Polypropylene tubes	Greiner	227261

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
50 mL Serological pipettes	Greiner	768180
5 mL Serological pipettes	Greiner	606180
Aspiration pipettes	Carl Roth	NA41.2
Barrier OP Towel (50 cm × 50cm)	Barrier	706400
Bone Micro Probe	FST	10030-13
Burrs for Micro Drill	FST	19008-07
Cell culture Flask, 50 mL, 25 cm^2, PS, Red standard screw cap, Clear, CELLSTAR® TC, Sterile	Greiner	690160
Cell culture Flask, 250 mL, 75 cm^2, PS, Red standard screw cap, Clear, CELLSTAR® TC, Sterile	Greiner	658170
Cell culture Flask, 50 mL, 25 cm^2, PS, Red filter screw cap, Clear, CELLSTAR® TC, Sterile	Greiner	690175
Cell culture Flask, 50 mL, 75 cm^2, PS, Red filter screw cap, Clear, CELLSTAR® TC, Sterile	Greiner	658175
CellTrics™ 50 µm Sterile	Sysmex	04-004-2327
Disposable pasteur pipette, with cotton plug	VWR	612-1799
Dissecting Chisel	FST	10095-12
Ethilon polyamide 6 (3.0) 19 mm 75 cm 3/8C	Ethicon	EH7665
Extra Fine Bonn Scissors	FST	14085-08
Fine Scissors - Sharp	FST	14060-09
Graefe Forceps	FST	11051-10
Halsey Micro Needle Holder	FST	12500-12
High glass petri dish, 2 mm high, 100 mm diameter	VWR	391-2840
High Speed Rotary Micromotor Kit	Foredom	K.1070
LS column	Miltenyi Biotec	130-042-401
MediHeat warming cabinet (Customized)	PECOSERVICE	N/A
Mouse Ear Bar for SR Series	NARISHIGE	EB-3B
Mouse Homeothermic Blanket with YS451 Rectal Probe	Harvard Apparatus	HB101SM451
MRI 3 Tesla	MR Solutions	N/A
NovaFlex	World Precision Instruments	F0-150
PrimeSurface 384 well	FUJIFILM Wako Chemicals Europe GmbH	628-01449
QuadroMACS™ Separator	Miltenyi Biotec	130-091-051
Scalpel Handle - #3	FST	10003-12
Semken Forceps	FST	11009-13
Stereotaxic instrument	NARISHIGE	SR-5R
Stereotaxic microinjector	NARISHIGE	ISM-3
Stereotaxic micromanipulator	NARISHIGE	SM-15
Sterile Scalpel Cutfix®, 24	Carl Roth	X006.1
STERIPLAN Petri dish 100 mm	Fisher Scientific	11750844
Surgical blades carbon steel	Swann-Morton	203
Surgical patties	Codman	80-1402
Syringe 5 µL, Model 75 RN SYR, Small Removable NDL, 32 ga, 2 in	Hamilton	7105KH
Temperature control unit	Harvard Apparatus	HB101
Thermo Scientific™ Nalgene™ System 100™ Cryogenic Vials 1.5 mL	Thermo Fisher Scientific	5000-1020

MATERIALS AND EQUIPMENT

Organoid medium		
Reagent	Final concentration	Amount
DMEM 4.5 g/L glucose w/o L-Glu		420 mL
Fetal Bovine Serum	10% (v/v)	50 mL
Pen-Strep	100 U/mL	5 mL
UltraGlutamine I	2 mM	5 mL
NEAA	100 μM	20 mL
Total	**n/a**	**500 mL**
Store at 4°C for two months maximum.		

Freezing medium		
Reagent	Final concentration	Amount
DMEM 4.5 g/L glucose w/o L-Glu	70% (v/v)	70 mL
Fetal Bovine Serum	20% (v/v)	20 mL
DMSO	10% (v/v)	10 mL
Total	**n/a**	**100 mL**
Aliquot and store at −20°C for six months maximum.		

MACS buffer		
Reagent	Final concentration	Amount
HBSS or PBS w/o Ca^{2+}/Mg^{2+}		95 mL
10% (w/v) BSA in HBSS	1. (v/v)	5 mL
Total	**n/a**	**100 mL**
Prepare fresh for each experiment. Store at 4°C until use.		

STEP-BY-STEP METHOD DETAILS

Derivation of organoids from glioma patient tumors

Timing: 15 days

This part describes the detailed step-by-step derivation of tumor organoids from viable tumor tissue obtained during patient surgery. This includes the processing of the tumor tissue after reception from the hospital, organoid culture and biobanking. The processes described below must be performed under Biosafety level 2 (BSL2) and sterile conditions.

Note: Prior to tissue manipulation, pre-warm organoid medium to 37°C in the water bath. Prepare sterile BSC II with required material and transfer agar flasks to BSC II prior tumor tissue collection.

Tumor tissue processing

Timing: 1 h

1. The resected viable tumor tissue (from male and female) remaining after neuropathology sample is collected is placed in 10 mL ice-cold DMEM in the operating theater and stored at 4°C in the

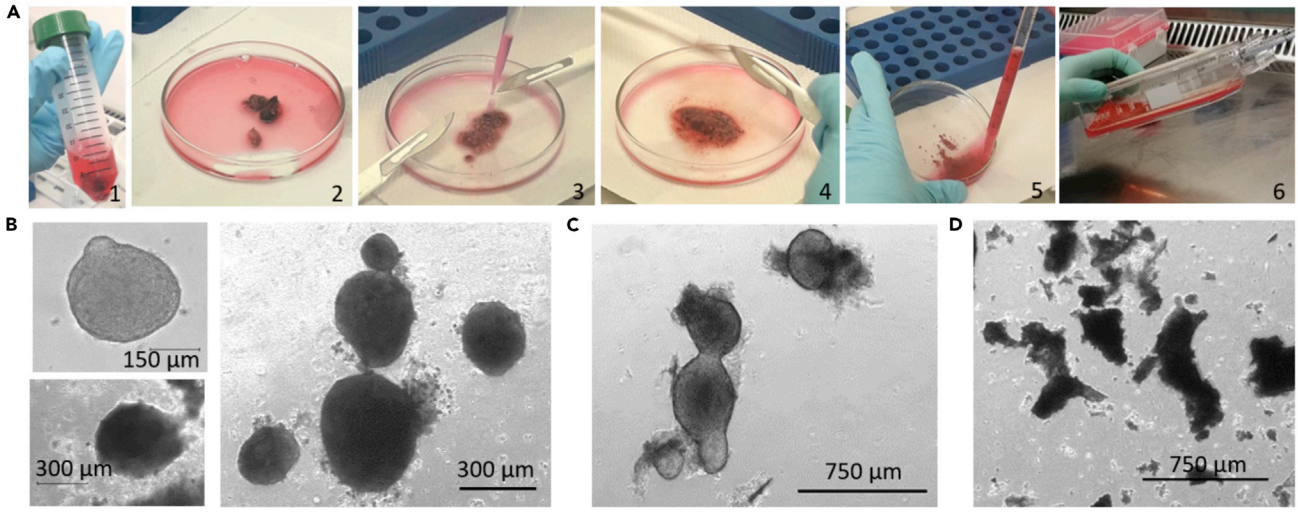

Figure 1. Derivation of organoids from tumor tissue

(A) Tumor tissue processing: (1) Tumor reception in DMEM, (2) Tissue transfer to a glass petri dish before cutting, (3–4) Mechanical mincing with scalpels, (5–6) Transfer of tissue fragments to culture flasks coated with agar.

(B) Examples of good quality organoids derived from brain tumor tissue.

(C) Examples of fused organoids after prolonged culture.

(D) Brain tumor tissue fragments that did not form organoids.

hospital until collection. Short time between surgery and collection is advised (Figure 1A). We regularly collect the tumor tissue within 30 min after end of the surgery.

2. Transfer tumor tissue to a glass petri dish under BSC II sterile conditions. Remove excess medium.

Note: We find most comfortable to cut tissue in glass petri dishes of 100 mm in diameter with 2 mm high glass borders. Cutting in plastic petri dishes leads to production of small plastic pieces floating in the culture. If 5-Aminolevulinic Acid (5-ALA) is used during surgery, we recommend working in the dark to limit a potential fluorescence-associated toxicity.

3. Add 0.5 - 1 mL of fresh organoid medium to ensure sufficient moisture of the tissue during processing.
4. If desired cut small tissue fragments and snap freeze for downstream molecular analysis.
5. Mince tumor tissue for approximately 10 - 20 min at 20°C–22°C with sterile scalpels until obtaining small tumor fragments invisible by eye (approximately 0.5 mm × 0.5 mm). Add more medium if needed. See troubleshooting 1 for problems during mincing.

Note: Visible bone fragments, fixed tissue or blood clots should be removed prior mincing. Certain tumor types can be difficult to cut and longer time may be required for larger samples.

6. Transfer tissue fragments into a 50 mL falcon tube. Rinse the petri dish with organoid medium to collect all fragments.
7. Allow tissue fragments to sediment at 20°C–22°C for approximately 5 min at the bottom of the falcon tube. Do not centrifuge!
8. Aspirate the medium and resuspend tissue fragments in fresh organoid medium. Transfer cultures to agar flasks. Volume needs to be adjusted to reach high culture density. On average 1–2 T75 flasks are sufficient (15–20 mL per flask). More than one flask may be needed for larger tumors. For smaller tumor tissue use one T25 flask (4–5 mL per flask).
9. Incubate the culture flasks in a standard cell culture incubator at 37°C with 5% CO_2 and 21% O_2. Keep the vent/close cap open or replace with a filter cap.

10. Discard remaining material according to internal rules for the BSL2 waste.
11. After 1 - 2 days, verify cultures under the microscope for contamination and tissue fragments size. If tissue fragments appear too large see troubleshooting 2.
12. A high amount of blood in the tissue may also cause a change in pH. Proceed with medium change after 1 day in culture if pH change is observed. We do not recommend performing red blood cell lysis as we have noticed that glioma cells are particularly sensitive to the classical erythrocyte lysis buffers based on ammonium chloride.

Organoid culture

Timing: 7–14 days

13. Organoid cultures should be monitored on a regular basis. Change medium after 5 - 7 days of culture. If cultures appear too dense (e.g., change in pH indicated by yellow Phenol red) or high number of debris is observed proceed with medium change earlier.
14. Gently collect the tissue fragments and organoids in a 50 mL falcon tube.
15. Sediment organoids for approximately 5 min. Do not centrifuge.
16. Aspirate medium and resuspend organoids in an appropriate volume of pre-warmed organoid medium. Transfer cultures to fresh agar flasks. Culture for 10–14 days before freezing.

Note: If well-structured organoids are already formed after one week of culture (Figure 1B), freeze cultures following protocol described below. If organoids start to fuse, proceed with freezing (Figure 1C). Fused organoids can be re-minced with scalpels prior cryopreservation.

⚠ CRITICAL: Quality and quantity of organoids rely on size and quality of tumor tissue obtained. This step allows preselecting tissue with high tumor content, organoid derivation is limited from necrotic areas and normal brain with low tumor content. See troubleshooting 3 for organoid cultures with high amount of debris.

Organoid biobanking

Timing: 1 h

Organoids should be used directly for implantation or frozen after maximum 10 - 14 days in culture. Organoids will start to fuse after prolonged culture.

Freezing protocol

17. Gently collect the organoids in a 50 mL falcon tube. In case of cultures containing high amount of debris and dead tissue pieces (Figure 1D), collect good quality organoids under a binocular microscope. If possible, place the binocular under the BSCII and collect manually the healthy organoids.
18. Sediment organoids at the bottom of the falcon tube for approximately 5 min. Do not centrifuge.
19. Aspirate medium and resuspend organoids in an appropriate volume of ice-cold freezing medium.
20. Transfer 1 mL of organoids into cryogenic vials. Aim to freeze 35–40 organoids per 1 mL freezing medium in one cryogenic vial.
21. Place cryogenic vials in a Mr. Frosty freezing container (or equivalent) and transfer to −80°C for 12 - 24 h.
22. After 12–24 h, transfer cryogenic vials to a liquid nitrogen tank for long-term storage.

Note: The organoids derived from patient material correspond to the generation 0 (G0). Organoids obtained at later stages upon serial transplantation of PDOXs must be denoted with appropriate generation number (generation of organoids = generation of PDOX they were derived from).

Defrosting protocol

23. To defrost organoids for implantation or functional assays, thaw cryogenic vials with organoids rapidly at 37°C.
24. Gently transfer the defrosted solution with organoids to 15 mL tube. Add slowly drops of pre-warmed organoid medium up to 3 mL (1 mL per minute).
25. Shake gently up and down and incubate 5 min.
26. Add organoid medium up to 10 mL.
27. Sediment organoids for approximately 5 min, aspirate the medium containing DMSO.
28. Resuspend organoids in fresh organoid medium, transfer to agar flask and culture at 37°C in the incubator for 24 h to 72 h before surgery/experiment. For IDH mutated models showing compromised survival of organoids during cryopreservation see Troubleshooting 4.

Note: Organoid culture can be obtained from brain tumors of different types and grades, including gliomas, meningiomas and brain metastases. We have successfully obtained high quality organoids from 79% of GBMs and 68% of grade II-III gliomas. The growth of organoids depends on patient tumor and is generally limited after 7 days in culture. Viable organoids with a diameter between 400 μm and 1000 μm are selected for intracerebral implantation in mice. Our cultures can be adapted to the serum-free media supplemented with the growth factors (Christensen et al., 2010). However, we have not compared systematically organoids cultured in the two different media and we have not used organoids cultured in serum-free conditions for the implantation. For more discussion on organoid media composition see (Klein et al., 2020).

Establishment of patient-derived orthotopic xenografts (PDOXs)

Timing: up to 16 months

This part describes implantation of tumor organoids and mouse monitoring during development of PDOX models.

Cerebral implantation of organoids

Timing: 3 h

Intracortical implantation of organoids in immunodeficient mice is performed to induce tumor growth *in vivo* and to establish PDOX models. Animals must be housed in individually ventilated cages in a Specific Pathogen Free (SPF) facility, under controlled environment (temperature 22 ± 2°C, humidity between 45% and 65% and 12 h light / 12 h dark cycle) with free access to autoclaved and acidified water and irradiated food *ad libitum*.

Preparation of glioma organoids for implantation

29. Collect viable organoids of 400 - 1000 μm under binocular microscope or equivalent.

⚠ CRITICAL: This step requires gentle handling to avoid mechanical breaking of organoids.

30. Gently transfer selected organoids to a 50 mL falcon tube using 2 mL to 10 mL pipette.

31. Sediment organoids at the bottom of the falcon tube for approximately 5 min.
32. Aspirate the medium and wash organoids in 10 mL of DMEM w/o supplements. Repeat 2 times.
33. Resuspend organoids in 15 mL of DMEM and incubate on ice until surgery.

Optional: Cryopreserved organoids can be used for implantation. Defrost organoids as described above and culture in organoid medium for 24 - 72 h before surgery.

Intracortical implantation

Note: We routinely use NSG mice (NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ, males and females, between 8 and 16 weeks) for PDOXs development due to their strong immunodeficiency and long survival. In initial experiments, we used NOD SCID mice (NOD.CB17-Prkdc^{scid}/NCrCrl, male and female) or eGFP^{+} NOD SCID (Niclou et al., 2008). However, the NOD SCID strain have high incidence of thymic lymphoma, which limits mice survival and experimental time frame. Nude mice can also be used for implantation of organoids obtained from well-established PDOX models for large-scale experiments. In general, we do not observe tumor growth differences between these strains.

34. Inject buprenorphine subcutaneously (volume of injection 10 mL/kg, dose 0.1 mg/kg) to NSG mice to ensure analgesia for 4 - 6 h time period.
35. Pre-warm the recovery chamber and the cover to 37°C.
36. Prepare the sterile field with all surgical instruments and reagents needed (Figure 2A).
37. Anesthetize the mouse with an intra-peritoneal injection (volume of injection 10 mL/kg) of a mixture of Ketamine (dose 100 mg/kg) and Xylazine (dose 10 mg/kg).

Note: If available, gaseous anesthesia by isoflurane decreases probability of mouse death due to adverse respiratory effect of Ketamine and Xylazine.

38. Shave and disinfect the skin using a sterile surgical pad and 70% ethanol.
39. Protect eyes of the mouse with a drop of Ocry-gel.
40. Place the head in a stereotaxic frame (Figure 2B).
41. Inject subcutaneously a local anesthetic (Bupivacaine (0.25% Marcain with Adrenalin), volume of injection 2 mL/kg; dose 5 mg/kg) on top of the skull.
42. Disinfect the Hamilton syringe with several flushing of Ethanol followed by 0.9% NaCl.
43. Fill the Hamilton syringe with organoids. Transfer organoids to the petri dish and manually aspirate 6 organoids in maximum volume of 2 μl. For problems during aspiration procedure, see Troubleshooting 5.
44. Fix the syringe in the syringe holder of the stereotactic frame.
45. Perform a short incision (~ 0.7 cm) and open the skin in order to expose the bregma, the sagittal and coronal sutures (Figure 2C).
46. Keep the needle at an angle of 90° to the skull and place it directly on the bregma to set the 0 position for the X and Y axis on the frame's ruler (Figure 2C).
47. Place the needle on the surface of the brain to set the 0 position for Z axis.
48. Using the stereotaxic ruler, move the needle to the X (2 mm right) and Y (1 mm front) coordinates to locate the drill site (Figure 2D). See Troubleshooting 6 for possible injection to the lateral ventricle.
49. Drill a small hole of 0.7 mm diameter.
50. Using the micro-scalpel, remove the remaining piece of bone and perform cross incision of the dura mater.
51. Place the needle on the surface of the brain to set the 0 position for Z axis on the frame's ruler and slowly lower the needle 0.5 mm deeper than the Z depth (2 mm).
52. Wait for 2 min, slightly retract (0.5 mm) the needle and inject slowly the organoids (1 min for 1 μl) (Figure 2E).

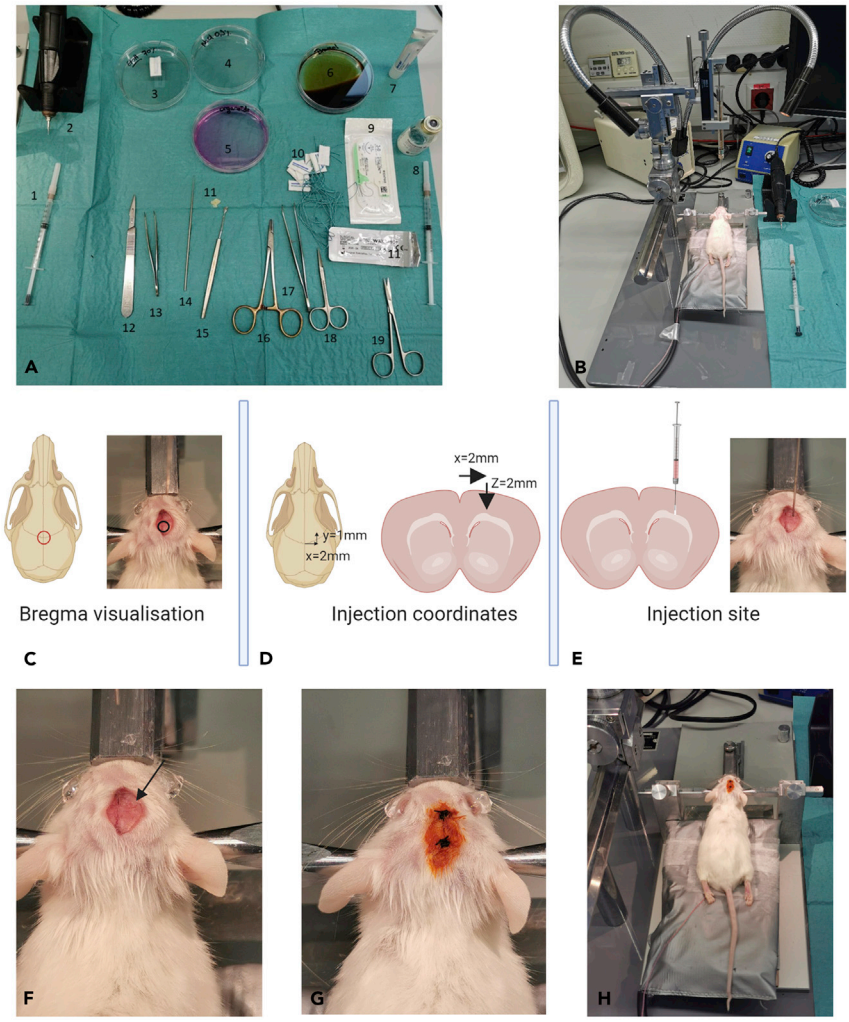

Figure 2. Intracranial operation procedure

(A) Sterile field with all materials. 1: Syringe with anesthetic mix, 2: Drill, 3: Ethanol 70%, 4: NaCl 0.9%, 5: Organoids on glass petri dish, 6: Braunol, 7: Ocry-gel, 8: Syringe of local anesthesia, 9: Ethilon polyamide 6 (3.0), 10: Surgical patties, 11: Bone wax, 12: Scalpel, 13: Graefe forceps, 14: Bone micro probe, 15: Bonn scissors, 16: Needle holder, 17: Semken forceps, 18: Extra Fine Bonn Scissors, 19: Fine scissors.

(B) Mouse placed on a warming cover, head fixed in the stereotaxic frame.

(C) Bregma visualization: Junction between sagittal suture and coronal suture (red circle), Bregma corresponds to the zero position.

(D) Drill site. Coordinates are applied from the Bregma position, 2 mm to the right side (X) and 1 mm to the front (Y).

(E) Insertion of the needle after meninges puncture with the bone micro probe. Insert first at 2.5 mm depth, then retracted to 2 mm depth.

(F) After injection, needle is removed and the hole is closed with bone wax (arrow).

(G and H) Skin is closed and disinfected, end of surgery. Illustration created with Biorender.com.

53. Keep the needle in place for 2 min and slowly withdraw the needle.
54. Wash the skull with 0.9% NaCl, verify if all organoids were implanted. For incomplete injection of organoids see Troubleshooting 7. Close the hole with the bone wax (Figure 2F) and close the skin with separated knots (Ethilon 3-0) (Figures 2G and 2H).
55. Place animal in a recovery chamber until it recovers from anesthesia.
56. Carefully monitor the scar for at least 5 - 7 days until loss of knots.

Animal supervision and verification of tumor growth in vivo

Timing: up to 16 months

Development of glioma tumors in immunodeficient mice is expected to take between several weeks to several months after implantation. The growth depends on the neuropathological features of tumors (proliferation index, level of angiogenesis and invasion) (Bougnaud et al., 2016; Golebiewska et al., 2020). Animals are supervised daily for common behavioral patterns and specific symptoms indicating tumor growth. Magnetic resonance imaging (MRI) is applied to detect and quantify tumor growth.

MRI

Timing: 15–30 min

57. Anesthetize the mouse with 2.5% isoflurane / oxygen mix in the induction chamber.
58. Transfer the mouse to a pre-warmed Minerve bed adapted for mouse head imaging. The bed allows for constant body temperature at 37°C, and breath monitoring using a motion sensor pillow placed under the abdomen. Anesthesia is maintained through a nose mask with 2.5% isoflurane / oxygen mix.
59. Place the Minerve bed in the mouse head coil and insert the imaging cell (half pipe that contains the animal bed and the coil) in the magnet tunnel so that the head of the animal reaches the isocenter of the magnet.
60. Check the breathing rate before the start of the acquisition (PC-SAM software). Breathing rate must be between 50 and 100 per min. The anesthesia mix is adjusted during the scan session to maintain breathing in this range.
61. Acquire a multislices brain scout (Preclinical software MR solutions). This short scan allows verifying that the head of the animal is correctly positioned in the isocenter using 3 different plans (sagittal, coronal and transverse) and serves to correctly position slices packages for the next imaging protocols.

Note: Multi-slices brain scout (Gradient echo - GRE) is defined by an echo time (TE)/repetition time (TR) = 8/190 ms, 30 × 30 mm field of view, 9 slices, 1 mm slice thickness, and 256 × 192 matrix (spatial resolution = 0.1171 × 0.1563 mm), number of averages = 1, receiver bandwidth (BW) = 40 kHz, duration: 36 seconds.

⚠ CRITICAL: The parameters of the sequences described in this section are adapted for the 3T MRI system with a 3T horizontal bore 3017 magnet from MR Solutions, equipped with head and body quadrature coils for mouse and rats imaging. Parameters need to be adapted to the magnet with a different field strength.

62. Perform tuning and matching of the coil and magnetic field shimming to ensure high quality of the scans.
63. Select the Axial Fast Spin Echo T2-weighted MRI sequence (FSE T2w Scan).
64. Properly position the slices packages of the scan. Anatomical landmarks can be used to reproducibly position slices and facilitate comparisons in time series. The top of the frame should be aligned with the top of the brain in the coronal plan. The frame is then rotated in the Axial plane to correct for animal head side tilting by aligning symmetrical landmarks visible in the scout images parallel to the slices. In the sagittal plane, the left side of the frame must be aligned with the olfactory bulb and the cerebellum. The first 3 slices are placed to cover the olfactory bulbs.
65. Start the acquisition of the FSE T2w scan.

Note: Axial FSE-T2w scan is defined by echo time (TE)/repetition time (TR) = 68/3000 ms, 25 × 25 mm field of view, 15 Slices, 1 mm slice thickness, and 256 × 248 matrix (spatial resolution = 0.0977 × 0.1008 mm), echo train length = 8, number of averages = 4, receiver bandwidth (BW) = 20 kHz, duration: 6 min 12 seconds.

66. If Tumor contrast enhancement is desired, T1w scan with contrast is applied. Copy the slice position of the pervious FSE T2w scan, select the Axial Fast Spin Echo T1-weighted MRI sequence (FSE T1w scan) and paste the slices position.
67. Start the acquisition.
68. After the first acquisition, take out the imaging cell from the MRI. Avoid displacing the bed or the coil to keep the animal in the exactly the same position.
69. Inject subcutaneously 100 µl of Dotarem (Gadoterate 0.5 mmol/mL), put back the imaging cell in the machine and wait for 5 min.
70. Start the acquisition of the FSE T1w scan.

Note: Axial FSE-T1w scan is defined by echo time (TE)/repetition time (TR) = 17/1400 ms, 25 × 25mm field of view, 15 Slices, 1 mm slice thickness, and 256 × 256 matrix (spatial resolution = 100 × 100 µm), echo train length = 4, number of averages = 4, receiver bandwidth (BW) = 20 kHz, duration: 5 min 42 seconds.

71. Remove the imaging cell from the machine after the acquisition, then remove the mouse from the bed, place directly in the cage and supervise until awoken.

Note: Frequency of the MRI scans should be adapted according to the PDOX model. MRI can be performed once a week for PDOX with fast tumor growth. For PDOXs with slow tumor growth MRI once every 2 months is sufficient. For new PDOX models, MRI scan is advised upon first neurological symptoms.

⚠ CRITICAL: Highly invasive tumors are not detectable by MRI, close and strict supervision of each mouse is mandatory to recognize first neurological symptoms and avoid pain.

Tumor quantification based on MRI images (Figure 3).

72. Open MRI-obtained DICOM files in the ImageJ software.
73. Adjust the brightness of the images.
74. Delineate the tumor to define the area of the tumor on each slice.
75. Tumor volume in mm^3 is defined by the sum of the tumor area on each slice (slice thickness is 1 mm).

Note: Similarly to patient tumors, the invasive part of PDOX tumors is undetectable by MRI, thus immunohistochemistry is essential to confirm the size and the histopathological phenotype of the tumor. See (Bougnaud *et al.*, 2016) for detailed analysis of histopathological phenotypes.

Mouse supervision and PDOX tumor tissue processing

76. Verify the mouse every day and fill in the score sheet. The score sheet (Table 1) should contain observation regarding common behavior of each mouse and specific symptoms linked to brain tumor. Scoring criteria:
    Score 0 = no symptoms, normal behavior
    Score 1 = mild symptoms, start & body weight loss $\leq$ 5%,
    Score 2 = established symptoms & body weight loss >5% and <15%,
    Score 3 = severe symptoms, body weight $\geq$ 15% and tumor size bigger than 100 mm^3.

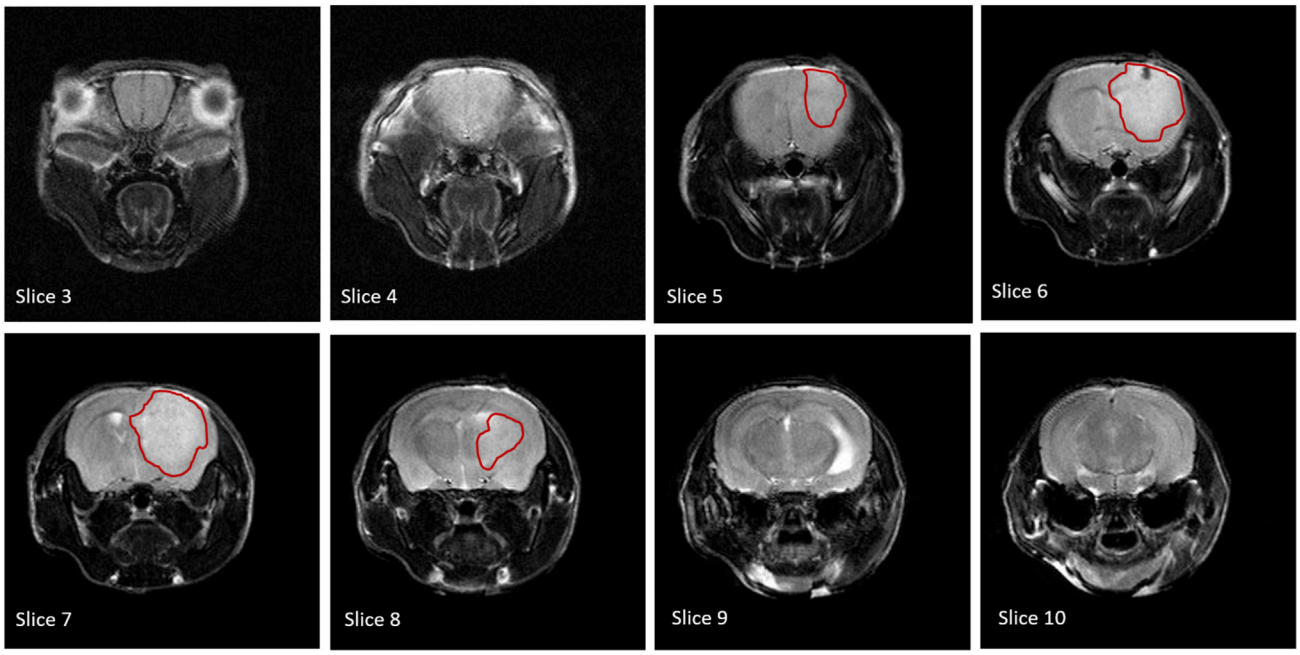

Figure 3. MRI Imaging of GBM tumor in PDOX brain

MRI slices of mouse head from the anterior part to the posterior part. Tumor visible as brighter area is delineated on each slice (red line). The area of each slice (mm^2) is summed to obtain the tumor volume in mm^3.

77. The mouse is euthanized when three symptoms reach a score of 2 or one symptom reaches a score of 3.
78. Euthanize the mouse by cervical dislocation.
79. Gently extract the brain.
80. Fresh mouse brain can be used to obtain organoids with the same protocol as described for patient tumor tissue. Remove the midbrain. Mechanically mince brain hemispheres with scalpels.

Note: If desired, two hemispheres can be processed separately to measure efficiency of organoid derivation from the tumor core (right hemisphere) and invasive zone (left hemisphere). For well-established PDOX models with known histopathological phenotype, select only part of the hemisphere(s) where tumor cells are expected.

81. Generated PDOX-derived organoids (G1) can be implanted directly for derivation of Generation 2 PDOXs or cryopreserved using the protocols described above.

Note: Serial transplantation involves subsequent intracranial implantations and derivation of organoids from PDOXs. We consider generation 3 (G3) PDOXs as stable models, which can be used for preclinical application. We have observed that genetically heterogeneous tumors may undergo clonal selection in the first two passages *in vivo* (Stieber et al., 2014). Although PDOXs at generation 1 can directly be used for experiments (see e.g., (Golebiewska et al., 2013)), this compromises significantly the number of organoids available for further implantation and maintenance of the PDOX models long term.

⚠ CRITICAL: 6 mice are commonly used per implantation series. This allows for detailed characterization of the PDOX model and biobanking of sufficient organoids for further use. Use of several mice per implantation series increases the success rate of the first generation, particularly in case of slowly growing tumors, as mice can be lost due to

Table 1. Score Sheet for establishment of PDOX models

	Common behavior					Symptoms link to brain tumor						Others		
Mouse number	Appearance of hair	Grooming	Nest building	Eating	Socialization	Abnormal movement or immobility	Inflammation at the injection site	CNS symptoms (seizure, vestibular disorder)	Kyphosis	Head shape	Weight loss	Swollen abdomen	Comment	Decision

0 = No symptoms , normal behavior, no body weight loss ; 1 (starting) = Starting symptoms and body weight loss $\leq$5% ; 2 (established) = Established symptoms, abnormal behavior and body weight loss between 5 and 15% ; 3 (strong)= Strong symptoms , body weight loss $\geq$15% and tumor size (if quantifiable $\geq$100% mm^3). Perform euthanasia if: 3 criteria = 2 or 1 criteria = 3

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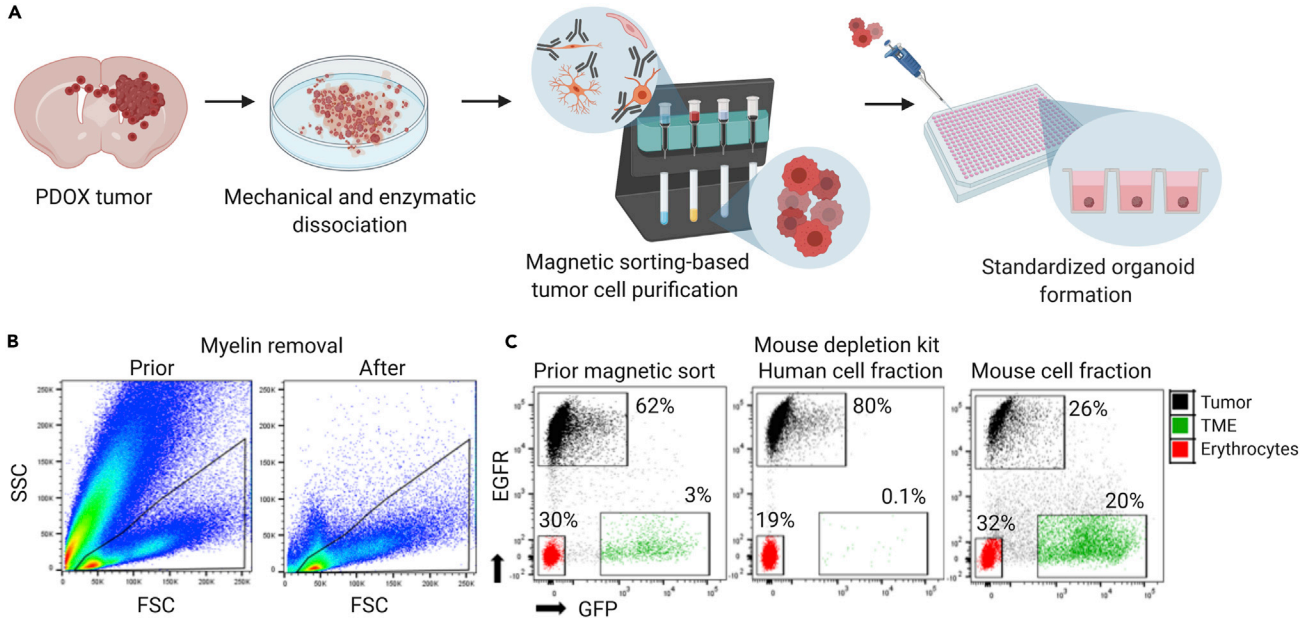

Figure 4. Tumor tissue processing for *ex vivo* functional assays

(A) Experimental workflow for purification of human tumor cells from PDOX-derived tumor tissue. Illustration created with Biorender.com.

(B) Flow cytometry-based analysis of PDOX-derived single cell suspension prior and after myelin removal. Magnetic sorting allows for efficient removal of myelin (recognized as FSC^{low}/SSC^{high} events), but not small cell debris ($FSS^{low}SSC^{low}$).

(C) Flow cytometry-based analysis of single cell suspension prior and after depletion of mouse TME cells. Analysis was performed on PDOX P8 implanted in $eGFP^{+}$ NOD/SCID mice (Golebiewska et al., 2013). Tumor cells are recognized as $EGFR^{+}GFP^{-}$ (black events). Mouse TME cells are $EGFR^{-}GFP^{+}$ (green events). Erythrocytes appear as $EGFR^{-}GFP^{-}$ (red events). Human cell fraction contains mouse erythrocytes. Mouse cell fraction is significantly contaminated by the tumor cells, due to non-specific capture at LS columns. Percentages of single viable cells are displayed for each fraction.

independent health problem and/or aging. In certain PDOX models, delay in tumor growth was observed if organoids were cultured for more than 15 days or due to sensitivity upon freezing-thawing process. Histological verification of tumor features is advised at each PDOX generation.

PDOX tissue processing and derivation of standardized organoids for *ex vivo* functional assays and drug testing

Timing: 3–5 h

Mechanical dissociation of PDOX tumor tissue and subsequent organoid culture limits the usage of the PDOX models for functional assays and preclinical drug testing requiring single cells and/or standardized organoids. In this part, we describe a protocol allowing for dissociation of brain tissue into single cells followed by purification of human tumor cells and xenograft-based murine cells constituting tumor microenvironment (TME) (Figure 4A). Isolated cells can be applied for varying functional assays and reconstitution of standardized organoids.

Enzymatic dissociation of PDOX-derived tumor tissue

Timing: 1 h

To dissociate glioma PDOX-derived tumors, enzymatic digestion based on papain and DNAse I is recommended. Here we describe the protocol based on the Neural Tissue Dissociation Kit (Miltenyi), however, other equivalent protocols are also available.

82. Preheat a water bath to 37°C.
83. Prepare enzyme mix 1 (EM1) by adding components specified in the Neural Tissue Dissociation Kit product leaflet and pre-warm to 37°C to ensure optimal enzymatic activity.
84. Remove olfactory bulbs (myelin-rich) and midbrain. Collect part of the brain hemisphere(s) containing tumor cells.
85. Moist tissue with ice-cold HBSS w/o Ca^{2+}/Mg^{2+}.
86. Cut tumor tissue with scalpels for several min and transfer tissue fragments into a 50 mL falcon tube, wash by adding ice-cold HBSS up to 20 mL.
87. Centrifuge at 300 *g* for 3 min at 4°C and discard the supernatant.
88. Resuspend tissue pellet in EM1 and incubate for 15 min at 37°C in water bath, mix tube gently every 5 min.
89. Prepare enzyme mix 2 (EM2) by adding components specified in the product leaflet, add EM2 to the tissue.
90. Dissociate tissue mechanically using a glass pipette with cotton plug. Pipet up and down 10 times, avoid air bubbles.
91. Incubate for 10 min at 37°C. Mix tube gently every 5 min during the incubation period.
92. Repeat mechanical dissociation with a glass pipette.

Optional: If tissue fragments are still visible, incubate another 10 min at 37°C.

93. Add 10 mL HBSS to the dissociated tissue and filter cells through a 50 μm sterile filter. Collect single cell suspension into a 15 mL falcon tube.
94. Centrifuge single cell suspension at 300 *g* for 3 min at 4°C.

Note: Single cells can be incubated on ice for a short period before next steps.

⚠ CRITICAL: For experiments requiring subsequent cell culture perform tissue dissociation under sterile conditions. For experiments requiring molecular analysis of isolated cells, except for tissue dissociation at 37°C, perform remaining steps at 4°C.

Optional: Myelin removal

Timing: 1 h

Amount of myelin depends on the tissue collected and histopathological features of each PDOX model. E.g., dissociation of angiogenic tumors leads to low amount of myelin fibers, invasive tumors may still show relatively high amount of myelin fibers after tissue dissociation. Removal of myelin prior to depletion of mouse cells is only needed if the experiment requires isolation of mouse TME cells. Myelin can be removed with magnetic sorting using MACS® Myelin Removal Beads II.

95. Resuspend tissue pellet in 1800 μl ice-cold MACS buffer per PDOX tumor. Add 200 μl of Myelin Removal Beads II.

Note: Indicated volumes are sufficient for up to 500 mg tissue.

96. Mix gently, do not vortex. Incubate for 15 min in the refrigerator (4°C, do not incubate on ice).
97. Wash cells by adding 13 mL ice-cold MACS buffer and centrifuge at 300 *g* for 10 min. Aspirate supernatant completely.
98. Add 3 mL of MACS buffer per 200 μl of Myelin Removal Beads II.
99. Use 3 LS columns per PDOX tumor. Place 50 μm sterile filter on top of each column (if not filtered before). Place LS columns in the magnetic field of a suitable MACS® magnetic cell separator.
100. Prepare LS columns by rinsing with 3 × 1 mL ice-cold MACS buffer per column. Collect buffer in 15 mL falcon tubes. . Discard tubes and collected buffer.

101. Place new 15 mL collection flacon tubes under the columns on ice.
102. Transfer 1 mL of single cell suspension through each filter and LS column. Collect flow-through fraction containing single cells.
103. Wash columns with 3 × 1 mL of ice-cold MACS buffer, collect to the same falcon tubes.
104. Pool collected cells from 3 falcon tubes. Adjust volume to 20 mL with ice-cold MACS buffer. Count the cells.

Note: Myelin fibers will be retained in the LS columns. The flow-through fraction should contain pure single cell suspension. Small debris will still be visible (Figure 4B).

Purification of human tumor cells

Timing: 1 h

Human tumor cells can be purified by depleting mouse cells constituting TME with magnetic sorting using Miltenyi's Mouse Cell Depletion kit.

105. Continue with single cell suspension obtained directly after tissue dissociation (collection of tumor cells only) or after myelin removal (for experiments requiring collection of mouse cells).
106. Determine total cell number or tumor cell number with a hemocytometer. Centrifuge at 300 *g* for 5 min at 4°C.
107. Aspirate buffer and resuspend cell pellet: 80 µl ice-cold MACS buffer per 2 × 10^6 tumor cells or 10^7 total cells including red blood cells.

Note: Visual discrimination between human tumor and mouse TME cells during counting is not always possible. Tumor cells often present similar size as certain mouse cells within TME.

108. Add 20 µl cell depletion cocktail per cell suspension in 80 µl ice-cold MACS buffer. Incubate for 15 min at 4°C in the fridge (do not incubate on ice).

Note: Cell number obtained from one mouse brain vary across PDOX models. 3 - 5 × 10^6 tumor cells per brain can be expected from small or highly invasive tumors, whereas larger and highly proliferative tumors contain > 30 × 10^6 tumor cells. Volume of the cell depletion cocktail and the number of LS columns needs to be adjusted accordingly.

109. Use one LS column per cell suspension in 80 µl ice-cold MACS buffer. If filtering was not performed before, place a 50 µm filter on top of each column. Place LS columns in the magnetic field of a suitable MACS® magnetic cell separator.
110. Prepare LS column(s) by rinsing with 3 × 1 mL MACS buffer per column. Collect buffer in 15 mL falcon tubes. Place new 15 mL collection flacon tubes under the columns on ice.
111. Adjust volume to 500 µl using ice-cold MACS buffer for up to 2 × 10^6 tumor cells or up to 10^7 total cells. Apply cell suspension to the LS column(s). Collect flow through fraction containing human tumor cells.
112. Wash LS column(s) with 5 × 1 mL of ice-cold MACS buffer and collect flow through fraction. This fraction represents human tumor cells.
113. If desired, collect mouse cells from the LS columns by washing the column with 3 mL ice-cold MACS buffer. Remove the column from the magnetic cell separator and push buffer with the plunger.

Note: Purification is not 100% efficient. Human fraction is contaminated with mouse erythrocytes, which lost mouse-specific membrane epitopes during tissue dissociation. Mouse cell fraction still contains human cells captured physically in the column (Figure 4C). If higher purification is required for functional assays, replace magnetic sorting with fluorescent-activated cell sorting (Bougnaud *et al.*, 2016; Golebiewska *et al.*, 2013).

⚠ CRITICAL: If the mouse is sacrificed too early due to other health problems, the number of tumor cells obtained from the brain of not fully developed PDOX can be limited.

114. If applicable, pool human tumor and mouse cell fractions from separate LS columns, respectively. Count cells with hemocytometer.
115. Centrifuge cell suspension at 300 *g* for 3 min at 4°C. Resuspend cell pellet in the buffer or medium adequate for the downstream application.
116. Single tumor and mouse cells can also be cryopreserved. Follow the protocol described for organoids.

⚠ CRITICAL: To ensure specific antibody binding, this procedure should be performed at 4°C. It is key to apply ice-cold MACS buffer to LS columns and collect cells on ice.

Formation of standardized organoids

Timing: 2 days

Standardized tumor organoids can be reformed from isolated single tumor cells. This allows also for co-cultures of tumor cells with subpopulations of TME cells in a standardized ratio (Bougnaud et al., 2016).

117. To reform standardized organoids use either freshly isolated or cryopreserved single tumor cells from above described workflow.
118. After defrosting, incubate tumor cells in 50 mL falcon tube in organoid medium for 20–30 min. Count cells to determine number of viable cells.

Note: Single tumor cells show variable viability after cryopreservation depending on the PDOX model. Dead cell staining should be used for cell counting (e.g., Trypan Blue). Adapted freezing media may be need to increase viability of single cells after cryopreservation.

119. Seed 1000 cells per well in 25 µl organoid medium in a 384-well u-shaped, low adherence plate. If using 96-well plates, adapt volume to 50 µl.

Note: Cell number can be adapted to the experimental needs. 1000 cells will allow for creation of small organoids of size between 100 - 300 µl (depending on the size of the tumor cells), applicable for preclinical drug testing protocols. Larger organoids, of size equivalent to organoids obtained by mechanical dissociation, can be grown from 10 - 50 * 10^4 cells.

120. Fill unused wells with 90 µl HBSS (or equivalent) according to the plate layout.

⚠ CRITICAL: Avoid seeding of cells in outer wells and edge well regions due to potential edge effect phenomenon on organoids during longer experimental protocols.

121. Centrifuge 384-well plates to pellet cells softly at maximum 300 *g* for 3–5 min at 4°C. If cells do not assemble at the bottom of the plate, repeat centrifugation.
122. Place plates on a 3D shaker in a standard incubator and shake at high speed (approximately 30 – 40 rpm) for 48 h to allow for organoid formation.
123. Proceed to the experimental protocol. Add 25 µl of organoid medium containing desired compound(s) to reach total volume of 50 µl.

EXPECTED OUTCOMES

This protocol allows for efficient establishment of clinically relevant glioma models, which can be further applied for functional studies and preclinical drug testing. Derivation of organoids from glioma patient tumors has high success rate (Golebiewska et al., 2020). Contrary to cell lines, organoids can be derived also from less aggressive lower grade gliomas (Fack et al., 2017). The growth of organoids is limited *in vitro*. PDOX models allow for further propagation of organoids *in vivo* and multiplication of the patient material. We have shown that our organoids and PDOX models derived thereof recapitulate very well the histological, genetic, epigenetic and transcriptomic features of patient tumors (Golebiewska et al., 2020). Similarly to patient tumors, glioma cells create transcriptomic gradients of cells in different cell cycle phases, hypoxia and phenotypic states (Golebiewska et al., 2020). GBM organoids reaching a diameter of 300–1000 μm recapitulate hypoxic and phenotypic heterogeneity, and retain blood vessels and other TME cells to a certain extent (Bougnaud et al., 2016; Christensen et al., 2010). Despite replacement of human TME by the mouse counterparts, PDOX models recapitulate well histopathological features of patient GBMs such as invasion and angiogenesis in the mouse brain (Bougnaud et al., 2016). Organoids and PDOXs can be applied directly for a variety of functional assays including preclinical drug testing (Abdul Rahim et al., 2017; Golebiewska et al., 2020; Johansson et al., 2013; Keunen et al., 2011; Sanzey et al., 2015), biomarker discovery (Demeure et al., 2016), assessment of tumor heterogeneity (Bougnaud et al., 2016; Dirkse et al., 2019; Golebiewska et al., 2013; Stieber et al., 2014), or metabolism (Fack et al., 2015; Fack et al., 2017). Although immune component is compromised *in vivo* and *ex vivo*, PDOXs and organoids can be further developed towards immunocompetent conditions (Klein et al., 2020).

LIMITATIONS

Quality and quantity of patient tumor tissue represent the main limitation of the protocol. Close collaboration with the neurosurgeons and/or pathologists responsible for tumor removal and storage are key for high quality tissue. Poor organoid quality is the main reason for failure of *in vivo* growth of glioblastoma tumors. IDH1 mutated gliomas suffer from low yields of organoids, which are fragile during cryopreservation. Implantation of fresh organoids or tissue fragments increases tumor take.

TROUBLESHOOTING

Problem 1

Certain tumors are difficult to cut with scalpels. If too much medium is added to the tissue, mincing becomes difficult as tissue pieces float in the liquid.

Potential solution

Sediment tissue in the falcon tube for approximately 5 min. Remove excess medium. Transfer back to glass petri dish and proceed with mincing. Do not use plastic pipette, as larger fragments will be blocked inside. Use scalpels or sterile spoons to move tissue pellet. For difficult to cut tissue, additional mincing may be needed after 1–2 days in culture.

Problem 2

Tissue fragments are too large after 1 - 2 days in culture.

Potential solution

Sediment tissue in the falcon tube for approximately 5 min. Remove excess medium. Transfer back to glass petri dish and proceed with mincing. Do not use plastic pipette, as larger fragments will be blocked inside. Use scalpels or sterile spoons to move tissue pellet.

Problem 3

Limited amount of organoids and high amount of dead tissue fragments and debris.

Potential solution

The common reason for limited organoid growth is the original tissue quality. Derivation of organoids from necrotic tissue and brain regions with limited number of viable tumor cells is compromised. Discuss optimal tissue collection with the neurosurgeons. For cultures with high amount of debris, proceed with manual collection of good quality organoids with use of a binocular microscope.

Problem 4

Compromised survival of IDH1 mutated glioma organoids during cryopreservation.

Potential solution

Organoids obtained from IDH1 mutated gliomas are in general more fragile and very sensitive to cryopreservation. Implantation of cryopreserved organoids often lead to delayed growth *in vivo* and high variability in mice survival. Serial transplantation of fresh organoids allows for more reliable PDOX derivation. If organoid integrity is compromised, small fresh tissue fragments can be used for intracranial implantation directly after mechanical cutting. For implantation, aspirate 2 μl of mix of tissue fragments. Tissue fragments can also be cryopreserved following the protocol described for organoids.

Problem 5

Difficult to aspirate larger organoids in the Hamilton syringe.

Potential solution

Cut larger organoids mechanically shortly before surgery. Optionally, aspirate larger organoids separately and pipette back to the medium. Organoids will adapt the shape and second aspiration in smaller medium volume (2 μl) will be easier.

Problem 6

If the meninges are not properly punctured, the flat needle cannot enter straight in the soft brain tissue, potentially leading to the injection to the lateral ventricle. This could impact tumor growth and lead to more invasive tumor growth. Ventricular injection can be suspected if visible liquid (cerebrospinal fluid) is detected after needle removal at the injection site or by MRI.

Potential solution

Puncture dura mater and other meninges in straight direction under the injection hole. In case of quantitative *in vivo* study, mice injected in the lateral ventricle need to be excluded from the study.

Problem 7

Incomplete implantation of 6 organoids. Unimplanted organoid(s) is/are visible on the syringe plunger after implantation.

Potential solution

Implantation of < 6 organoids per brain should not impact directly tumor growth and derivation of the PDOX model. In case of quantitative study, animal should be excluded or tumor growth should be validated by MRI.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Anna Golebiewska (anna.golebiewska@lih.lu).

Material availability

To facilitate the access to established glioma PDOX models, detailed information is available via PDXFinder (https://www.pdxfinder.org) and the EurOPDX Consortium (https://www.europdx.eu). Well-established models are available from the corresponding author.

Data and code availability

No exclusive data or code was used or generated for this method.

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AUTHOR CONTRIBUTIONS

V. Barthelemy, E.K., M.D., M.W., C.F., C.D., A.B., R.B., S.P.N., and A.G. optimized patient tumor processing and organoid biobanking. A.O., V. Baus, and S.P.N. optimized *in vivo* experiments. E.K., A.-C.H, A.B., A.M., Y.A.Y., and A.G. optimized tissue processing and organoid derivation for functional assays. A.O., G.K., and O.K. optimized MRI protocol. R.B. and S.P.N. provided initial protocols. A.M., O.K., R.B., S.P.N., and A.G. supervised the work and obtained funding. A.O. and A.G. prepared the first manuscript draft. All authors reviewed and edited the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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