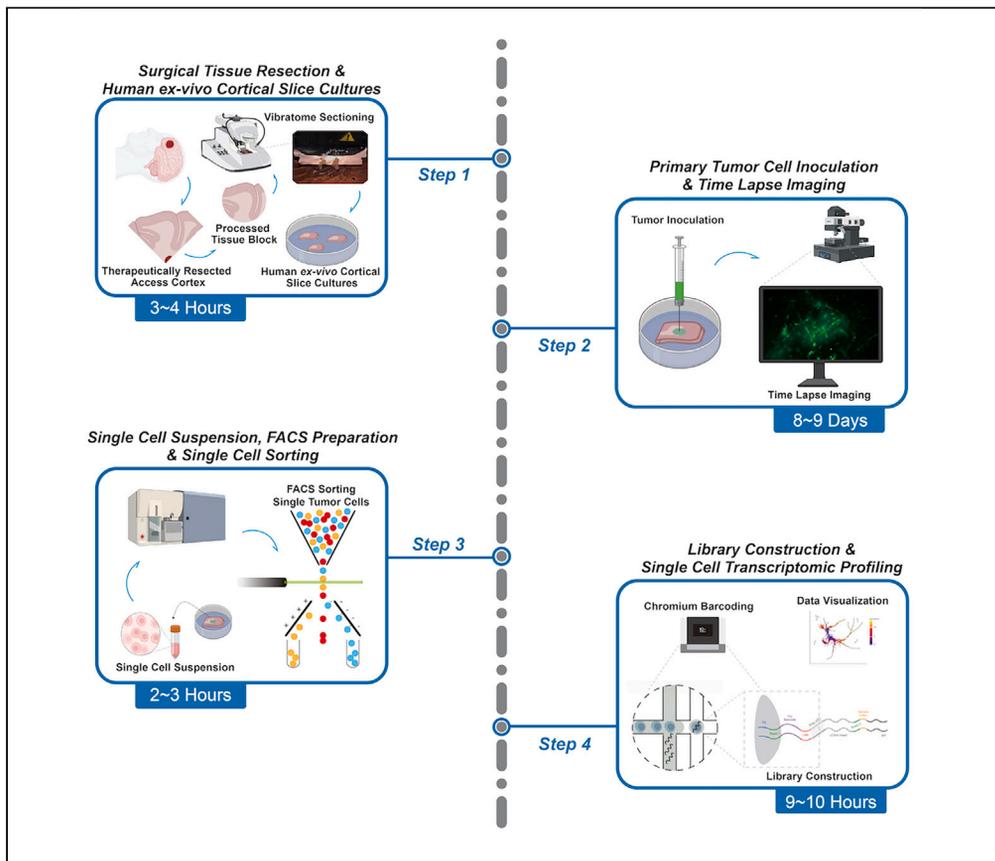


Protocol

Isolation and profiling of viable tumor cells from human *ex vivo* glioblastoma cultures through single-cell transcriptomics



Junyi Zhang, Jakob Straehele, Kevin Joseph, ..., Oliver Schnell, Dieter Henrik Heiland, Vidhya M. Ravi

vidhya.ravi@uniklinik-freiburg.de

Highlights

Describes methods to re-purpose therapeutically discarded human cortical tissue

Protocol is adaptable to brain tissue from different ages of human or rodent subjects

Outlines steps for isolating single-tumor cells from human *ex vivo* cortical cultures

Details quality control measures and library preparation for Illumina sequencing

Single-cell RNA-sequencing (scRNA-seq) is becoming a ubiquitous method in profiling the cellular transcriptomes of both malignant and non-malignant cells from the human brain. Here, we present a protocol to isolate viable tumor cells from human *ex vivo* glioblastoma cultures for single-cell transcriptomic analysis. We describe steps including surgical tissue collection, sectioning, culturing, GBM inoculation, growth tracking, fluorescence-based cell sorting, and population-enriched scRNA-seq. This comprehensive methodology empowers in-depth understanding of brain tumor biology at the single-cell level.

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

Zhang et al., STAR Protocols 4, 102383
September 15, 2023 © 2023
The Author(s).
<https://doi.org/10.1016/j.xpro.2023.102383>



Protocol

Isolation and profiling of viable tumor cells from human *ex vivo* glioblastoma cultures through single-cell transcriptomics

Junyi Zhang,^{1,3,4,6,7,18,19} Jakob Straehle,^{4,5,6,18} Kevin Joseph,^{2,4,5,6,7,18} Nicolas Neidert,^{4,6} Simon Behringer,^{4,6} Jonathan Göldner,^{1,3,4,6,7} Andreas Vlachos,^{8,9,10,11} Marco Prinz,^{6,10,11,12,13} Christian Fung,^{4,6} Jürgen Beck,^{4,5,6,10,14} Oliver Schnell,^{4,5,6,7} Dieter Henrik Heiland,^{3,4,5,6,7,14,15,16} and Vidhya M. Ravi^{1,4,5,6,7,17,19,20,*}

¹3D-Brain Models for Neurodegenerative Diseases, Medical Center, University of Freiburg, Freiburg, Germany

²NeuroEngineering Laboratory, Medical Centre, University of Freiburg, Freiburg, Germany

³Microenvironment and Immunology Research Laboratory, Medical Center, University of Freiburg, Freiburg, Germany

⁴Department of Neurosurgery, Medical Center, University of Freiburg, Freiburg, Germany

⁵Center of Advanced Surgical Tissue Analysis (CAST), University of Freiburg, Freiburg, Germany

⁶Faculty of Medicine, University of Freiburg, Freiburg, Germany

⁷Translational NeuroOncology Research Group, Medical Center - University of Freiburg, Freiburg, Germany

⁸Department of Neuroanatomy, Institute of Anatomy and Cell Biology, Faculty of Medicine, University of Freiburg, Freiburg, Germany

⁹BrainLinks-BrainTools Center, University of Freiburg, Freiburg, Germany

¹⁰Center for Basics in Neuromodulation (NeuroModulBasics), Faculty of Medicine, University of Freiburg, Freiburg, Germany

¹¹Center for NeuroModulation (NeuroModul), University of Freiburg, Freiburg, Germany

¹²Institute of Neuropathology, Medical Center - University of Freiburg, Freiburg, Germany

¹³Signalling Research Centres BIOSS and CIBSS, University of Freiburg, Freiburg, Germany

¹⁴Comprehensive Cancer Center Freiburg (CCCF), Faculty of Medicine and Medical Center - University of Freiburg, Freiburg, Germany

¹⁵German Cancer Consortium (DKTK), Partner Site Freiburg

¹⁶Department of Neurological Surgery, Lou and Jean Malnati Brain Tumor Institute, Robert H. Lurie Comprehensive Cancer Center, Feinberg School of Medicine, Northwestern University, Chicago, IL, USA

¹⁷Freiburg Institute of Advanced Studies (FRIAS), Freiburg, Germany

¹⁸These authors contributed equally

¹⁹Technical contact: vidhya.ravi@uniklinik-freiburg.de

²⁰Lead contact

*Correspondence: vidhya.ravi@uniklinik-freiburg.de
<https://doi.org/10.1016/j.xpro.2023.102383>

SUMMARY

Single-cell RNA-sequencing (scRNA-seq) is becoming a ubiquitous method in profiling the cellular transcriptomes of both malignant and non-malignant cells from the human brain. Here, we present a protocol to isolate viable tumor cells from human *ex vivo* glioblastoma cultures for single-cell transcriptomic analysis. We describe steps including surgical tissue collection, sectioning, culturing, primary tumor cells inoculation, growth tracking, fluorescence-based cell sorting, and population-enriched scRNA-seq. This comprehensive methodology empowers in-depth understanding of brain tumor biology at the single-cell level. For complete details on the use and execution of this protocol, please refer to Ravi et al.¹



BEFORE YOU BEGIN

This protocol describes the specific steps required to carry out single-cell RNA sequencing from human organotypic cortical glioblastoma models. Transcriptomic profiling of all cell types on a single-cell level is a powerful tool to understand the intra-tumoral heterogeneity of these tumors. The readers can target any specific cell populations in the microenvironment by customizing FACS sorting markers.

Institutional permissions

The local ethics committee of the University of Freiburg approved data evaluation, imaging procedures and experimental design (protocol 1000020/09 and 472/15_160880). Human brain tissue specimens were obtained with informed consent under a Declaration of Helsinki, as requested by the local ethics committee (No. 187/04). All methods were carried out in accordance with the approved guidelines. Written informed consent was obtained from all patients. The studies were approved by an institutional review board.

We hereby remind the readers who will carry out the same experiment to follow the advice of their local ethics committee and get an approval from their institutional review board before you begin.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
rLV.EF1ZsGreen1-9	Takara Bio	0037VCT
Biological samples		
Human neocortical access tissue/GBM tissue samples	Department of Neurosurgery, Medical Centre, University of Freiburg	N/A
Experimental models: Cell lines		
BTSC_#233	Uniklinik Freiburg	Primary Cell Line
Chemicals, peptides, and recombinant proteins		
SPRSelect reagent	Beckman Coulter	B23318
Bovine serum albumin (BSA)	Sigma-Aldrich	A3059
4',6-Diamidino-2-phenylindole (DAPI)	Merck KGaA	32670#5MG
Hibernate™ -A Medium	Gibco	12087586
N-methyl-D-Glucamin (NMDG)	Sigma-Aldrich	M2004
Antibiotic-Antimycotic (100×)	Gibco	15240062
Neurobasal™ Medium	Gibco	11570556
L-Glutamine	Gibco	25030149
B-27 Supplement, serum-free (50×)	Gibco	11530536
MgSO ₄	Sigma-Aldrich	M3409
HEPES	Sigma-Aldrich	H0887
D-Glucose	Sigma-Aldrich	RNBG7039
Potassium chloride (KCl)	Merck KGaA	1.04936.0500
Potassium dihydrogen phosphate (KH ₂ PO ₄)	Merck KGaA	1.04873.0250
Sodium chloride (NaCl)	VWR	27810.295
Disodium hydrogen phosphate dihydrate (Na ₂ HPO ₄)	VWR	28029.292
4% Paraformaldehyde solution	Thermo Fisher Scientific	J61899.AK
O.C.T. Tissue-tek	Sakura	SA62550-01/-12
Incidin™ Plus	Ecolab	3011520
Fetal bovine serum (FBS)	PAN Biotech	1502
Ethylenediaminetetraacetic acid (EDTA)	Carl Roth	8043.1
Critical commercial assays		
KAPA SYBR FAST qPCR Master Mix	Roche	KK4600
Chromium Next GEM Single Cell 3' GEM, Library & Gel Bead Kit v3.1	10× Genomics	PN-1000121

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chromium Next GEM Chip G Single Cell Kit	10x Genomics	PN-1000120
Single Index Kit T Set A	10x Genomics	PN-1000213
Fragment Analyzer 5200 HS NGS Kit	Agilent	DNF-474
Qubit 1x dsDNA HS Kit	Thermo Fisher Scientific	Q33231
NextSeq 500/550 High Output Kit v2.5 (150 cycles)	Illumina	20024907
NextSeq 500/550 High Output kit v2.5 (75 cycles)	Illumina	20024906
Neural Tissue Dissociation Kit (T)	Miltenyi Biotec	130-093-231
Software and algorithms		
R/R Studio software	Posit	https://posit.co/downloads/
Cell Ranger analysis pipeline	10x Genomics	https://support.10xgenomics.com/single-cell-gene-expression/software/pipelines/latest/installation
Cranial Map Neuro Navigation Cart 2	Stryker Corporation	N/A
Other		
Leica VT1200 semi-automatic vibratome	Leica	1491200001
Millipore insert	Merck KGaA	PIHP03050
6-well plate	Greiner BIO-ONE	657160
Cryostar™ NX70 cryostat	Epredia	15380755
NIO laser imaging system	Invenio Imaging	01-0224
Hamilton micro syringe	Hamilton	80330
EVOS M7000 microscope	Thermo Fisher Scientific	AMF7000
EVOS onstage incubator	Thermo Fisher Scientific	AMC1000
C-Tube	Miltenyi Biotec	130-093-237
Chromium controller	10x Genomics	N/A
Qubit™ 4 Fluorometer	Thermo Fisher Scientific	Q33238
5200 Fragment Analyzer	Agilent	M5310AA
BD FACSAria™ Fusion	BD Biosciences	N/A
BD FACSAria™ III	BD Biosciences	N/A
MoFlo Astrios EQ	Beckman Coulter	N/A

MATERIALS AND EQUIPMENT

Preparation Medium (PM) for Slicing – 160 mL

Reagent	Final concentration	Amount
Hibernate Medium	N/A	147.72 mL
D-Glucose (1 M)	13 mM	2.08 mL
NMDG (1 M)	30 mM	6 mL
L-Glutamine (200 mM)	0.5 mM	1 mL
Antibiotic-Antimycotic (100x)	2%	3.2 mL
Total	N/A	160 mL

△ **CRITICAL:** Following sterile filtration, it is recommended to divide the 160 mL preparation medium into 50 mL aliquots to prevent contamination. These aliquots should then be stored at a temperature of -20°C to maintain the integrity of the medium. It is crucial to avoid freeze-thaw cycles as they can compromise the quality of the medium. Moreover, it is essential to use the aliquoted medium within 20 days of preparation to ensure its effectiveness. Each sectioning procedure with a vibratome requires approximately 100 mL of the preparation medium. Therefore, it's important to plan the required amount of medium carefully to avoid any shortages during the experimental procedure.

Note: To prepare 1M N-Methyl D-Glucosamine take 1.95 g of the NMDG powder and mix it in 40 mL of Sterile H₂O. Please filter the solution before using.

Growth Medium (GM) for Slice Culture – 160 mL

Reagent	Final concentration	Amount
Neurobasal Medium	N/A	147.80 mL
D-Glucose (1 M)	13 mM	2.08 mL
Magnesium Sulfate (1 M)	2 mM	320 μ L
L-Glutamine (200 mM)	0.5 mM	1 mL
HEPES (1 M)	15 mM	2.4 mL
B-27 Serum free (50 \times)	2%	3.2 mL
Antibiotic-Antimycotic (100 \times)	2%	3.2 mL
Total	N/A	160 mL

△ CRITICAL: To ensure the consistency and reproducibility of cell culture experiments, it is essential to store the growth medium correctly. We recommend dividing the 160 mL medium into 50 mL aliquots and storing them at -20°C to prevent contamination and maintain the quality of the medium. To avoid any loss of efficacy, it is crucial to avoid freeze-thaw cycles. However, if thawing is necessary, we suggest storing the isolated medium at 4°C and using it within 3 days. By following these guidelines, researchers can maintain the effectiveness of the growth medium and obtain reliable and reproducible experimental results.

PBS (10 \times) – 1 L

Reagent	Final concentration	Amount
Milli-Q Water	N/A	1 L
KCl	26.8 mM	2.0 g
KH_2PO_4	14.7 mM	2.0 g
NaCl	1.396 M	80.0 g
Na_2HPO_4	64.6 mM	11.5 g
Total	N/A	1 L

Note: The PBS solution is prepared in the absence of Mg^{2+} or Ca^{2+} ions. A long-term storage at room temperature (25°C) is allowed.

FACS Buffer – 100 mL

Reagent	Final concentration	Amount
PBS (1 \times)	N/A	97 mL
FBS (100%)	2%	2 mL
EDTA (0.5 M)	5 mM	1 mL
Total	N/A	100 mL

Note: We recommend to prepare FACS buffer shortly before use instead of keeping it in store for a long term. During the experiments, the FACS buffer should be kept on ice.

STEP-BY-STEP METHOD DETAILS

Tissue collection, transport, sectioning, and preservation

⌚ Timing: 3–4 h

Here we describe best practices to collect, preserve and carry out the slicing of neocortical access tissue obtained from the resection of brain tumors and from epilepsy surgery. The neocortical access tissue used is not required for neuropathological diagnostics and would otherwise be discarded during the usual course of surgery. Depending on the point of entry and the extent of resection, the

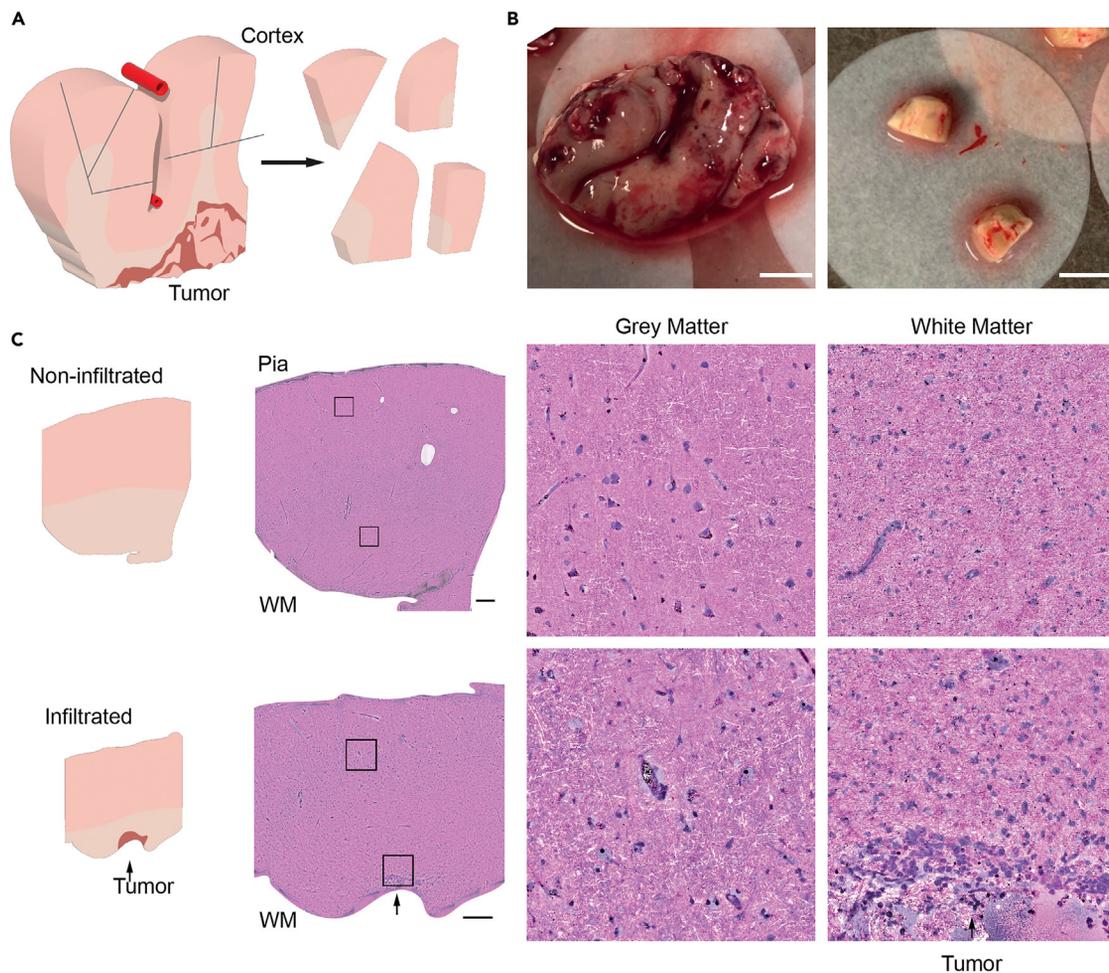


Figure 1. Neurosurgical cortical tissue sampling and pathological evaluation

(A) Illustration of tissue dissection for tissue culture experiments.

(B) The example of a neocortical access tissue sample from a neurosurgical operation (left) which is processed and cut into blocks (0.5–1 cm³, right), Scale bar: 1 cm.

(C) Stimulated Raman histology of a representative slice of non-infiltrated neocortical access tissue (top row) vs. a slice of infiltrated (arrow) access tissue (bottom row) from the same sample. Note, that slices from a visibly tumor infiltrated block would not be used for tissue culture experiments. WM: white matter, Boxes and Scale bar: 500 μm.

neurosurgeon removes a piece of neocortical access tissue, using the shortest non-eloquent trajectory as usual. The dissection is ideally performed with a scalpel and a sharp dissector, with only minimal cauterization and avoiding mechanical damage.^{1–5} The anatomical location of the resected tissue is recorded in the neuronavigation software (Cranial Map Neuro Navigation Cart 2; Stryker). In the operating theater, the resected tissue is immediately immersed in pre-cooled (0°C) and oxygenated preparation medium (95% O₂, 5% CO₂) and transported to the laboratory on ice as quickly as possible (<5 min).

1. Set up of the tissue processing platform, tissue sectioning, and tissue culture.
 - a. Keep the cortical tissue always immersed in preparation medium saturated with carbogen (95% O₂, 5% CO₂) at 0 ± 4°C before tissue processing.
 - b. Process the resected tissue.
 - i. Prepare a cooled metal platform (0° ± 4°C) that is generously flooded with preparation medium.

- ii. Extract the resected tissue from the transport chamber and place it on the cooled platform (Figure 1B).
- iii. Dissect away all visibly cauterized and damaged parts.
- iv. Further dissect the processed tissue block into smaller blocks (size from 0.5–1 cm³) for sectioning (Figure 1B).

Note: We recommend that each processed and dissected smaller block of tissue contains portions of the neocortex as well as underlying white matter.

- c. Section the prepared tissue blocks into 300 μm slices with a dimension of 6 mm by 6 mm using a tissue vibratome (Leica V1200 vibratome) at a speed of 0.16 mm/s, in preparation medium.

Note: The temperature of the sectioning chamber is monitored and is maintained between 0–4°C with the help of crushed ice. During sectioning, the vibratome containing the preparation medium must be bubbled using carbogen.

- d. Process the resected tissue.

Note: The temperature of the sectioning chamber is monitored and is maintained between 0–4°C with the help of crushed ice. During sectioning, the vibratome containing the preparation medium must be bubbled using carbogen.

- e. Transfer 300 μm tissue sections onto Millipore inserts in 6-well plates, with 1.2 mL of growth medium in each well.

Note: The first two tissue sections from each block are generally discarded due to lack of consistency of section thickness (>300 μm).

Note: Each block of tissue allows for the generation of 20–25 tissue sections for technical replicates, allowing for the usage of tissue sections across multiple experimental paradigms.

Note: We recommend a maximum of 3 sections/well. Increase in the number of sections/well requires an increase in the frequency of medium exchange due to increased cytokine/chemokine release during culture. The medium can be collected for further experimental workplan with ELISA to detect metabolites from within the culture medium such as cytokines and neurotransmitters, as has been previously described by us.²

△ CRITICAL: For proper pre-recovery of tissue sections before culture, it is essential to transfer them using a glass pasteur pipette into ice-cold incubation medium in 60 mm × 15 mm petri dishes that are saturated with carbogen. The sections should be allowed to incubate for 10–15 min before being placed onto the inserts for culture. This step is critical to ensure the viability of the tissue sections during the subsequent culture process.

- f. Refresh the growth medium at periodic intervals to ensure maximal viability: The first medium refresh must be carried out 6 h post plating, followed by medium refreshes every 48 h, until the end of the culture period.

Note: For infiltration within the cortical tissue sections is assessed using conventional histopathological staining methods (e.g., Hematoxylin and Eosin (H&E)) or alternatively using label-free simulated Raman histology (SRH).

Note: For H&E staining and immunohistochemistry, tissue sections are embedded in O.C.T. (Tissue-TEK, SA62550-01/-02) and re-sectioned using a cryotome (NX70, ThermoFischer Scientific) for a final thickness of 10 μm .

Note: For SRH imaging, no re-sectioning is required. An entire 300 μm tissue section is briefly (15 min) fixed in 4% paraformaldehyde (PFA) and subsequently washed in PBS. The section is then mounted on a specialized microscopy slide (NIO Slide, Invenio Imaging #PRT 01-0224). Imaging is performed using the NIO Laser Imaging System (Invenio Imaging) as described^{6,7} and tumor infiltration is assessed over the neocortical depth (Figure 1C).

△ CRITICAL: Sterile processing guidelines must be upheld during the entire protocol to ensure tissue viability, avoiding any/all sources of contamination. To ensure proper tissue processing, it is necessary to clean the vibratome with 100% alcohol and wash all spatulas and scalpels with Incidin™. The vibratome can be placed outside the flowhood during this process. And lastly, to prevent any potential contamination, it is mandatory to wear both lab coats and face masks. Additionally, preparation and growth medium should be sterile filtered.

Primary Glioblastoma cell inoculation, tumor growth, and monitoring

⌚ Timing: 8–9 Days

This section describes the isolation of tumor cells from our human organotypic cortical glioblastoma (GBM) model for single-cell transcriptional profiling.

Note: It is necessary to ensure the viability of the cultured sections using electrophysiology or immunostaining protocols, during the culture period.²

2. Generate fluorescently labeled GBM cells.

Note: Here we use patient derived GBM cells. Cells were transduced with lentiviral particles (rLV.EF1.ZsGreen1-9 (0037VCT), Takarabio; Clontech). The concentration of viral particles required was calculated according to the manufacturer's instructions (M.O.I. = 10, 10^9 TU/mL).

- a. Seed 5×10^5 cells per well and incubated overnight in an incubator at 37°C and 5% CO₂.
 - b. Prepare the transduction mix by adding the required volume of thawed viral particles and Hexadimethrine bromide (Polybrene, 800 $\mu\text{g}/\text{mL}$).
 - c. Refresh culture medium after 24 h.
 - d. Assess the quality of transduction after 48 h using microscopy.
3. Inoculate 1 μL of GBM cells at the interface of gray and white matter using a 10 μL Hamilton syringe (80330; Hamilton Company, Bonaduz, Switzerland). Inoculate fluorescently tagged GBM cells into cultured sections.

Note: We suggest to carry out this step 48 h post tissue plating to allow the tissue to recover from the trauma of sectioning.

- a. Trypsinize GBM cells and dilute the suspension to a concentration of 20,000 cells/ μL .
- b. Inoculate 1 μL of GBM cells at the interface of gray and white matter using a 10 μL Hamilton syringe (80330; Hamilton Company, Bonaduz, Switzerland).

△ CRITICAL: Inoculation using the Hamilton micro syringe must be performed delicately to avoid damage to the tissue section.

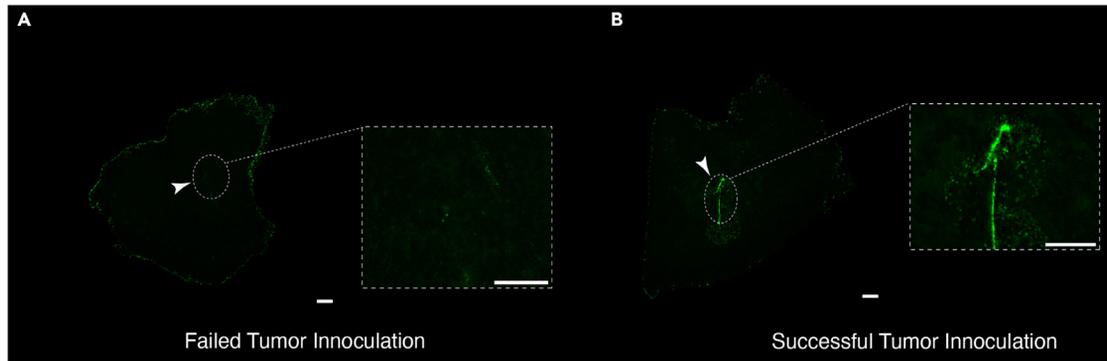


Figure 2. Representative images of tumor inoculation

The inoculation of tumor cells is checked under EVOS M7000 microscope.

(A and B) (A) Failed Tumor Inoculation and (B) Successful Tumor Inoculation in human cortical slice cultures. Scale bar: 0.02 inch.

△ **CRITICAL:** To prevent cross contamination, the Hamilton micro syringe must be washed using methanol, ethanol, and then PBS ~20 times, between inoculations.

Note: To obtain reliable and informative results when injecting primary tumor cells into a tissue section of 6mm-by-6mm dimension, it is essential to optimize the injection volume. Based on our experience, we recommend injecting no more than 1 μL (20,000 cells/ μL) of primary tumor cells to prevent compromising the viability of the cortical tissue sections. Injecting excessive cells can cause cell expansion and migration, leading to inaccuracies in the experimental results.

4. Rinse the sections with growth medium immediately after GBM inoculation.

△ **CRITICAL:** This step is essential to remove surface tumor cells outside the inoculation spot, to ensure that the tumor grows in an infiltrative fashion and not on the surface of the tissue sections.

△ **CRITICAL:** Hamilton syringe must be checked on a regular basis for potential clog to avoid failed inoculations (as shown in Figure 2A).

Note: If any clog is noticed in the Hamilton syringe. It is advisable to flush the syringe with 100% ethanol.

5. Monitor the tumor growth every 24 h, using a humidified and temperature-controlled incubating microscopic platform (EVOS M7000 with onstage incubator, Thermo Fisher Scientific, USA).

Note: Culture conditions are maintained as before GBM inoculations.

△ **CRITICAL:** It is recommended to preserve a few sections of interest by fixing them in 4% paraformaldehyde (PFA) overnight. This step will enable effective fluorescence staining of the tumor microenvironment, providing valuable insights into cellular behavior (as shown in Figure 3F).

Tissue preparation for FACS sorting of viable single cells

⌚ Timing: 2–3 h

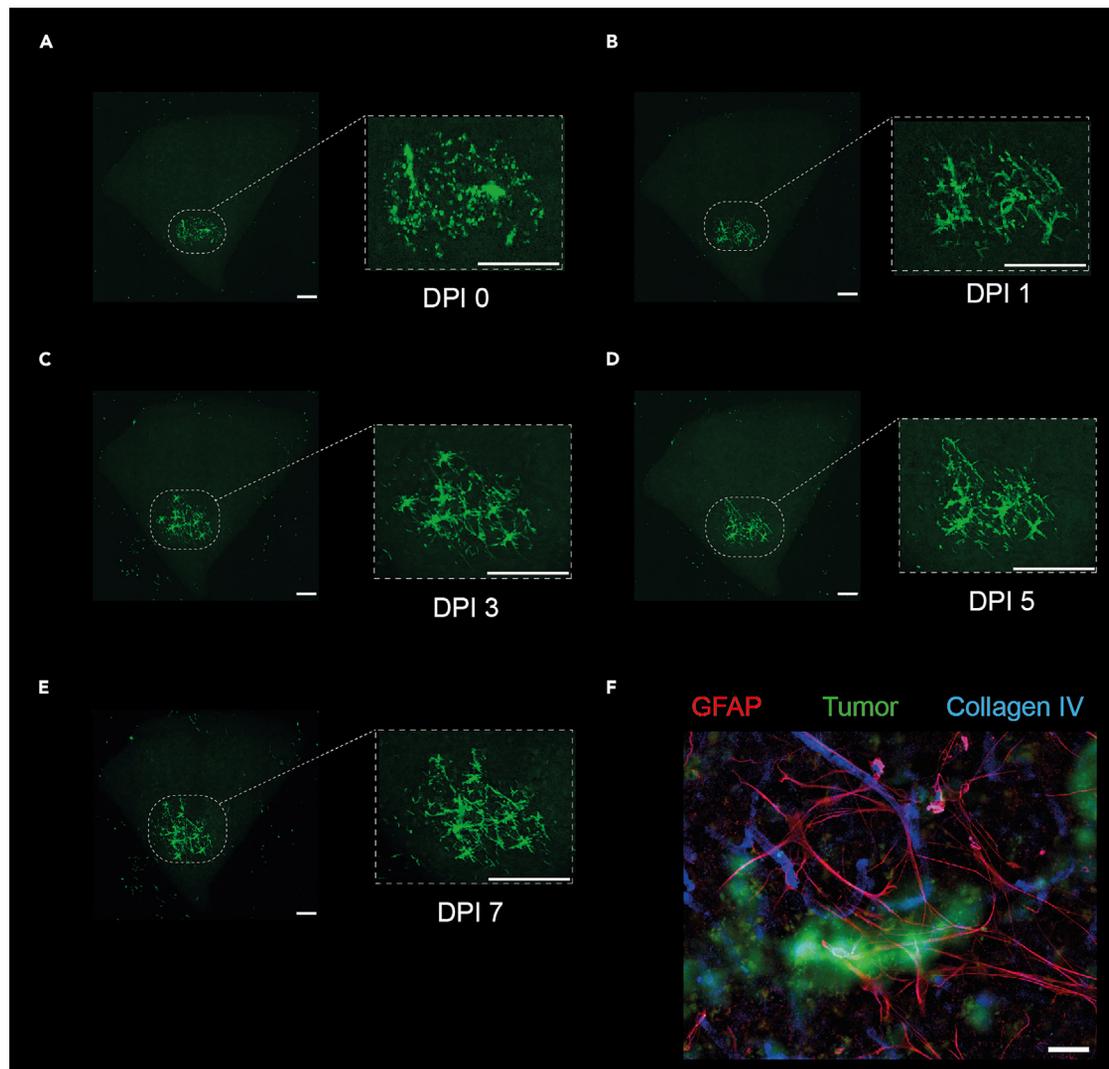


Figure 3. Inoculated GBM growth pattern live monitored using EVOS microscope

(A–E) Representative live imaging of GBM inoculated cortical tissue at 0,1,3,5,7 days post inoculation (DPI). Scale bar: 0.02 inch.

(F) Immunostaining showing tumor cells interacting with the microenvironment: Tumor cells are tagged with ZsGreen, GFAP was used to mark Astrocytes, DAPI was used to mark nucleus. Scale bar: 50 μm .

This section describes the methods including tissue dissociation, single-cell suspension and FACS sorting from cortical sections for single-cell sequencing.

Note: A minimum 3 of 300 μm thick tissue sections are required for obtaining sufficient viable cells to proceed with 10 \times Chromium Single Cell Library Preparation protocol.

6. Tissue dissociation (using Neural Tissue Dissociation Kit) and single-cell suspension.
 - a. Prepare the enzyme mix 1 (200 μL Enzyme T in 1750 μL Buffer X for every 500 mg of tissue).
 - b. Prepare the enzyme mix 2 (10 μL Enzyme A in 20 μL Buffer Y for every 500 mg of tissue).
 - c. Transfer tissue into C-tube, add enzyme mix 1 and incubate at 37°C for 5 min.
 - d. Dissociate the sample within the C-tube for 2 min at 37°C under slow and continuous rotation.
 - e. Add enzyme mix 2, incubate at 37°C for 5 min.
 - f. Repeat the dissociation step as described in step 6 d.
 - g. Centrifuge sample for 1 min at 350 g, 4°C.

- h. Remove supernatant and resuspend pellet in 9 mL growth medium.
 - i. Prepare 50 mL falcon tube with a 100 μm strainer at the opening. Apply 1 mL growth medium to moisten the strainer.
 - j. Filter the resuspended pellet solution from step 6 h bit by bit through the strainer tube setup from step 6 i.
 - k. Collect and transfer the sample into a 15 mL falcon tube.
7. FACS preparation and DAPI staining.
- a. Centrifuge the suspension in a 15 mL falcon tube at 300 g and 4°C for 10 min.
 - b. Aspirate the supernatant leaving approximately 100 μL in the falcon and resuspend the pellet gently.
 - c. Add the resuspended sample to a FACS tube filled with FACS buffer, and centrifuge at 300g and 4°C for 5 min.
 - d. Repeat steps 7 b and 7 c to wash the samples, aspirate the supernatant and keep approximately 100 μL . Simultaneously, prepare FACS incubation buffer with DAPI (DFIB) by diluting DAPI into FACS buffer for a final concentration of 1 $\mu\text{g}/\text{mL}$.
 - e. Add 450 μL DFIB mix from step 7 d into the washed sample and resuspend gently. This step needs to be carried out on ice, avoiding direct light.
 - f. Collect 70,000 cells that are DAPI⁻ and ZsGreen⁺ by collecting two sets of 35,000 cells each into pre-coated Eppendorf tubes.

Note: To ensure optimal collection efficiency and cell viability, pre-coat the Eppendorf tubes with 4% BSA overnight at 4°C, and fill each tube with 100 μL of FACS collection buffer before use.

8. Single-cell barcoding preparation.
- a. Measure and confirm one set of collected samples with a 1000 μL pipette, and further filter with 70 μm FlowMi Cell Strainers.
 - b. Centrifuge the filtered sample at 300 g and 4°C for 10 min. Aspirate the supernatant, leaving a final volume of 45 μL .

△ CRITICAL: The viability of the sorted cells is determined by microscopic observation. The second collection tube of 35,000 cells per condition is used to check the viability of cells. Resuspend the pellet and use 10 μL of collected cell suspension for a 1:10 dilution of in total 100 μL solution. Mix 10 μL of diluted GBM cells with 10 μL 0.4% Trypan Blue. After a 5 min incubation in room temperature, observe under the microscope count live/dead cells and calculate the viability.

- c. Resuspend the pellet from step 8 b in the 45 μL supernatant. Use 43.2 μL to proceed 10 \times Genomics Single Cell Library Preparation protocol (CG000204 Rev D) and Illumina Sequencing protocol (# 15048776 v09).

Single-cell library construction and sequencing

⌚ Timing: 8–9 h

In this section, we briefly describe the single-cell library preparation and sequencing procedure. Detailed step-by-step protocol could be found from 10 \times Genomics (CG000204 Rev D) and Illumina Inc. (# 15048776 v09).

9. GEM generation and barcoding.
- a. Prepare Master Mix on ice.
 - b. Assemble Chromium Next GEM Chip G.
 - c. Load Chromium Next GEM Chip G row labeled 1 with 70 μL Master Mix + cell suspension.
 - d. Load Chromium Next GEM Chip G row labeled 2 with 50 μL Gel Beads.

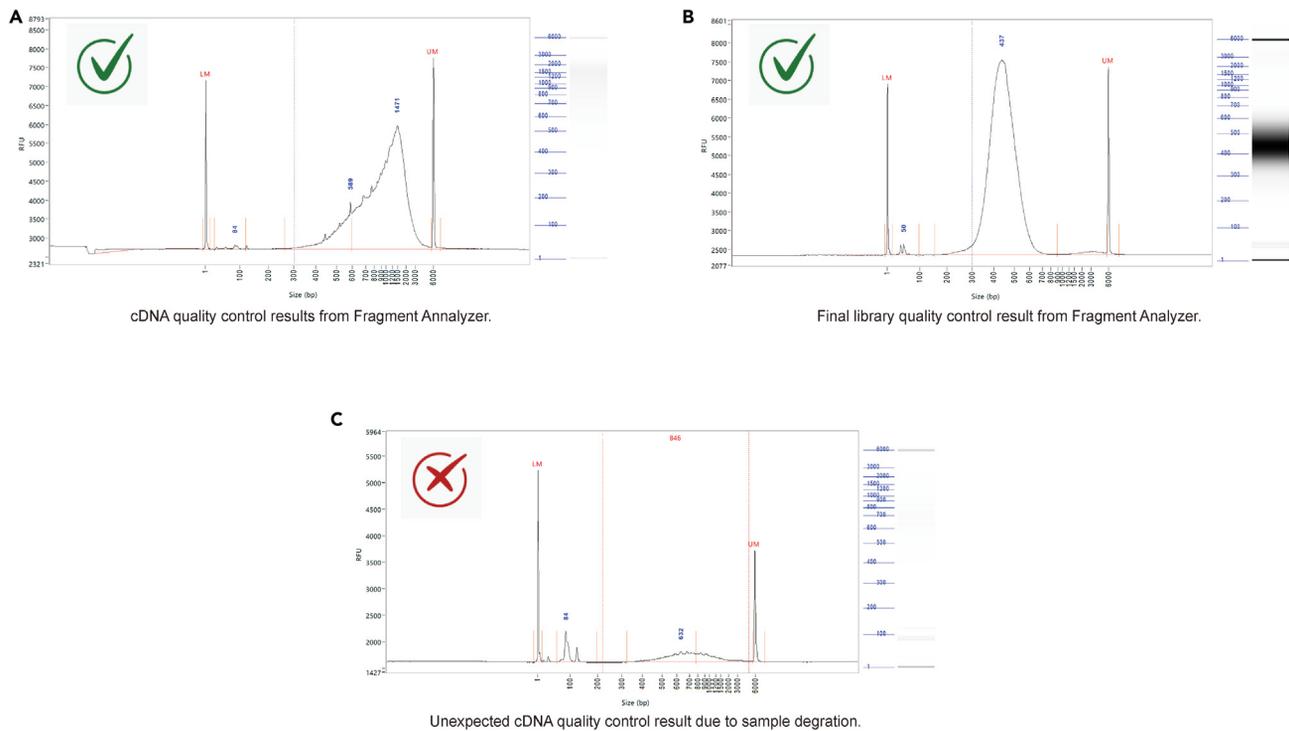


Figure 4. Representative library quality control results using Fragment Analyzer

(A–C) Representative cDNA library (A), final library (B) quality control performed using Fragment Analyzer. As well as low quality cDNA library (C) quality control result using Fragment Analyzer.

- e. Load Chromium Next GEM Chip G row labeled 3 with 45 μ L Partitioning Oil.
 - f. Run the Chromium Controller (about 18 min).
 - g. Immediately transfer GEMs and proceed GEM-RT Incubation.
Store at 4°C for up to 72 h or –20°C for up to 1 week, or proceed to the next step.
 - h. Add 125 μ L Recovery Agent without pipetting mix or vortexing.
 - i. Carefully discard Recovery Agent and Partitioning Oil from the bottom of the tubes.
10. Post GEM-RT Clean-up and cDNA Amplification.
- a. Prepare Dynabeads Cleanup Mix and proceed to cleanup.
 - b. Wash samples 2 times in 80% ethanol using a magnetic separator.
 - c. Prepare Elution Solution I based on the protocol.
 - d. Immediately add Elution Solution I, incubate and transfer supernatant to new strip tubes using a magnetic separator.
 - e. Prepare cDNA Amplification Mix, mix with samples.
 - f. Incubate in a thermal cycler following 10 \times Genomics protocol for cDNA Amplification.
Store at 4°C for up to 72 h or –20°C for up to 1 week, or proceed to the next step.
 - g. Add SPRIselect and incubate for a cDNA cleanup.
 - h. Remove supernatant after magnetic separation and wash 2 times with 80% ethanol.
 - i. Resuspend beads in Buffer EB, incubate and magnetic separate again.
 - j. Collect and transfer 40 μ L samples to a new tube strip.
Store at 4°C for up to 72 h or –20°C for up to 4 weeks, or proceed to the next step.
 - k. Run 1 μ L of sample (Dilution Factor 1:10) on Fragment Analyzer with High Sensitivity NGS (1–6000 bp) kits for cDNA quality control.
11. Gene expression library construction.
- a. Prepare a thermal cycler and Fragmentation Mix.
 - b. Transfer 10 μ L cDNA samples and proceed the following part of protocol.
 - c. Add Buffer EB and Fragmentation Mix to sample strip, pipette mix.

- d. Transfer samples into the pre-cooled thermal cycler and proceed incubation.
- e. Apply SPRIselect (0.6×), incubate, magnetic separate and collect supernatant.
- f. Apply SPRIselect (0.8×), incubate, magnetic separate and collect beads.
- g. Wash 2 times with 80% ethanol, resuspend in Buffer EB and collect 50 μL supernatant using a magnetic separator.
- h. Prepare Adaptor Ligation Mix and well mix with samples.
- i. Set up a thermal cycler and proceed incubation following 10× Genomics protocol.
- j. Apply SPRIselect (0.8×), incubate, magnetic separate and collect beads.
- k. Wash 2 times with 80% ethanol, resuspend in Buffer EB and collect 30 μL supernatant using a magnetic separator.
- l. Prepare Sample Index PCR Mix and add the mix to sample tubes.
- m. Add individual sample index (i7 Multiplex Index, Plate SI-GA) to tubes and pipette mix.
- n. Incubate in a thermal cycler with proper cycle numbers based on cDNA quality control results from step 10 k.
Store at 4°C for up to 72 h, or proceed to the next step.
- o. Apply SPRIselect (0.6×), incubate, magnetic separate and collect supernatant.
- p. Apply SPRIselect (0.8×), incubate, magnetic separate and collect beads.
- q. Wash 2 times with 80% ethanol, resuspend in Buffer EB and collect 35 μL supernatant using a magnetic separator.
Store at 4°C for up to 72 h or –20°C for long-term storage.
- r. Run 1 μL of sample (Dilution Factor 1:10) on Fragment Analyzer with High Sensitivity NGS (1–6000 bp) kits for final library quality control.

Note: A quality control is required by the end of step 10 (Figure 4A) and step 11 (Figure 4B) to continue the sequencing procedure (Refer also to 10× protocol). A low-quality cDNA library (Figure 4C) is not recommended to proceed with further library construction (Starting from step 11).

12. Library normalization, denaturation, dilution, and sequencing.
 - a. Normalize each single library to a “starting concentration” and combine single libraries to a library mixture.
 - b. Denature combined library with a corresponding amount (see the table from Illumina protocol) of 0.2 N NaOH and 200 mM Tris-HCl pH 7.
 - c. Dilute the denatured library to the final concentration with HT1.

△ CRITICAL: The entire library construction is recommended to proceed on ice as possible to minimize the degradation of samples.

Note: A Phix control for the final sequencing library could be planned and included if needed.

- d. Load the samples in cartridges and run Illumina NextSeq 550 with the following cycle numbers (Read 1 – i7 – i5 – Read 2: 28–8 – 0–56 cycles).
- e. Following sequencing, the Cell Ranger analysis pipeline (Version 7.1.0, Manufacturer) will align reads and count barcodes and UMIs to map transcripts in single cells.
- f. The primary output of Cell Ranger is a count matrix, consisting of columns of every cell barcode and rows for all measured features, such as genes for transcriptome analysis.
- g. The final files were exported to R for further analysis and visualization using standard pipelines.

EXPECTED OUTCOMES

This protocol will provide a comprehensive single-cell RNA sequencing data set from the isolated glioblastoma cells. Given the expected yield of approximately 35,000 to 70,000 single cells from three 300 micron tissue sections, we anticipate generating a robust and detailed map of the transcriptomic landscape of these cells.

In addition, the negative flow-through collection should yield a rich diversity of non-glioblastoma cell types including neurons, oligodendrocytes, oligodendrocyte progenitor cells, astrocytes, microglia, etc., This will enable the study of the brain's microenvironment and the interactions between glioblastoma cells and other cell types, offering new insights into disease pathogenesis and possible therapeutic targets.

When this protocol is adapted for the enrichment of specific cell types, it should provide reliable and reproducible outcomes regarding the selected target cell population. The resultant RNA sequencing data will enhance our understanding of the individual cell types and their unique roles within the human ex-vivo cortical cultures. This will empower researchers to investigate the roles and interactions of specific cell types in more detail, and potentially unveil previously unrecognized cell states or functions. Details of scRNA-seq analysis can be found in our recent publication Ravi, et al., 2022 *Cancer Cell*.¹

LIMITATIONS

Single-cell sequencing has emerged as a powerful tool to understand the complexity and heterogeneity of biological systems. However, several challenges and limitations are associated with this approach, particularly when studying human ex-vivo cortical cultures. Obtaining viable single cells from such cultures can be challenging due to factors such as tissue quality, time constraints, and technical variability. Moreover, single-cell sequencing protocols may result in the loss of certain cell types or a decrease in the number of cells due to several filtering steps during the procedure. The complexity and heterogeneity of human ex-vivo cortical cultures pose additional challenges, including the identification and isolation of specific cell populations for single-cell sequencing. Furthermore, the variability in the culture conditions, the genetic background of the donors, can affect the accuracy and reproducibility of the single-cell sequencing data.

Therefore, careful considerations and quality controls are required when designing single-cell sequencing experiments to obtain meaningful and reproducible results from human ex-vivo cortical cultures.

TROUBLESHOOTING

Problem 1

Unequal slice thickness during sectioning or blocks detaching from vibratome plate (Related to step 1 b).

Potential solution

- Make sure tissue blocks are correctly glued onto the preparation table.
- Remove blood clots from tissue blocks before gluing them onto the vibratome plate.
- Remove the arachnoid membrane and blood vessels from the pial surface of the block.
- Rather detach and re-mount a block than try to section a sub optimally attached tissue block.
- Consider changing cutting direction from perpendicular to orthogonal to the pial surface.

Problem 2

Reduced viability of ex-vivo human cortical cultures (Related to step 1 e).

Potential solution

- Ensuring proper hydration of human ex-vivo cortical sections is a critical consideration for successful culture. To this end, it is recommended to add 30 μ L of growth medium on top of the sections during medium changes to prevent tissue desiccation.
- We recommend checking for bacterial or fungal contamination in the incubator. Use sterile solutions and equipment, work in a laminar flow hood, and perform rigorous cleaning and disinfection of the work area and equipment.

- Make sure that the sections are not thicker than 300 μm as it will significantly affect the viability of the cultures.

Problem 3

Drifting of tissue samples during live cell imaging (Related to step 5).

Potential solution

- Optimizing live tissue imaging of tissue sections in 3D culture experiments requires careful attention to the liquid environment. Specifically, we recommend adding no more than 30 μL of medium on top of the section and limiting the medium below the insert to 1.2 mL. Excess liquid can cause section drift, compromising image registration with the previous time point and undermining the quality of live tissue imaging.

Problem 4

Low output of ZsGreen tagged GBM cells from FACS Sorting (Related to step 7 f).

Potential solution

- Inappropriate working temperature, and extended working times are significant contributing factors to low single-cell viability. Low single-cell viability, in turn, leads to a low output of ZsGreen tagged cells. To address this issue, it is recommended that users reduce the number of samples used per run. The optimal number of samples for each run is four. By reducing the number of samples, users can maintain the appropriate working temperature, and shorten the working time, which will result in higher single-cell viability.
- Sections with low tumor growth as determined by live cell imaging should be excluded from tissue sampling. This ensures that the resulting cell suspension will consist of viable and healthy cells, leading to a higher likelihood of successful downstream applications. Additionally, it is crucial to perform regular checks on the Hamilton syringe used for injection to ensure proper functionality. This quality control step is essential for obtaining reliable and reproducible results and will improve the overall success rate of the experiment.

Problem 5

Less viable tumor cells in the single-cell suspension (Related to step 7 b).

Potential solution

- Single-cell suspension has a time dependence for storage. Thus, the downstream processing must be done as soon as possible. In our experimental workflow, we never kept the suspension for more than two weeks at -80°C .

Problem 6

Low yield of genes or transcripts following sequencing (Related to step 12).

Potential solution

- Poor cell quality and mRNA degradation are the most common causes of low transcript recovery in scRNA-seq experiments. To minimize these issues, it is essential to use reagents and consumables that are free of nucleases. In addition, to maintain cell viability, it is crucial to process samples quickly and keep the cells on ice throughout the protocol, minimizing exposure to ambient temperatures.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Vidhya Madapusi Ravi (vidhya.ravi@uniklinik-freiburg.de).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate either specific, unique data sets or code. For detailed information about data sets and codes generated using the protocol described in this study, please refer to Ravi, et al. *Cancer Cell* 2022¹ or contact the authors.

ACKNOWLEDGMENTS

We express our gratitude to the patients who generously participated in this study, as well as Jonathan Göldner for all the cell culture work and assistance of bio-banking. This work was supported in part by the Freiburg Institute for Advanced Studies (FRIAS) for V.M.R. and Else Kroner-Fresenius Foundation for D.H.H. as well as by MEPHISTO project BMBF (German Ministry of Education and Research, project number 031L0260B, D.H.H. and D.D.).

We thank 10X Genomics and Dietmar Pfeifer for helpful advice. We thank [Biorender.com](https://biorender.com).

AUTHOR CONTRIBUTIONS

V.M.R. designed and optimized the protocol. J.Z. and K.J. carried out slice culture experiments. A.V., J.S., and N.N. carried out Raman experiments. J.B., O.S., N.N., M.P., S.B., C.F., J.S., and D.H.H. formed surgical and neuropathological teams. V.M.R., K.J., and J.Z. wrote the manuscript. V.M.R., K.J., D.H.H., O.S., and J.B. reviewed the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

1. Ravi, V.M., Will, P., Kueckelhaus, J., Sun, N., Joseph, K., Salié, H., Vollmer, L., Kuliesiute, U., von Ehr, J., Benotmane, J.K., et al. (2022). Spatially resolved multi-omics deciphers bidirectional tumor-host interdependence in glioblastoma. *Cancer Cell* 40, 639–655.e13.
2. Ravi, V.M., Joseph, K., Wurm, J., Behringer, S., Garrelfs, N., d'Errico, P., Naseri, Y., Franco, P., Meyer-Luehmann, M., Sankowski, R., et al. (2019). Human organotypic brain slice culture: a novel framework for environmental research in neuro-oncology. *Life Sci. Alliance* 2, e201900305.
3. Maier, J.P., Ravi, V.M., Kueckelhaus, J., Behringer, S.P., Garrelfs, N., Will, P., Sun, N., von Ehr, J., Goeldner, J.M., Pfeifer, D., et al. (2021). Inhibition of metabotropic glutamate receptor III facilitates sensitization to alkylating chemotherapeutics in glioblastoma. *Cell Death Dis.* 12, 723.
4. Ravi, V.M., Neidert, N., Will, P., Joseph, K., Maier, J.P., Kückelhaus, J., Vollmer, L., Goeldner, J.M., Behringer, S.P., Scherer, F., et al. (2022). T-cell dysfunction in the glioblastoma microenvironment is mediated by myeloid cells releasing interleukin-10. *Nat. Commun.* 13, 925.
5. Buehler, M., Yi, X., Ge, W., Blattmann, P., Rushing, E., Reifenberger, G., Felsberg, J., Yeh, C., Corn, J.E., Regli, L., et al. (2023). Quantitative proteomic landscapes of primary and recurrent glioblastoma reveal a protumorigenic role for FBXO2-dependent glioma-microenvironment interactions. *Neuro Oncol.* 25, 290–302.
6. Neidert, N., Straehle, J., Erny, D., Sacalean, V., El Rahal, A., Steybe, D., Schmelzeisen, R., Vlachos, A., Reinacher, P.C., Coenen, V.A., et al. (2022). Stimulated Raman histology in the neurosurgical workflow of a major European neurosurgical center - part A. *Neurosurg. Rev.* 45, 1731–1739.
7. Straehle, J., Erny, D., Neidert, N., Heiland, D.H., El Rahal, A., Sacalean, V., Steybe, D., Schmelzeisen, R., Vlachos, A., Mizaikoff, B., et al. (2022). Neuropathological interpretation of stimulated Raman histology images of brain and spine tumors: part B. *Neurosurg. Rev.* 45, 1721–1729.