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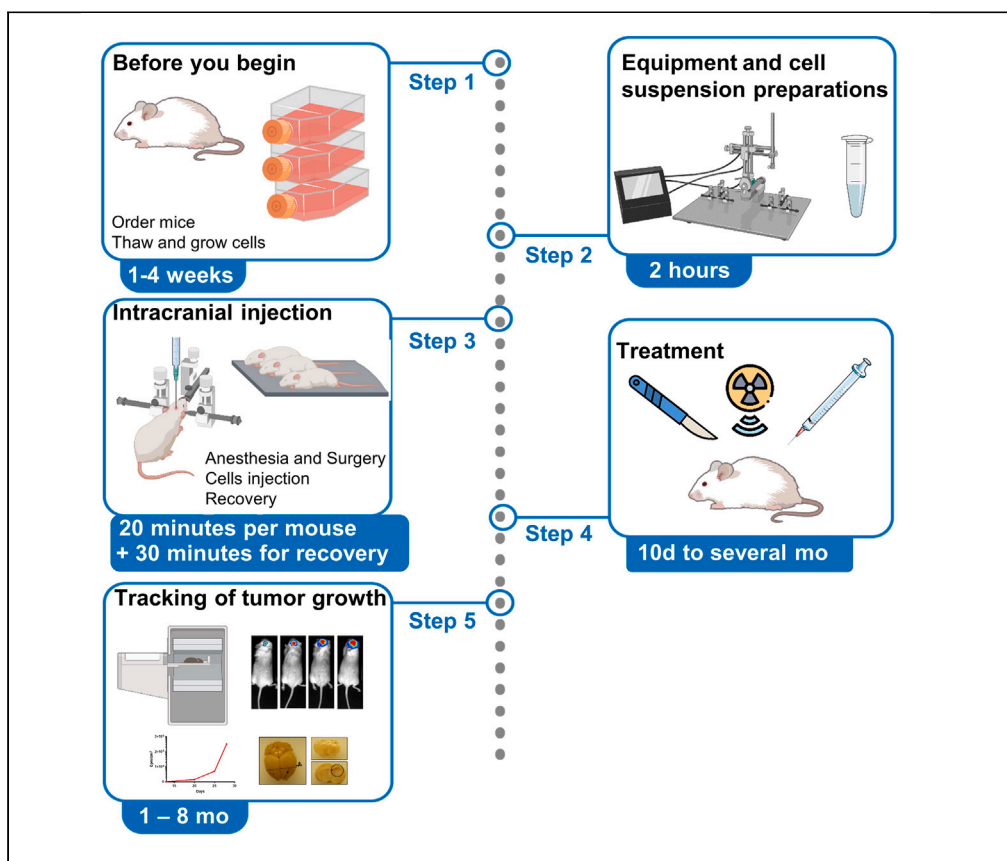
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Protocol

Protocol to generate two distinct standard-of-care murine glioblastoma models for evaluating novel combination therapies



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Highlights

Detailed steps for engineering GB cells to express luciferase for bioluminescence imaging

Generation of xenograft and syngeneic GB models via direct intracerebral injection

Comprehensive instructions for tumor resection, including video guidance for precision

Techniques for evaluating new combination therapies in preclinical glioblastoma studies

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In cancer research, murine models play a crucial role as highly valuable preclinical tools. Here, we present a protocol to generate a murine model of glioblastoma through the direct intracranial injection of tumor cells. We describe steps for cell culture, intracranial implantation, and standard-of-care treatments. We then detail procedures for monitoring tumor growth using bioluminescent imaging.

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

Protocol

Protocol to generate two distinct standard-of-care murine glioblastoma models for evaluating novel combination therapies

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SUMMARY

In cancer research, murine models play a crucial role as highly valuable preclinical tools. Here, we present a protocol to generate a murine model of glioblastoma through the direct intracranial injection of tumor cells. We describe steps for cell culture, intracranial implantation, and standard-of-care treatments. We then detail procedures for monitoring tumor growth using bioluminescent imaging.

For complete details on the use and execution of this protocol, please refer to Pelizzari-Raymundo et al.¹

BEFORE YOU BEGIN

The protocol described here lists the specific steps to obtain two murine models of established glioblastoma (GB) cell lines from both human and mouse using immuno-compromised and immuno-competent mice respectively, which can be used to probe the anti-tumor and anti-relapse potential of chemical compounds/drugs. We use the human GB line U87 isolated from a male glioma patient (U87; HTB-14; ATCC) that displays an epithelial morphology; and a mouse GB line GL261 derived from original tumor induced by intracranial injection of 3-methylcholanthrene into C57BL/6 mice.² Of note, we have successfully used this protocol with other GB cell lines including human primary GB cultures enriched in GB stem-like cells³ and the murine GB line CT-2A.⁴

Note: The U87 and GL261 GB cells used in this protocol are engineered to express firefly luciferase as described in materials and methods, which allows non-invasive tracking of tumor growth *in vivo*.

Institutional permissions

This protocol requires the use of animals. All animal experiments should be performed in accordance with relevant governmental and institutional guidelines. All experiments described here met the European Community Directive guidelines (Agreement C35-238-40 Biosit Rennes, France/ No DIR 13480) and were approved by the local ethics committee (University of Rennes). This ensured that the breeding and the daily monitoring of the animals were conducted under optimal welfare



conditions according to the European laws on the protection of animals used for scientific purposes ([Directive 2010/63/EU](#) and [Regulation \(EU\)2019/1010](#)) and the 3R principle (Reduce-Refine-Replace). The concept of the Three Rs was launched in the early 1960s by two English biologists, Russel and Burch, in their book *The Principle of Humane Experimental Technique*.⁵ The user should check the applicable laws in their country of origin.

Generation of established reporter-labeled (luciferase) GB cells

⌚ Timing: 2–3 weeks

This section outlines the generation of glioblastoma cell lines labeled with luciferase reporters.

The U87 and GL261 GB cell lines were cultured in warm complete medium (detailed recipe in the [materials and equipment](#) section) and were respectively transduced and transfected with a firefly luciferase open reading frame using viral particles ([Figures 1A](#) and [1C](#), RediFect Red-Fluc-puromycin lentiviral particles, PerkinElmer, Waltham, MA) or the pGL4-Luc plasmid ([Figures 1B](#) and [1D](#); Addgene plasmid # 64034; <http://n2t.net/addgene:64034>; RRID: Addgene_64034).

1. Thaw U87 and/or GL261 cells in 10 mL of warm complete medium at 37°C and 5% CO₂ in a humidified incubator.
2. Centrifuge the cells suspension at 300 g for 5 min.
3. Discard the supernatant.
4. Wash the cells with PBS (Phosphate Buffered Saline, 1X, sterile Thermo Fisher Scientific).
5. Repeat **steps 2** and **3**.
6. Resuspend the pellet of cells in 10 mL of complete medium and transfer to a Cell Culture Flask 75 cm² (STEMCELL Technologies).
7. Grow U87 and/or GL261 cells until the cells reaches 80% confluency.
8. Split the cells.
 - a. Wash the monolayer of cells once with 5 mL of PBS 1X.
 - b. Discard the supernatant and add 1 mL of Trypsin-EDTA (Solution de trypsin 0,25%-EDTA 0,02%, Sigma-Aldrich).
 - c. Incubate for 5 min at 37°C.
 - d. Tap the flask until the cells detach.
 - e. Add 10 mL of complete medium and gently mix thoroughly to disrupt any cell lumps.
 - f. Centrifuge the cells suspension at 300 g for 5 min.
 - g. Gently re-suspend cell pellet in warm medium.
 - h. Perform 1:10 cell split as needed. Discard the rest of the cells.
 - i. Incubate the cells at 37°C with 5% CO₂ in a humidified incubator until they reach 80% confluency or as needed.

Note: Cells should be checked daily to identify their grow rate or possible contaminations. In our lab and with the dilution protocol performed above, U87 and GL261 usually should be split every 3 days.

9. For **lentiviral protocol**, proceed with **step 10**. For cells expressing the pGL4-Luc plasmid, proceed with **step 18**.
10. On day 0, count the cells using a hemocytometer or Neubauer counting chamber and plate 5 × 10⁴ cells/mL in complete medium into 1 well of a 24 well-plate.
11. Incubate cells for 16 h.
12. On day 1, thaw a vial with lentiviral particles, gently spin down the solution and place it on ice.
13. Replace the medium with 500 µL of fresh complete medium containing hexadimethrine bromide (polybrene) at a final concentration of 4 µg/mL.

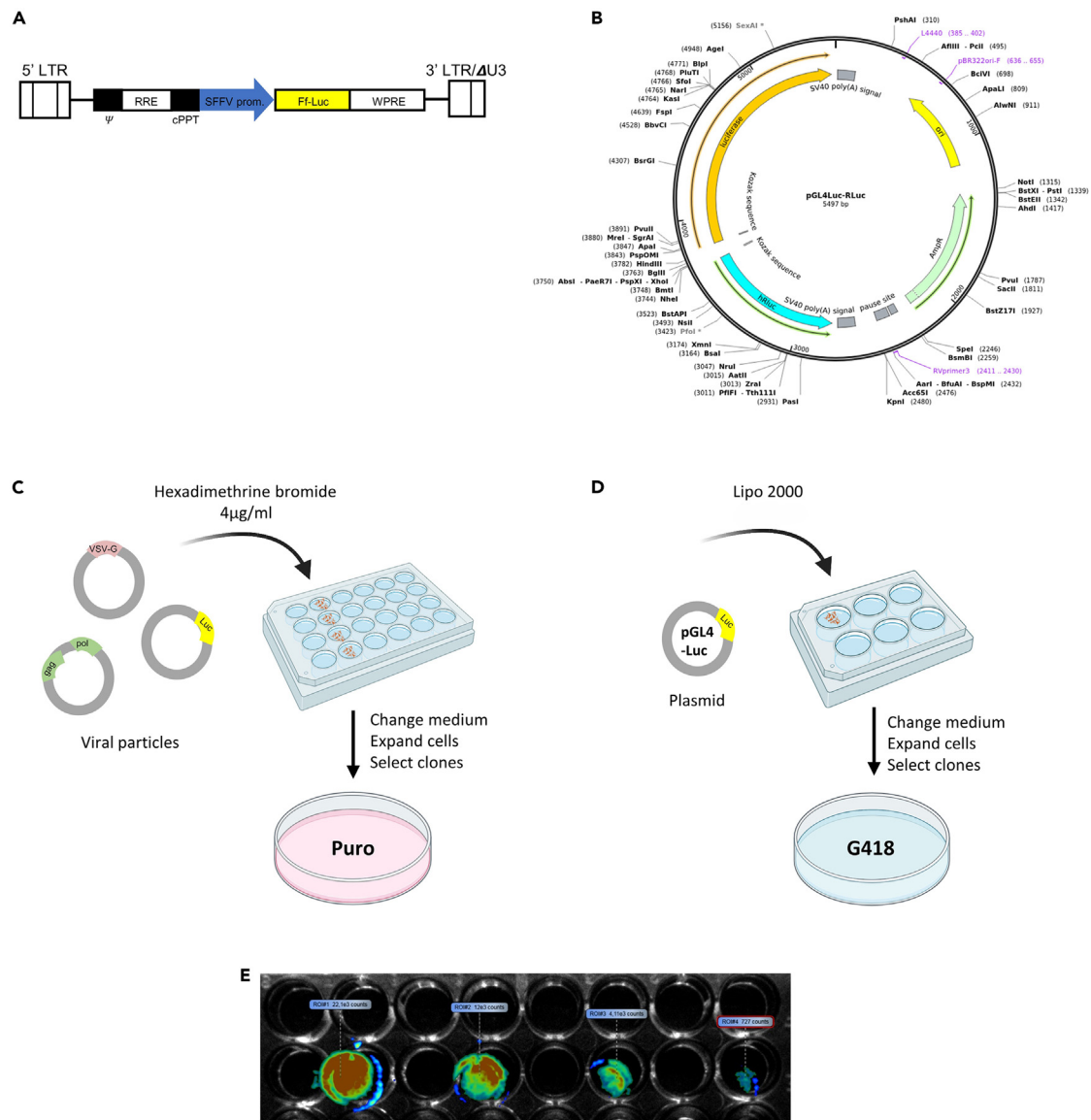


Figure 1. Generation of established reporter luciferase-labeled cells and confirmation of luciferase expression

(A) Diagram of the lentiviral vector key regions including the Ff-Luc transgene. Transgene expression is driven by the SFV promoter (spleen focus-forming virus promoter). U5 (LTR unique 5' region), R (LTR repeat region), Rev responsive element (RRE), central polyurine tract (cPPT), Luc (firefly luciferase), WPRE (wood-chuck hepatitis virus posttranscriptional regulatory element), 3' LTR/ΔU3 (self-inactivating LTR).

(B) Diagram of plasmid vector key regions including the Luciferase gene.

(C) Lentiviral particles are incubated with the cells in fresh complete medium containing hexadimethrine bromide (polybrene) infection agent at a final concentration of 4 μg/mL for 24 h. After this period, the virus containing medium is replaced and the cells are incubated for more 24 h before expansion and selection of the clones. Several clones are tested by luciferase activity using an In Vivo Imaging System (IVIS).

(D) The plasmid pGL4-Luc is mixed with Lipofectamine 2000 transfection reagent prior the incubation with the cells for 6 h. After this period, the plasmid containing medium is replaced and the cells are incubated for more 24 h before expansion and selection of the clones. Several clones are tested by luciferase activity using IVIS.

(E) Imaging of GL261 glioblastoma selected clones. Cells are seeded at an initial concentration of 2×10^5 cells/mL in 96-well plates with a total volume of 100 μL per well. Serial 1:2 dilutions are performed starting from the first well. The cells are then tested for luciferase activity using IVIS following the addition of D-luciferin.

14. Incubate cells with the viral particles for 24 h.
15. On day 2, split the cells if confluency exceeds 80% of the total well area.
16. Add fresh complete culture medium with 2.5 μg/mL of puromycin to select the transduced cells.

Note: A kill curve experiment is recommended when the appropriate amount of puromycin is unknown for the cell line.

Note: For a more detailed transfection protocol, please see the “[RediFect Lentiviral Particles Transfection Protocol](#)” available on the supplier website (PerkinElmer, Waltham, MA) or by contacting their Global Technical Support team (global.techsupport@perkinelmer.com). In case the user decides for another supplier, please see the specific detailed protocols for the preferred supplier.

17. Expand puromycin resistant cells to assay for luciferase expression.

Note: Puromycin is stable in culture medium. Maintain the cells in a medium containing the antibiotic for 3–5 days. After this period, replace the medium with fresh puromycin and continue the selection for 2 weeks or until 3 cell passages have been completed.

18. Transfect GL261 cells with 10 µg/mL of the plasmid using Lipofectamine 2000 according to the [manufacturer’s instructions](#). Briefly,
- On day 0, count the cells using a hemocytometer or Burker camera and seed 2×10^6 cells in complete medium into 1 well of a 6 well-plate. Incubate cells for 16 h.
 - On day 1, dilute 15 µL of Lipofectamine 2000 into 150 µL of Opti-MEM medium. In another tube, dilute DNA into 150 µL of Opti-MEM medium for a final concentration of 10 µg/mL.
 - Add diluted DNA to diluted Lipofectamine 2000 reagent (ratio 1:1). Incubate at room temperature for 5 min.
 - Add DNA-lipid complex to cells dropwise.
 - Incubate cells for 24 h.
 - Add 100 µg/mL of G418 for selection and incubate 1–3 days at 37°C. Once cells become 70%–90% confluent, proceed to **step 19**.

Note: G418 is a stable reagent. Cells can be kept with the medium containing antibiotic for 3–5 days. After this period, replace the medium containing G418 and keep the selection for 2 weeks or 3 cells passages.

19. Expand resistant cells to assay for expression of luciferase, add 100 µg/mL of G418 for selection.

Note: A polyclonal population of luciferase-positive GL261 cells named GL261-Luc and a clonal population of U87 named U87-Luc were used in this protocol.

D-luciferin potassium salt preparation

⌚ Timing: 1 h

This part details the preparation of D-luciferin solution. The full procedure should be performed in a chemistry hood in the dark to avoid the compound degradation.

Working in chemistry hood without light, prepare the luciferin solution.

20. Dissolve 1 g of D-luciferin in 63 mL of sterile water for a final concentration of 50 mM of stock solution.
- Once solved, filter the solution using a 0.22 µm filter.
 - Aliquot the solution in 5 mL microtubes.
 - Store the aliquots at –20°C covered from light, do not use longer than the expiry date of the powder indicated by the supplier. The solution can be kept for up to 6 months at –20°C.

Monitoring of the luciferase activity of established luciferase GB cells

⌚ Timing: 1 h

This section describes the procedure to test the luciferase activity in the stable luciferase-expressing U87-Luc and GL261-Luc glioblastoma cells by measuring the emitted luminescence.

The stable luciferase-expressing U87-Luc and GL261-Luc GB cells were tested for the luciferase activity by detecting the emitted luminescence using Photon imager Biospace lab (Nesles-la-vallée, France).

21. Grow U87 and/or GL261 cells.
22. Count the cell and plate 2×10^4 cells per well in 96-well plates in 100 μ L of complete medium.

Note: Serial dilutions in the ratio 1:2 from the initial concentration (step 22) can be performed in order to have a better quantification of the bioluminescence signals (Figure 1E).

23. Grow U87 and/or GL261 cells for 24 h.
24. Add 25 μ L of D-luciferin solution per well.
25. Analyze the luciferase activity using a luminescence analyzer (Figure 1E).

Note: D-luciferin is rapidly metabolized by cultured cells. Therefore, there is no need to incubate the cells after adding D-luciferin before analyzing luminescence

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
RediFect Red-Fluc-Puromycin lentiviral particles	PerkinElmer, Waltham, MA	Cat#CLS960002
pGL4-Luc plasmid	Addgene ⁶	Cat# 64034; RRID: Addgene_64034
Chemicals, peptides, and recombinant proteins		
DMEM, high glucose (4.5 g/mL)	Invitrogen	Cat#11965092
Fetal bovine serum active	PAN-Biotech	Cat#P04-96650
L-glutamine	Gibco	Cat#25030-081
PBS (phosphate-buffered saline), 1X, sterile	Thermo Fisher Scientific	Cat#J61196.AP
Solution de trypsin 0.25%-EDTA 0.02%	Sigma-Aldrich	Cat#T4049
Polybrene infection/transfection reagent	Thermo Fisher Scientific	Cat#TR-1003
Puromycin dihydrochloride from <i>Streptomyces alboniger</i>	Sigma-Aldrich	Cat#P7255
Opti-MEM reduced serum medium	Gibco	Cat#31985062
Lipofectamine 2000 transfection reagent	Thermo Fisher Scientific	Cat#11668027
Geneticin (G418 sulfate)	Thermo Fisher Scientific	Cat#11811023
D-luciferin (potassium salt)	Cayman Chemical	Cat#14681
Xylazine	Elanco	Rompun 2%
Ketamine	Boehringer Ingelheim	Imlagen 1000
Buprenorphine	Ecuphar	Buprecare
Medical surface detergent	Anios	Surfa Safe Premium
0.5% alcoholic chlorhexidine	GILBERT	Cat#6997C
Ocular solution (Ocry-Gel)	Domes Pharma	N/A
Trizma base	Sigma-Aldrich	Cat#T1503
NaCl (sodium chloride)	Sigma-Aldrich	Cat#S9888
HCl (hydrochloric acid) fuming 37%	Sigma-Aldrich	Cat#1.00317
Isoflurane (2%)	Dechra	NDC: 66794-020
Sodium fluorescein	Faure	ID: ChEMBL177756
Fibrin-collagen gel	Principal investigator: Eric Chevet ⁷	N/A

(Continued on next page)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Thrombin from bovine plasma	Sigma-Aldrich	Cat#T7513
Temozolomide	Thermo Fisher Scientific	Cat#466760010
MKC8866	Ambeed	Cat. No.: A1003533
Z4P	Enamine	Z409477496
Experimental models: Cell lines		
U-87MG	ATCC	RRID: CVCL_0022
GL261	Principal investigator: Maria G Castro ²	N/A
Experimental models: Organisms/strains		
Mouse NMRI-nude male	Janvier Laboratories	NMRI-Foxn1nu/Foxn1nu 8-week-old male mice
Mouse NSG	Charles River	NOD.Cg-Prkdc ^{scid} Il2rg ^{tm1Wjl} /SzJ 8-week-old male mice
Mouse C57B/6	Janvier Laboratories	C57BL/6JRj 8-week-old male mice
Software and algorithms		
Software bioluminescence visualization	Biospace Lab	PhotonIMAGER Optima
Software bioluminescence quantification	Biospace Lab	M3 Vision
GraphPad Prism	https://www.graphpad.com/	RRID: SCR_002798
Other		
Falcon 50 mL high clarity PP centrifuge tube, conical bottom, sterile, 25/rack, 500/case	Corning Life Sciences	Cat#352098
Microtubes size 1.5 mL, clear	Corning Life Sciences	Cat#AXYMCT150CS
CO ₂ incubator	Thermo Scientific	Forma Series II HEPA Class 100
Cell culture flask 75 cm ²	STEMCELL Technologies	Cat#200-0501
Cell culture flask 175 cm ²	STEMCELL Technologies	Cat#38073
Centrifuges	Thermo Scientific	Sorvall X Pro Series
Neubauer counting chamber 0.1 mm	Marienfeld	Cat#0640110
Inverted cell culture microscope	Olympus	CX31
6-well for adherent cells; flat bottom; clear; sterile	Sarstedt	Cat#83.3920
24-well for adherent cells; flat bottom; clear; sterile	Sarstedt	Cat#83.3922
96-well for adherent cells; flat bottom; clear; sterile	Sarstedt	Cat#83.3924
Chemical fume hood	Thermo Scientific	Cat#MSC1.2
Photon imager	Biospace Lab	Model Optima
Stereotactic frame	Stoelting	Model 963
Microinjector	Stoelting	Legato 130
Fine forceps #1	Stoelting	Cat#11006-12
Forceps #2	Stoelting	Cat#11018-12
Fine scissors #1	Stoelting	Cat#14569-09
Fine scissors #2	Stoelting	Cat#14568-09
Needle holder	Stoelting	Cat#12500-12
Suture thread prolene 6/0	Ethicon	Cat#EH7471H
Care drape	Tetra Medical	Cat#16202
Non-woven swabs	Medicomp	Cat#411052
Syringe with needle	BD Biosciences	Cat#050835
Needle 26Gx	Terumo	Ref AN*2613R1
Syringe 10 µL	Hamilton	Cat#549-1137
Latex surgical gloves	GAMMEX	Cat#330048070
Filter (sterile) 0.22 µM	Millipore	SLGVR33RS
Stoelting rodent warmer X2 with mouse heating pad	Stoelting	Cat#53850M
pH meter	METTLER TOLEDO	FiveEasy FE20
UV light 395–400 nm	Berger and Scröter	88305931
Neuro-Patch	B. Braun	N/A
Irradiator	Faxitron	CellRad

MATERIALS AND EQUIPMENT

Complete medium

Reagent	Final concentration	Amount
DMEM high glucose	N/A	445 mL
L-Glutamine	N/A	5 mL
FBS	N/A	50 mL
Total	N/A	500 mL

Store at 4°C under sterile conditions for up to 3 months.

Analgesic solution

Reagent	Final concentration	Amount
Buprenorphine	0.1 mg/kg	1 mL
NaCl solution	150 mM	9 mL
Total	N/A	10 mL

Store at 4°C for a maximum of 2 weeks.

Note: We recommend to calculate a total volume of 100 μ L per mouse with 20% additional volume.

Anesthetic solution

Reagent	Final concentration	Amount
Xylazine	10 mg/kg	1.5 mL
Ketamine	60 mg/kg	3.2 mL
NaCl solution	150 mM	5.3 mL
Total	N/A	10 mL

Store at 4°C for a maximum of 2 weeks.

Note: We recommend calculating a total volume of 80 μ L per mouse with 20% additional volume.

Tris-buffered saline (TBS) (1X)

Reagent	Final concentration	Amount
Trizma base	50 mM	6.05 g
NaCl	150 mM	8.76 g
HCl	N/A	N/A
ddH ₂ O	N/A	800 mL
Total	N/A	1,000 mL

Adjust pH to 7.5 with 1 M HCl and make volume up to 1 L with H₂O. TBS is stable at 4°C for 3 months.

△ CRITICAL: Hydrochloric acid is a strong acid and a highly corrosive liquid. This chemical is highly volatile and is a high-risk inhalation hazard. Wear appropriate protective gloves and clothing to prevent skin exposure. Handle it under a chemical hood.

STEP-BY-STEP METHOD DETAILS

This protocol describes the generation of two distinct murine models with U87-Luc and GL261-Luc GB cells injected in the brain of immunocompromised (NMRI-Foxn1nu/Foxn1nu or NSG NOD.Cg-Prkdcscidll2rgtm1Wjl/SzJ) and immunocompetent (C57BL/6JRj) mice, respectively. The treatment

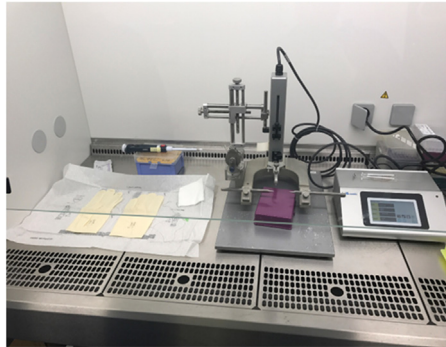


Figure 2. Set up stereotaxic surgery for the tumor injection in the mouse brains

regimen for the murine model using GL261-Luc cells^{8,9} includes both irradiation and chemotherapy,⁷ whereas the regimen for the U87-Luc model includes only chemotherapy.¹

Preparation of U87-Luc and GL261-Luc GB cells

⌚ Timing: 7–10 days

In this section, we describe the cell culture steps required to prepare the cells for injection into experimental models.

1. Thaw U87-Luc and GL261-Luc GB cells in complete medium.
2. Maintain GB cells in culture at 37°C in 5% CO₂ until they are prepared for injection.

Note: Before tumor cell injection we suggest to split the GB cells at least once after thawing for recovering and resuming the normal cell cycle. We recommend reaching approximately 80% confluency prior to injecting GB cells. It is not recommended to use GB cells that are too confluent as this could compromise cell viability.

Note: The stably-transfected cells are maintained in culture with the selection media (puromycin or G418, depending on the construct). There is an agreement that the number of passages should be minimized to reduce the possibility of phenotypic variations, genetic drift, and contamination as much as possible, but standards organizations differ as to how many passages are acceptable. The ATCC recommends that this number should not exceed seven passages from the original reference culture.

Preparation of the anesthesia/analgesia solutions and equipment in the animal facility

⌚ Timing: 15 min of UV exposure after 30 min for solution and equipment preparation

This part includes the preparation of anesthesia and analgesic solutions for administration to mice before surgery.

3. Prepare the analgesic solution freshly as described in the [materials and equipment](#) section.
4. Prepare the anesthetic solution freshly as described in the [materials and equipment](#) section.
5. Prepare the hood ([Figure 2](#)).
 - a. Clean the hood with medical surface detergent.
 - b. Install the [stereotaxic frame \(Stoelting, Model 963\)](#) and [microinjector \(Stoelting, Model Legato 130\)](#) according to the manufacturer's instructions.

Note: For the specific model used in this protocol, refer to [Figure 2](#) for installation guidance. Detailed setup parameters for the microinjector are described in steps 24 and 25.

- c. Decontaminate all the equipment and solution tubes (anesthetics, analgesics, disinfectant and moisturizing eye gel) with medical surface detergent, followed by exposure of UV light for 15 min.
- d. Set up all the sterile surgical equipment inside the hood (forceps, scissors, needle holders, suture thread, care drapes, latex surgical gloves, Ocry-gel, 0.5% alcoholic Chlorhexidine, 1 mL syringes, 26Gx needles, Hamilton syringes).
- e. Filter analgesic and anesthetic solutions with a 0.22 μm filter.

GB cell suspension for injection

⌚ Timing: 1 h

This segment explains the preparation of cells suspension for injection into mice, detailing the cell culture steps, cell concentration, and short-term storage methods. We recommend the suspension preparation of stable luciferase-expressing U87-Luc and GL261-Luc GB cells during the set-up of the equipment in the animal facility.

6. Detach U87-Luc and/or GL261-Luc adherent GB cells from the tissue culture flasks.
 - a. Wash the cell monolayer with 5 mL sterile PBS.
 - b. Incubate GB cells with 1 mL of 0.25% trypsin solution for 3 min at 37°C.
 - c. Stop the trypsin reaction with 10 mL of culture medium.
7. Centrifuge the cell suspension at 300 g for 3 min at RT.
8. Remove the supernatant.
9. Resuspend cells in 10 mL sterile PBS.
10. Repeat **steps 7, 8, and 9** twice for a total of 3 washes.
11. After the last wash, resuspend cells in 500 μL of PBS.
12. Count cells and record cell viability.
13. Adjust cell suspension to a final concentration of 5×10^7 cells/mL in PBS.
14. Keep the cell suspension on ice before the injection.

Note: We recommend to repeat this preparation every 5–6 animals to avoid decrease of cell viability after the injections and to get further homogeneous tumor growth between animals

Tumor cell orthotopic implantation

⌚ Timing: 35 min on hold (steps 15 and 16)

⌚ Timing: 20 min per animal

This section outlines the detailed procedure for administering tumor cells into the brains of mice.

15. Inject 100 μL of freshly prepared analgesic solution (buprenorphine) (**step 3**) subcutaneously in immunocompromised (Mouse NMRI-Nude NMRI-Foxn1nu/Foxn1nu or NSG NOD.Cg-Prkdc^{scid}Il2rg^{tm1Wjl}/SzJ) for U87-Luc injection and immunocompetent (C57BL/6JRj) for GL261-Luc injection) mice 30 min before anesthesia.
16. Inject 80 μL of freshly prepared anesthetic solution (xylazine/ketamine) intraperitoneally (**step 4**).

Note: The expected time to obtain narcosis is of about 5 min.

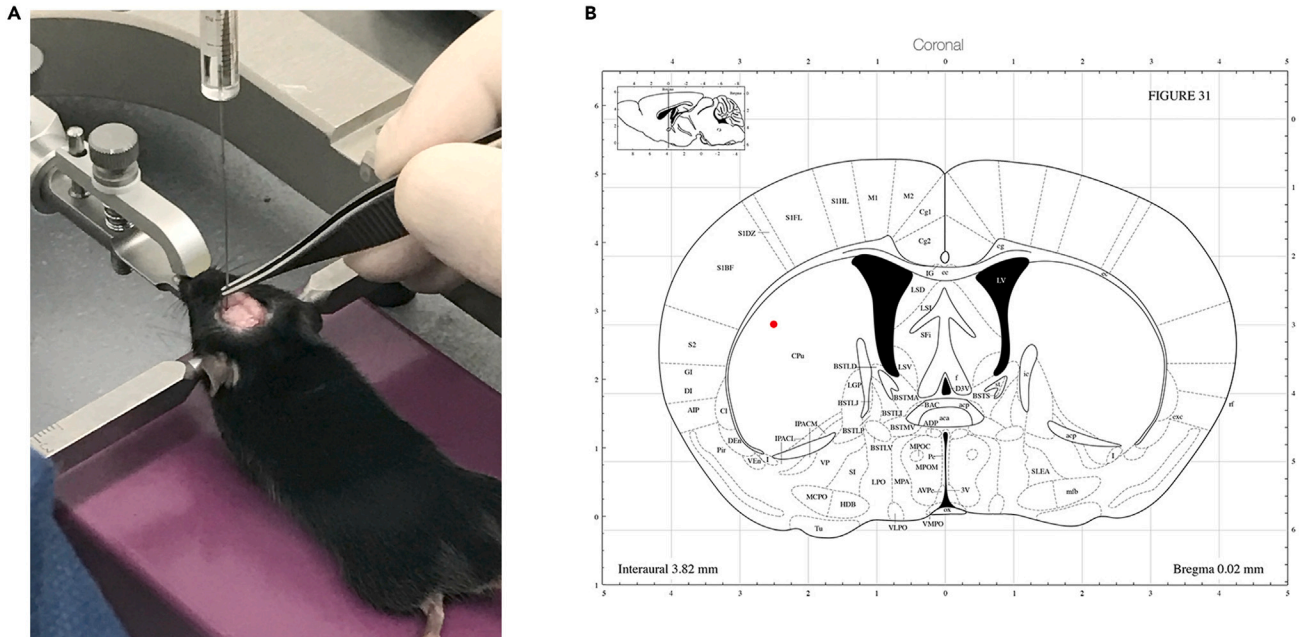


Figure 3. Stereotaxic surgery for tumor cells implantation

(A) Photograph of an anesthetized mouse held by the stereotaxic frame while being injected with tumor cells.

(B) Intracranial injection sites. Representative atlas diagram¹⁰ with the injection site (red dot) represented within the caudate putamen (CPu).

△ **CRITICAL:** Induce anesthesia in a single mouse at the time. To make sure the animal is completely anesthetized, pinch the tail. If the narcosis is sufficient, no reflex when pinching should be seen. If the mouse is not asleep, wait a few more minutes and/or adjust the anesthetic dose.

17. Transfer anesthetized mouse to the stereotaxic platform (Figure 2).
18. Place it on the stereotaxic frame.
19. Apply a veterinary ocular protection solution on the eyes to avoid lesion by dryness (Figure 3A).

△ **CRITICAL:** To make sure the animal is properly fixed on the stereotaxic frame position the ear bars at the bone located above the ear canal in order that the head does not move and especially not in the ear in order to avoid piercing the eardrum. The head must be parallel to the support to assure the correct injection position.

20. Incise the scalp with fine scissors #2 and clean any blood with 0.5% alcoholic chlorhexidine.
21. Mark the point of injection with a needle 26Gx at the desired stereotaxic coordinates calculated for injection of tumor cells into a specific point of the brain.
22. Fill the syringe with 1 μ L of cell suspension (corresponding to 5×10^4 cells per mouse in 1 μ L).
23. Position the syringe at the stereotaxic coordinates with the injector.

Note: In this procedure, the tumor cells were injected laterally at 2.2 mm to the left of the bregma, longitudinally at the bregma and with depth of 3.0 mm to perform the implantation in the striatum (caudate putamen - CPu) (Figure 3B).

24. For microinjector parameters, select the syringe model used (in this protocol, Hamilton 10 μ L).
25. Set the injector at a rate of 0.3 μ L/min for a total injection volume of 1 μ L.
26. Inject the GB cells. Take out the syringe slowly from the brain after injection.

Note: Generally, it should take about 30 s for the needle to be completely out from the tissue.

27. Transfer the anesthetized mouse to a preheated platform draped with an absorbent pad.
28. Perform the stitches. Re-apply a veterinary ocular protection solution on the eyes, if necessary.

Note: Once step 28 is completed, re-start the procedure (step 17) with the next animal.

29. Keep the mouse on the heated platform at 37°C until it awakes.
30. Transfer awakens animals to the cage.

Note: Be aware of the animals on the pad. Recently anesthetized mice recover completely their spatial senses in about 30 min.

Animal monitoring

⌚ Timing: 1–8 months

This section presents the criteria for monitoring the well-being of animals and provides guidance on how to implement them.

31. The day after the surgery, examine carefully the stitches.
32. Monitor every day mice behavior. Weigh every mouse individually daily. The animals should be monitored using the following criteria.
 - a. 10% loss of initial weight.
 - b. Abnormal behavior (social isolation).
 - c. Lack of grooming (ruffled fur).
 - d. High bioluminescence signal.

⚠ CRITICAL: When an animal is in pain or distress that cannot be relieved, the animal must be removed from the situation to prevent further suffering. This can involve removing the animal from an experiment or activity either temporarily or permanently, euthanizing the animal, ceasing the activity, or discontinuing observations. Under no circumstances should the animal's pain or distress be ignored

A standard-of-care treatment of GB mouse model

⌚ Timing: 10 days to few weeks (depending on the treatment regimen and drug used)

In this treatment section, two types of standard-of-care (SOC) murine models are described. The first, using GL261-Luc cells, includes resection, irradiation and chemotherapy treatments (Stupp-like),¹¹ mimicking clinical practices. The second SOC using human U87-Luc cells evaluates the effect of combining chemotherapy (new molecules or repurposed drugs) with temozolomide (TMZ) without surgical resection and irradiation. In both protocols, tumor development is tracked by bioluminescence, a process described in the subsequent section.

Resection of GL261-Luc tumor bulk (Stupp-like)

⌚ Timing: 36 min on hold (steps 33, 34, and 35)

⌚ Timing: 15 min per mouse

Here, we describe the resection of tumors derived from GL261-Luc cells injections.

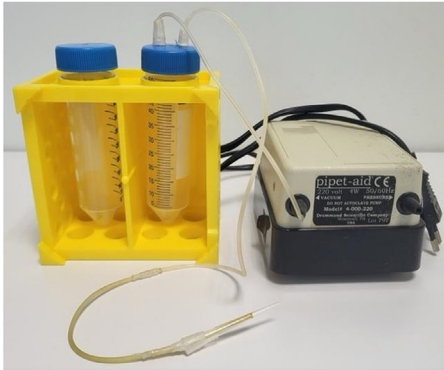


Figure 4. Suction device

33. Inject 100 μ L of freshly prepared analgesic solution 30 min before anesthesia.
34. Inject 100 μ L fluorescein (caudal injection) 1 min before anesthesia.

Note: Tumor visualization is enhanced by injecting fluorescein and using a UV source. This fluorophore enables visualization of the tumor following blood-brain barrier (BBB) disruption, a characteristic of glioblastoma (Methods video S1).

35. Inject 80 μ L of freshly prepared anesthetic solution intraperitoneally.

Note: The expected time to obtain narcosis is about 5 min.

36. Place the animal on the stereotaxic frame.
37. Make an incision with the Scalp to expose skull convexity and injection site (Methods video S1).
38. Open the bone with fine forceps #1, enabling minimal craniectomy (Methods video S1).

Note: The visual aid previously described facilitates the delineation of the tumor borders and minimizes the lesions on healthy parenchyma.

39. On day 14, tumors should be grown. Perform bioluminescent acquisition to confirm tumor growth (Figure 8C).
40. Resect the tumor with fine scissors #1 using a surgical microscope, and aspirate remaining tumor areas with the help of a suction device to gently remove it (Figure 4 and Video).

Note: For the entire removal of the remnant tumor, we built a suction device with a vacuum pump, 50 mL tubes and appropriate tubing (Figure 4).

Note: The vacuum pump used during the resection step had a flow rate of 40 mL/min.

41. Once the resection is achieved, fill the hole left by the tumor with a fibrin-collagen gel (Methods video S1).⁷

Note: For the fabrication of gel implants dissolve human fibrinogen in deionized water at a concentration of 30 mg/mL and make the dialysis overnight against tris-buffered saline (TBS – detailed recipe on Materials and equipment setup section). Dispense the gel mixture as 7 μ L droplets onto a hydrophobic surface (commercial Teflon tape) to form micro-droplets. Crosslink the gel droplets with human thrombin (40 U/mL) and incubate at 37°C for 20 min to ensure stable cross-linking. For more detailed information about the gels and the infusion of drugs on it please refer to Le Reste et al., 2020.⁷

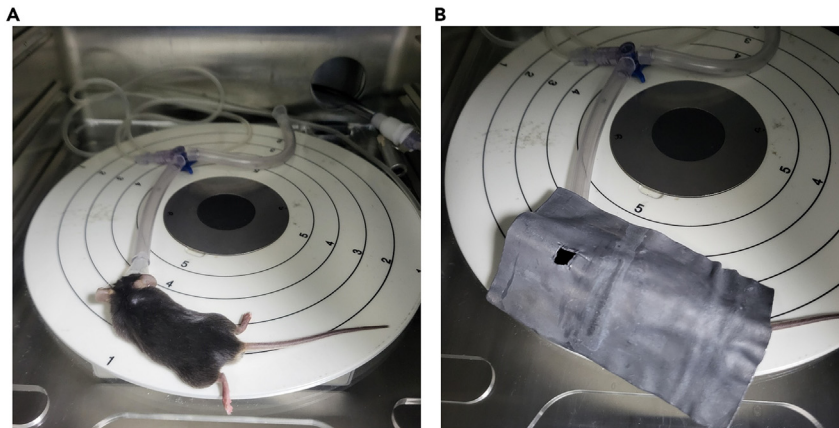


Figure 5. Irradiation apparatus

Mouse is kept under continuous anesthesia before (A) and after (B) the placement of the lead shield, which protects the rest of the animal during irradiation.

42. Slide the Teflon patch (Neuropatch) under the skull and fixed by the application of biocompatible acrylic glue.
43. Close the craniectomy with a fine forceps #2.

Note: The steps 42 and 43 allow maintenance of intracranial pressure and prevent tumors from developing in the subcutaneous space ([Methods video S1](#)).

44. Sew the skin with non-resorbable monofilament sutures at the end of the procedure.

Irradiation and chemotherapy treatments of GL261-Luc-injected mice (Stupp-like)

⌚ Timing: 3 min per mouse over 5 days

In this part we describe the irradiation and chemotherapy steps of the Stupp protocol.

45. Place the mice in the irradiator (Faxitron, CellRad) under continuous gas anesthesia.
46. Apply a lead shield with a small opening to irradiate the area of interest (tumor) and to protect the rest of the animal during irradiation.

Note: Perform a cycle of 2 Gy per day for 5 days ([Figures 5A and 5B](#)).

47. Inject the mice intraperitoneally once a day with TMZ 25 mg/kg for 5 days in the first week (simultaneously with radiotherapy) and with TMZ 50 mg/kg once a day for 4 weeks in a regimen of 5 days of treatment spaced with 2 days without TMZ injections.

Note: The pause with TMZ treatment avoids the severe side effects commonly associated with this chemotherapeutic agent.

Chemotherapy treatment of U87-Luc-injected mice

⌚ Timing: 10 days to several months (depending on the treatment regimen and drug used)

In this section we describe the chemotherapy treatment of mice who bearing tumors derived from U87-Luc cells using compounds that are BBB-permeable. To mimic tumors that cannot be resected

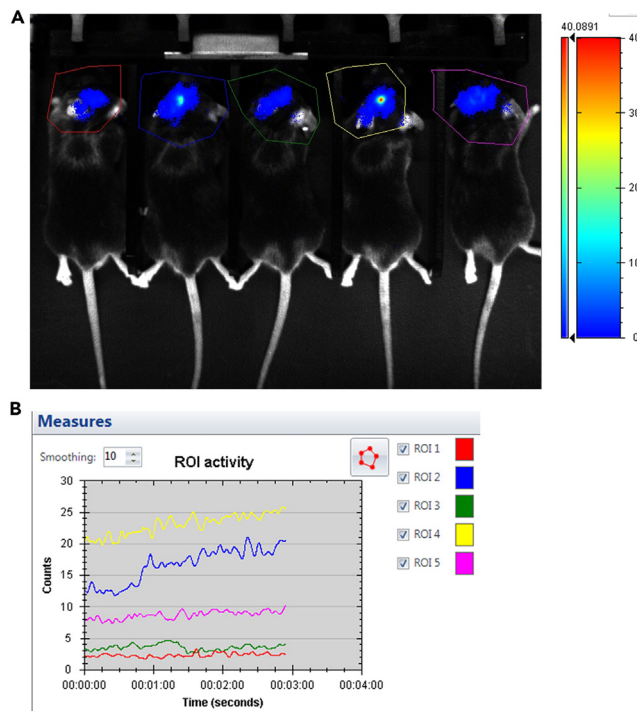


Figure 6. Bioluminescence signals acquisition

(A) The bioluminescence images of the tumor-bearing mice.

(B) Quantification of ROI activity for each mouse shown in (A), individually over time. ROIs 1 to 5, shown in (B), correspond to the mice from left to right in (A), following the color code.

or irradiated (which comprises between 20 to 30% of the patients), we performed a protocol in which we treated the mice with TMZ combined with an adjuvant treatment, a small molecule named Z4P. This compound targets IRE1, the most evolutionary conserved sensor of the Unfolded Protein Response (UPR). Z4P is able to cross the BBB¹ and, for this reason, could be administered intraperitoneally.

48. Inject co-adjuvant treatments from day 4 post-injection every day with a single dose (for Z4P, 300 $\mu\text{g}/\text{kg}$) of compound intraperitoneally.

49. Inject the mice once a day with TMZ 10 mg/kg starting 11 days-post injection for 10 days.

50. Monitor mouse behavior every day and weigh every mouse individually.

Note: Adjust chemotherapy doses according to the body weight.

Quantification of tumor growth and statistical analyses

⌚ Timing: 20 min per batch of 5 mice during several months (the number of animals per batch can vary according to different imager models)

In this step we detail the precise monitoring and quantification of tumor growth in mice over time using bioluminescence imaging, explaining the data acquisition process and the analysis of luminescence signals. Tumor growth is monitored every 4–7 days using a bioluminescence imaging device. In this protocol, the luminescence signals were analyzed with the image analysis software M3 Vision (Biospace Lab).

51. Inject 100 μL of luciferin (10 mg/kg) intraperitoneally into each mouse.

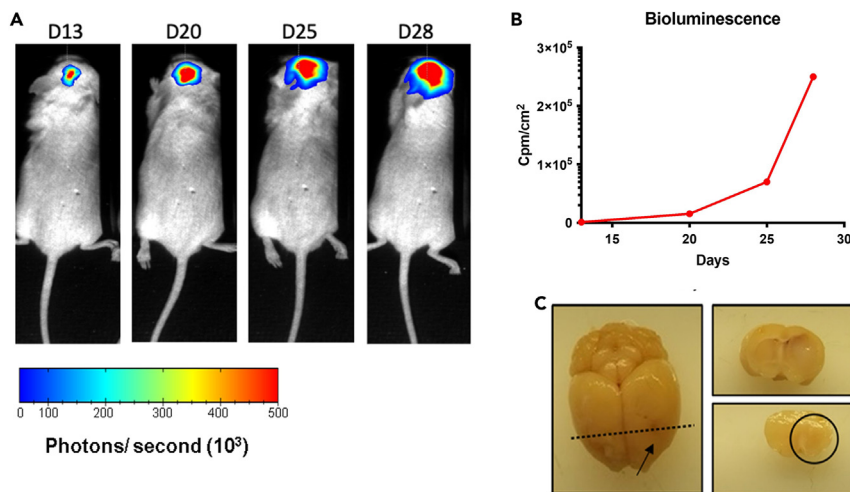


Figure 7. Tumor growth monitored by bioluminescence imaging

(A) Tumor growth derived from U87-Luc cells monitored by bioluminescence imaging. The bioluminescence images of the tumor-bearing mice at days 13, 20, 25, and 28 post-U87 cells implantation.

(B) Time-resolved quantitative analysis of bioluminescence imaging signal over 28 days of experiment.

(C) Macroscopic images of the brains following injection of U87-Luc cells. The entire brain is cut in the point injection (arrow) level. The tumor mass can be seen at necropsy.

52. About 5 min after injection, anesthetize the animals putting them in an induction box under isoflurane 2%–0.1 L/min warmed at 37°C.
53. Transfer anesthetized animals to the imager. Accommodate them on a warm plate and place a mask adapted for the muzzle of the mouse. The anesthesia mask releases isoflurane (2%) to maintain the animal anesthetized.

△ **CRITICAL:** The steps 51 to 53 should be performed in batches with the number of animals that could fit in the imager to avoid long periods of anesthesia.

54. Acquire the real-time image. Select each tumor area as a Region of Interest (ROI) (Figure 6A).

Note: The measurement in real time would be measured by counts per second and plotted on a graph in the software. Monitor the ROI activity over time for each mouse individually until it stabilizes (Figure 6B).

55. Once the bioluminescence signals reach a plateau, perform a new acquisition during 1 min.
56. Save the acquisition.
57. Open corresponding bioluminescence images with the M3 Vision software.
58. Select each tumor area as a ROI. The signal measurement here will be displayed in photons/seconds/cm² (Figure 7A).

△ **CRITICAL:** Use the same ROI size to analyze all images independently of the individual animal or the day post-implantation.

59. For each animal, keep a track of the bioluminescence intensities to monitor tumor growth.

Note: Black fur can impair bioluminescence signals. In this case, the area of interest should be shaved before the acquisition. If the skin becomes hyperpigmented after several days, combine other methods of tumor growth monitoring such as immunohistochemistry post-mortem and mouse behavior.

60. Draw identical ROIs for each condition and export the luminescence data.

Note: For quantifying luminescence, we recommend using identical ROIs for each condition and exporting the luminescence data in counts per minute for an user defined area (in square centimeter) calculated by the software and exported in cpm/cm². After acquisition, draw identical ROIs for every condition and change the software settings from photons/sec to cpm/cm². This will enable more accurate tracking throughout the entire experiment. For more details on the quantification procedure, please refer to the software's manual.

61. Plot bioluminescence values in xy plot (Figure 7B).

62. Test for statistical significance using the appropriate statistical test (i.e., t-test, ANOVA).

63. If the tracking of tumor growth is successful the analysis should correspond to the real tumor size (Figure 7C).

△ CRITICAL: Error on luciferin injection and tumor necrosis could decrease bioluminescence intensities. Each case should be evaluated individually based on the previous readings, tumor cell aggressiveness and mouse behavior.

EXPECTED OUTCOMES

If viable cells were correctly injected into the mice, small tumors can already be detected by bioluminescence as early as 4 days post-injection, allowing mice randomization. Bioluminescent imaging signals correlate with visible tumors in the brain at necropsy (Figures 7A and 7C). This protocol was used to assess the therapeutic efficacy of IRE1 inhibitors in the preclinical models of GB in mice. After the cell's implantation, 2 models can be followed to evaluate the SOC and effect of small molecules as monotherapy or adjuvant therapy alongside TMZ. In the first model (Figure 8A) we prioritize the similarity with the SOC used in the clinic. Due to the lack of a preclinical mouse model that accurately mimics how patients with GB respond to treatments, we aimed to create a new model that comprises surgery followed by radiotherapy and chemotherapy, similar to what patients experience in clinical setup. We designed an experimental approach utilizing the GL261 mouse GB cell line engineered for stable luciferase expression to enable real-time tumor imaging. These modified cells were then injected into the brains of C57BL/6 mice orthotopically, allowing tumors to develop over 12–13 days. Subsequently, tumors were surgically removed with the aid of fluorescein as a tumor tracer, and the mice were given a week to recover before undergoing a treatment regimen similar to the Stupp protocol^{7,11} (Figure 8A). This Stupp-like treatment involved radiation (2 Gy/day, 5 sessions) combined with chemotherapy (TMZ, 25 mg/kg, 5 doses), followed by TMZ treatment alone (30–50 mg/kg, 5 doses with 2 days without treatment for 4 weeks). The Stupp-like procedure can be combined with other treatments. In our case, we used the IRE1 inhibitor, MKC8866⁷ at 10 μM as a co-adjuvant therapy of TMZ. This inhibitor being unable to cross the BBB, treatment was carried out by implantation of a fibrin-collagen gel in the resection cavity.⁷

In the second model (Figure 8B), we evaluated BBB-permeable IRE1 inhibitors on the effect of chemotherapy (TMZ) in the absence of surgical resection and irradiation. This was carried out in the xenogeneic mouse model with U87 cells also engineered for stable luciferase expression to enable real-time tumor imaging. Mice were treated as described above with TMZ only and/or Z4P (a BBB-permeable IRE1 inhibitor¹) in an orthotopic mouse model (i.p. 300 μg/kg daily, starting on D4), 10 daily treatments of TMZ (10 mg/kg) at day 11 post tumor cell implantation.

In both models the tumor growth is monitored by bioluminescence. Quantitative analysis of the bioluminescence imaging signal in the control groups as those treated with TMZ alone or in combination with IRE1 inhibitors allow the evaluation of treatment efficacy and tumor development in both models presented here (Figures 8C and 8D). The Kaplan-Meier survival curves (Figures 8E and 8F)

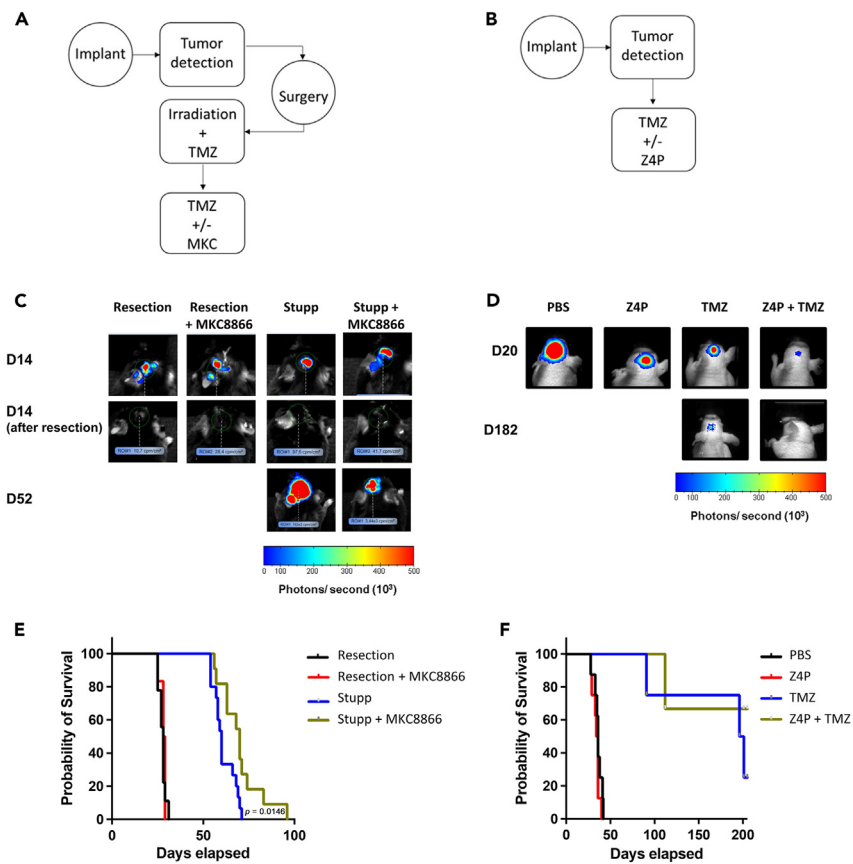


Figure 8. Preclinical development of GB model in mice

(A and B) Schematic representations of the experimental design for the 2 different models described in this protocol. The first model (A) uses the GL261-Luc cell line in immunocompetent (C57BL/6JRj) mice. This model comprises surgical resection followed by radio/chemotherapy that recapitulates the current GB patient standard of care (Stupp protocol) in mice. The second model (B) was used to evaluate the direct effect of chemotherapy in tumor growth. It involves the injection of U87-Luc cells into immunocompromised mice (Mouse NMRI-Nude NMRI-Foxn1nu/Foxn1nu or NSG NOD.Cg-Prkdcscidll2rgtm1Wjl/SzJ).

(C and D) Tumor growth derived from GL261-Luc (C) and U87-Luc (D) cells monitored by bioluminescence imaging. The bioluminescence images of the tumor-bearing mice from the groups treated with TMZ alone or in combination with IRE1 inhibitors in the indicated days post-implantation.

(E) Kaplan-Meier representation of mouse survival under this regimen (black: surgical resection ($n = 9$), red: surgical resection + MKC8866 10 μ M ($n = 6$), blue: Stupp only - surgical resection + radiotherapy + TMZ ($n = 15$), yellow: Stupp + MKC8866 10 μ M ($n = 11$)).

(F) Kaplan-Meier representation of mouse survival under this regimen (black: PBS ($n = 8$), red: Z4P 300 μ g/kg ($n = 8$), blue: TMZ ($n = 8$), yellow: TMZ + Z4P 300 μ g/kg ($n = 8$). Symbols and error bars represent mean values \pm SEM. p value between Stupp and Stupp + MKC8866 = 0.0146. Mantel Cox (log rank) test.

indicated that combination of MKC8866 or Z4P with TMZ treatment enhanced its efficacy, leading to arrest of tumor growth and to prolonged mouse survival (Figures 8C–8F).

LIMITATIONS

The brain tumors models in mice offer valuable platforms for conducting brain tumor research. However, limitations are unavoidable for many reasons. The use of tumor implantation models in brain research presents several important limitations. A primary concern is that the introduction of tumor cells into the brain can mechanically disrupt the surrounding brain tissue. This disruption could lead to a cascade of unintended effects that compromise the tissue's integrity and functionality of the BBB, making it difficult to accurately interpret experimental outcomes.^{12–14} Variations to drug

response are expected depending on the specific brain tumor type and cell line under investigation. The use of mouse cells can affect the responsiveness of drugs or other types of treatment targeted to humans. The choice of mouse species for xenografts of human brain tumors is also crucial in interpreting results due to factors such as the host's immune system. A mouse with an intact immune system may experience graft-versus-host disease, potentially masking the immune system's role in oncology. Conversely, using mice lacking an immune system means a significant element of the tumor microenvironment is absent. Apart from the model, the use of bioluminescence to track tumor growth has some drawbacks. Animals with black fur, errors during luciferin injection, and tumor necrosis can affect the readout. If these issues arise during the protocol, alternative methods should be used to track tumor growth such as immunohistochemistry post-mortem. Additionally, monitoring mouse behavior is essential to ensure the well-being of the animals.

TROUBLESHOOTING

Problem 1

Needle clogging when performing an intracranial injection (step 26).

Potential solution 1

Rinse the needle and syringe several times with sterile PBS. If the plunger continues to not move smoothly when expelling the liquid, repeat the washes in the syringe and needle more and at a faster rate. If the problem persists, replace the syringe and continue with the injections. After completing the procedure, soak the clogged syringe in methanol and sonicate it for 1 h and rinse the syringe as previously described. If clogging occurs during an intracranial injection, it is recommended to exclude the mouse from the analysis. Rinse the needle with PBS immediately after each injection. Thoroughly wash the needle and syringe with PBS immediately after the final injection.

Problem 2

Extensive death of tumor cells during injection (steps 14, 26, and 54).

Potential solution 2

Repeat the cell suspension protocol every 5–6 injections to prevent a decrease in cell viability and to ensure consistent tumor growth across all animals (check step 14 and note).

Problem 3

Mouse that wakes up during the positioning of stereotaxic frame or during intracranial injection (steps 17–26).

Potential solution 3

Ensure the mouse is adequately anesthetized before performing any procedures by checking the toe reflex and respiration rate. If the mouse has not achieved the desired level of anesthesia, wait a few minutes and/or administer an additional dose of Xylazine/Ketamine intraperitoneally. Do not exceed the maximum dose based on the animal's weight.

Problem 4

Inconsistent engraftment in mice and/or tumor grows extracranially (steps 26 and 54).

Potential solution 4

When performing intracranial cell injections, administer the injection slowly, at a rate of approximately 0.3 $\mu\text{L}/\text{min}$. Hold the syringe steady for 2–5 s after complete expulsion to ensure even dispersion of cells into the tissue. Using a guide screw for intracranial injection typically reduces extracranial tumor growth, primarily by preventing inadvertent cell suspension transfer outside the mouse skull through the guide screw hole. To avoid this, it is crucial to keep the needle within the mouse brain for 10 min post-injection and gradually withdraw it from the hole at intervals of 2–3 min. Rapid withdrawal of the syringe may create negative pressure, potentially causing cells

to escape from the cavity. Employing a slow injection technique helps achieve more uniform engraftment across mice.

Problem 5

Drop of bioluminescent signal across the tumor tracking (steps 54 and 61).

Potential solution 5

In vivo bioluminescence enables the visual representation of the bioluminescence generated by luciferase enzymes. Cells or animals expressing the luciferase enzyme can be imaged using sensitive charge-coupled device cameras for light detection, after injecting luciferin in the circulation. Some causes of low bioluminescence are.

- Failed luciferin injection: In this case re-inject the substrate and re-image the mouse.
- Low temperature: Since the injection requires an adequate mouse metabolism, check if the temperature of the room is ideal for the animals. Mice required room temperature is 23°C–25°C.
- Necrosis: If the area around the tumor is necrosed, luciferin will not reach (or reach less) the modified cells expressing luciferase enzyme. The signal will therefore be lower or absent.

RESOURCE AVAILABILITY

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Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate/analyze datasets/code.

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AUTHOR CONTRIBUTIONS

Conceptualization, D.P.-R., R.P., and E.C.; formal analysis, D.P.-R. and R.P.; funding acquisition, E.C.; investigation, D.P.-R. and R.P.; methodology, D.P.-R., R.P., and P.J.L.R.; project administration, D.P.-R. and E.C.; supervision, E.C.; validation, D.P.-R., R.P., and E.C.; visualization, D.P.-R., R.P., E.C., P.J.L.R., T.A., and U.J.; writing – original draft, D.P.-R.; review and editing, D.P.-R., R.P., E.C., T.A., and U.J.

DECLARATION OF INTERESTS

E.C. is the founder of Thabor Therapeutics; D.P.-R. and E.C. are authors of the patent PCT/EP2023/050228—Compounds containing a hydroxyphenyl moiety and their use, 2022.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.xpro.2024.103304>.

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