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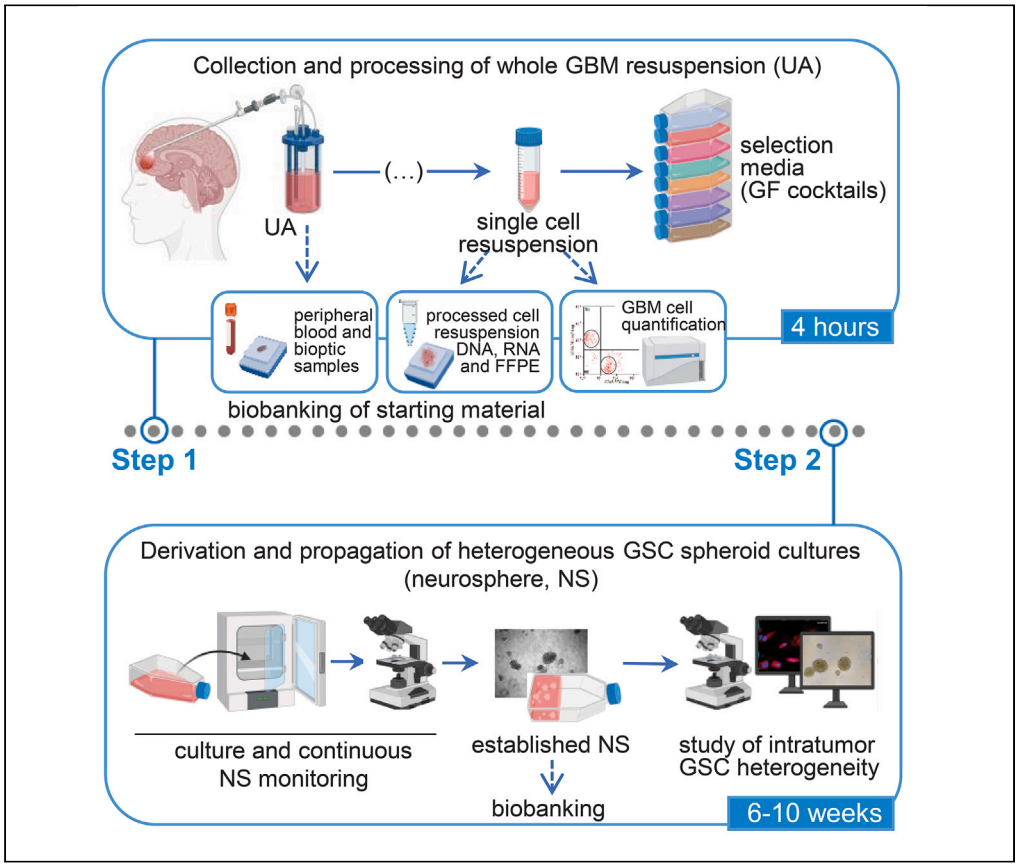
Protocol for *in vitro* establishment of heterogeneous stem-like cultures derived from whole human glioblastoma tumors



Cultures enriched in glioblastoma stem-like cells (GSCs) are prominent *in vitro* models to investigate molecular determinants and therapeutic targets of glioblastoma; however, conventional GSC derivation protocols fail to preserve GSC heterogeneity. Here, we present a protocol for the propagation of heterogeneous GSC cultures starting from cell resuspensions containing the entire tumor mass. We describe steps for isolation of GSCs and their maintenance and expansion in culture. We then detail procedures for preliminary analysis to be performed on freshly isolated material.

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

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Highlights

Processing of cell resuspensions containing the whole GBM tumor mass

Parallel application of different selective pressures (growth factor cocktails)

Monitoring of GBM stem cell (GSC) selection and propagation as neurospheres

Generation of distinct neurospheres to study intratumor GBM heterogeneity

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Protocol for *in vitro* establishment of heterogeneous stem-like cultures derived from whole human glioblastoma tumors

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SUMMARY

Cultures enriched in glioblastoma stem-like cells (GSCs) are prominent *in vitro* models to investigate molecular determinants and therapeutic targets of glioblastoma; however, conventional GSC derivation protocols fail to preserve GSC heterogeneity. Here, we present a protocol for the propagation of heterogeneous GSC cultures starting from cell resuspensions containing the entire tumor mass. We describe steps for isolation of GSCs and their maintenance and expansion in culture. We then detail procedures for preliminary analysis to be performed on freshly isolated material.

For complete details on the use and execution of this protocol, please refer to De Bacco et al.[1]

BEFORE YOU BEGIN

Glioblastoma stem cells (GSCs) have been successfully propagated as floating neurospheres or adherent cultures using protocols initially established for isolating neural stem cells.[2–8] GSCs have proven to be valuable for gaining a better understanding of the pathogenetic mechanisms and therapeutic vulnerabilities of glioblastoma (GBM).[9] Developing therapies that specifically target GSCs is recognized as one of the most significant challenges in current GBM drug discovery.[10,11] In recent years, it emerged that GBMs exhibit genetic and phenotypic heterogeneity at the single-cell level. This implies that GSCs should recapitulate such heterogeneity, and that GSC heterogeneity should be considered when developing targeted drugs.[12–14] However, conventional derivation protocols primarily yield genetically and phenotypically homogeneous GSC cultures (in particular in adherent conditions) from individual GBMs.[1,8] To address this limitation, we aimed to establish a protocol for deriving GSCs that represent different subclones coexisting within the same GBMs. The protocol outlined here utilizes GBM cell suspensions, ideally containing the entire tumor mass, and employs various positive selective pressures represented by different GFs for GSC culture establishment. The protocol describes essential steps for isolating GSCs, expanding their numbers, and establishing stable cultures as floating neurospheres. By this protocol, from each individual GBM, a family of potentially heterogeneous GSCs (each defined hereby as a 'family member') is derived.

1. Protocol should be performed under sterile conditions and by using pre-sterilized materials.
2. A class II Biological Safety Cabinet is essential to ensure sterility of experimental samples and to protect investigators from risks related to manipulation of biological materials.

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3. Tissue culture incubator adjusted at 37°C and 5% CO_2 is essential.
4. Conventional equipment for cell culture (vacuum system, pipettes and electronic pipettor) are required.
5. Before starting each one of the steps, investigators should prepare the solution specified in the "materials and equipment" paragraph and appropriately prepare Class II Biological Safety Cabinet.

Institutional permissions

Human glioblastomas used in this protocol were obtained in accordance with a study approved by the Ethical Committees of the Candiolo Cancer Institute (Candiolo, Italy) and Città della Salute e della Scienza (Torino, Italy). Informed written consent was obtained from all patients and studies were conducted according to the Declaration of Helsinki. All patient data and samples were de-identified before processing.

Patient de-identification, material collection, shipment and conservation

Timing: N/A

For generating a biobank of heterogeneous GSCs, coordination with the neurosurgery, neuro-oncology and pathological anatomy departments is essential for ensuring proper collection of both ultrasonic aspirates and related material [peripheral blood and formalin-fixed paraffin-embedded (FFPE) bioptic samples].

6. In agreement with the involved clinical departments, organize a patient de-identification system associating a unique number to patient's medical records, ultrasonic aspirate (UA) and all related materials.
7. Ultrasonic tumor removal is performed by neurosurgeons in the operating theater, and provides a resuspension of small tissue fragments and dissociated cells including most of the tumor. This material is collected in an appropriate receptal of surgical instrumentation.

⚠ CRITICAL: for optimal cell viability preservation, time between neurosurgical GBM collection and laboratory processing should be kept to the minimum. After collection, UA receptals can be shipped in a closed container on wet ice and, once received by the lab, stored at +4°C. GSC derivation efficiency decreases with time, starting to significantly decline 24 h after neurosurgery and becoming unfeasible after 48 h.

8. Collect 5–10 mL of peripheral blood before surgery in a blood collection tube containing K_2 EDTA at the concentration of 1.8 mg/mL.

Note: peripheral blood is required for collecting normal DNA from Peripheral Blood Mononuclear Cells (PBMCs) to be used for further analysis of tumoral DNA (see step-by-step method details, step 60). If possible, collection after surgery is not recommended as to avoid collection of tumoral DNA released in the peripheral system during surgery.

9. Collect, formalin fix and embed in paraffin a small intact fragment of tumor for further molecular and histopathological characterizations.

Note: tumor fragments obtained by conventional single biopsies likely do not capture GBM heterogeneity; therefore, parallel embedding of tumor fragments from UA is recommended (see step-by-step method details, step 58).

Class II biological safety cabinet preparation

Timing: 10–15 min

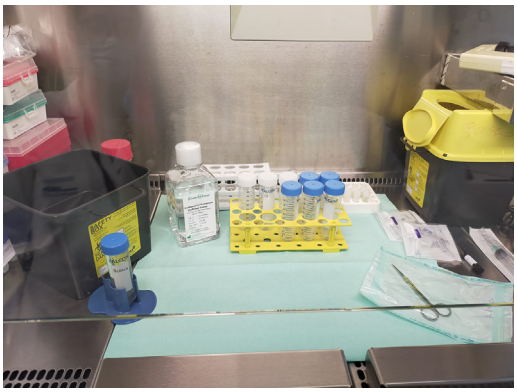

Figure 1. Biological safety cabinet
Biological safety cabinet with material required for UA processing.

10. Carefully prepare safety cabinet with the following materials before starting UA processing (Figure 1).
    a. disposable gown.
    b. disposable nitrile gloves (better to wear double glove pairs during the entire procedure).
    c. sterile cloth covering Cabinet surface (this is important to rapidly clean the Cabinet in case of accidental material leakage).
    d. conical tube containing 50 mL pure bleach for cleaning of vacuum system and instruments.
    e. UA receptal container.
    f. 50 mL conical tubes for material recovery from UA receptal.
    g. rack for 50 mL conical tubes.
    h. disposable pipettes (5–25 mL).
    i. 1 mL syringes.
    j. 18 G needles.
    k. 100 μm cell strainer.
    l. 70 μm cell strainer.
    m. 10 cm cell culture petri dish.
    n. 75 cm^2 ultra-low attachment flasks.
    o. 1.5 mL RNase-free Eppendorf tubes (for freezing material for DNA and RNA extraction).
    p. Cryogenic vials for live cells.
    q. Bio-cassettes with foam pads.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-CD45 PE-CF594 (HI30) antibodies (1:20 dilution)	BD Biosciences	cat#562312, RRID: AB_11154590
Anti-CD56 PE-Cy7 (N901) antibodies (1:20 dilution)	Beckman Coulter	cat#A21692, RRID: AB_2892144
Biological samples		
Human primary GBM sample, male, 67 years old	De Bacco et al.[1]	#GBM151
Human primary GBM sample, female, 62 years old	De Bacco et al.[1]	#GBM017
Chemicals, peptides, and recombinant proteins		
Bleach solution	Sigma-Aldrich	cat#1.05614
DMEM/F12 (1:1 ratio)	Sigma-Aldrich	cat#E1257
L-glutamine (200 mM)	Euroclone	cat#ECB3000D
B27 Plus supplement	Thermo Fisher Scientific	cat#17504044
Heparin (50 mg/mL)	Sigma-Aldrich	cat#H3149
BSA (35%)	Sigma-Aldrich	cat#A7979

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
EGF	Sigma-Aldrich	cat#E1257
FGF2	PeproTech	cat#100-18B
PDGFBB	PeproTech	cat#100-14B
HGF	PeproTech	cat#100-39H
Penicillin/Streptomycin/Fungizone	Sigma-Aldrich	cat#A5955
Collagenase type I (10 mg/mL)	Sigma-Aldrich	cat#C0130
NH_4Cl (MW: 53.49 g/mol)	Sigma-Aldrich	cat#1011430050
$KHCO_3$ (MW: 100.12 g/mol)	Sigma-Aldrich	cat#237205
Na_2EDTA (MW: 372.24 g/mol)	Sigma-Aldrich	cat#E4884
Sterile PBS (phosphate-buffered saline), pH 7.4	Euroclone	cat#ECB400L
Formalin	Sigma-Aldrich	cat#HT501128
DAPI	Roche	cat#10236276001
Trypan blue stain	Thermo Fisher Scientific	cat#15250061
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich	cat#4540
DNase I	Roche	cat#03724778103
Critical commercial assays		
Debris removal solution	Miltenyi Biotec	cat#130-109-398
VenorGeM Classic Mycoplasma Detection Kit, PCR-based	Voden	cat#11-1250
ReliaPrep gDNA Tissue Miniprep System	Promega	cat#A2050
Maxwell RSC miRNA Tissue Kit	Promega	cat#AS1460
PureLink Genomic DNA Mini Kit	Invitrogen	cat#K1820-00
Software and algorithms		
CytExpert software 2.4	Beckman Coulter	https://cytexpert.software.informer.com/2.4/
Kaluza Flow Analysis Software 1.3	Beckman Coulter	cat#A82959
Other		
K_2 EDTA blood collection tubes	Thermo Fisher Scientific	cat#10331254
Disposable gown	Sigma-Aldrich	cat#Z559008
Disposable nitrile gloves	Sigma-Aldrich	N/A
Sterile cloth	Essity	cat#190578
50 mL conical tubes	Corning	cat#352070
15 mL conical tubes	Corning	cat#352096
Disposable pipettes (5–25 mL)	Corning	N/A
5 mL syringes	Sigma-Aldrich	cat#Z116858
18 G needles	Sigma-Aldrich	cat#Z101141
10 cm cell culture Petri dish	Falcon	cat#353003
Falcon cell strainer, 70 μm	Corning	cat#352350
Falcon cell strainer, 100 μm	Corning	cat#352360
75 cm^2 ultra-low attachment flasks	Corning	cat#CLS3814
Falcon round-bottom polystyrene test tubes	Thermo Fisher Scientific	cat#14-959-5
Falcon round-bottom polystyrene test tubes with cell strainer snap cap, 5 mL	Thermo Fisher Scientific	cat#08-771-23
CellTrics 50 μm	Sysmex	cat#04-0042-2317
1.5 mL RNase-free Eppendorf tubes	Eppendorf	cat#0030108051
Cryogenic vials for live cells	Thermo Fisher Scientific	cat#5000-1020
Bio-cassettes	Bio-Optica	cat#07-7100
Biopsy pads blue (foam pads)	Bio-Optica	cat#07-7290

Alternatives: Chemical and regular laboratory reagents and plastic consumables can be obtained from other sources than those specified in the key resources table.

Note: B27 Plus supplement from Thermo Fisher Scientific is strongly recommended as critical reagent.

MATERIALS AND EQUIPMENT

ACK lysis solution

ACK lysis solution is required for red blood cells lysis for processing both ultrasonic aspirates and blood samples.

Reagent	Final concentration	Amount
NH_4Cl (MW: 53.49 *g*/mol)	150 mM	8.02 *g*
$KHCO_3$ (MW: 100.12 *g*/mol)	10 mM	1 *g*
Na_2EDTA (MW: 372.24 *g*/mol)	0.1 mM	37.2 *g*
ddH_2O	N/A	–
Total	–	**1000 mL**

Adjust pH to 7.2–7.4.

Filter the solution with 0.2 μM pore size membranes and store it at RT (+22°C–25°C) for up to 6 months.

Glioma stem cell (GSC) standard medium and growth factor supplementation

GSC standard medium is used as such or supplemented with different concentrations and combinations of growth factors (GFs), added immediately before use.

Suggested selective media are the following:

NO GF: No growth factor supplement.

E: Epidermal Growth Factor (EGF), 20 ng/mL.

F: Fibroblast Growth Factor 2 (FGF2), 20 ng/mL.

P: Platelet-derived Growth Factor BB (PDGFBB), 20 ng/mL.

H: Hepatocyte Growth Factor (HGF), 20 ng/mL.

EFPH2: EGF+FGF2+PDGF+HGF, each 2 ng/mL.

EFPH20: EGF+FGF2+PDGF+HGF, each 20 ng/mL.

Reagent	Final concentration	Amount
DMEM/F12 (1:1 ratio)	N/A	500 mL
L-glutamine (200 mM)	5 mM	5 mL
B27 Supplement (50X)	N/A	10 mL
Heparin (50 mg/mL)	4 μg/mL	40 μL
BSA (35%)	1%	5.75 mL
Total	–	**500 mL**

Filter the solution with 0.2 μM pore size membranes and store it at +4°C for up to 2 months.

Optional: Penicillin/Streptomycin/Fungizone solution (10 mL/L) can be added to GSC medium to prevent contamination.

Reagent	Final concentration	Amount
EGF	20 ng/mL (or 2 ng/mL)	–
FGF2	20 ng/mL (or 2 ng/mL)	–
HGF	20 ng/mL (or 2 ng/mL)	–
PDGFBB	20 ng/mL (or 2 ng/mL)	–

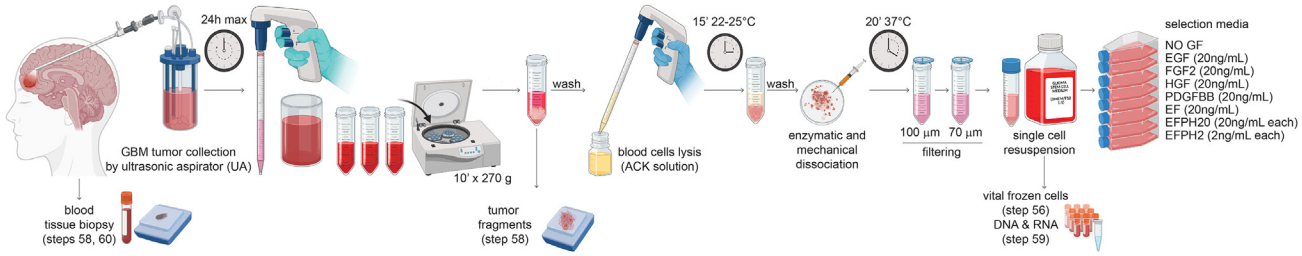

Figure 2. Overview of UA processing into multiple GSC cultures

Schematic showing key passages of GBM UA processing into single cell resuspension and preparation of multiple cell cultures with different selection media (single or GF cocktails).

⚠ CRITICAL: once added to the GSC Medium, GFs are stable for up to 2 weeks, after which they must be replenished at the original concentration.

Other required solutions

- Collagenase type I (10 mg/mL).

[store at −20°C for up to 12 months, prepare aliquots to avoid multiple freeze/thaw cycles].

- PBS 1X or other physiological saline solution.
- Formalin or other fixative solution.
- DAPI 0.2X solution (dilute 2 µL of 1000X DAPI in 10 mL of PBS 1X).

[store 1000X DAPI at −20°C for up to 12 months; fresh 0.2X DAPI should be prepared every time].

STEP-BY-STEP METHOD DETAILS

UA processing into GSC spheroid cultures (neurospheres)

Timing: 4 h

Starting from UA resuspension, whose reliability as a GSC source was previously shown,[15,16] the described method allows to obtain parallel cultures enriched in glioma stem cells (GSCs) preserving GBM heterogeneity by the use of different cocktails and concentration of GFs (Figure 2). If required, additional combinations can be assessed with the same methodology.

For biobanking, at the end of the described procedure part of cancer cell resuspension has to be collected (i) for histology, (ii) as vital frozen material, and (iii) for DNA and RNA extraction (see below, Figure 2).

1. Aliquot UA in the appropriate number of conical tubes (40 mL/tube), collecting the entire resuspension. See troubleshooting 1, 2.
2. Centrifuge for 10 min at 270 *g*, RT (+22°C–25°C).
3. Discard supernatant by vacuum aspiration.

Note: pellets may look different in distinct conical tubes, owing to uneven UA resuspension distribution. The best pellets are those containing considerable amount of tumor fragments in the intermediate ring (up to 10–15 mL mark) (Figure 3); ideally, 4 of such pellets are sufficient for the derivation process and for biobanking, thus the others can be discarded.

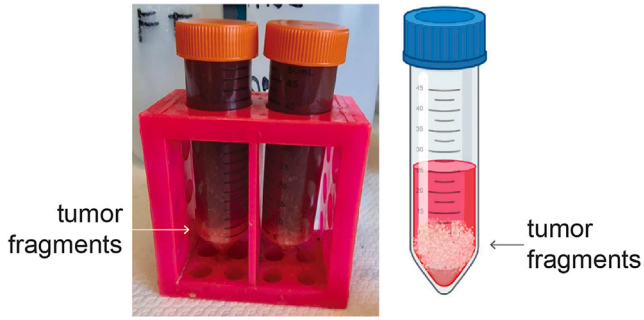

Figure 3. UA resuspension

Conical tube containing UA resuspension, tumor fragments and lysed blood cell bodies after centrifugation and before enzymatic and mechanical dissociation.

⚠ CRITICAL: if tumor fragments are to be collected also for histology, from this point one additional pellet is processed as described in step-by-step method details, step 58.

4. Wash pellets with 25 mL/tube PBS 1X.
5. Centrifuge for 10 min at 270 *g*, RT (+22°C–25°C).
6. Discard supernatant by vacuum aspiration.
7. Add 25 mL/tube ACK solution for blood red cell lysis.
8. Vigorously flip the tube 10–15 times or resuspend by pipetting with a 25 mL disposable pipette.
9. Incubate for 10–15 min at RT (+22°C–25°C) in the dark.
10. Centrifuge for 10 min at 270 *g*, RT (+22°C–25°C).
11. Discard supernatant by vacuum aspiration.

Note: if blood red cells are still detectable (as a residual red coloration of supernatant), ACK treatment can be repeated once.

12. Wash pellets with 15 mL/tube PBS 1X.
13. Centrifuge for 10 min at 270 *g*, RT (+22°C–25°C).
14. Discard supernatant by vacuum aspiration.
15. Pull together up to 4 pellets. Adding 5 mL PBS 1X to the first tube and sequentially transferring resuspension in the other tubes can be helpful.
16. Transfer pellets in 10 cm cell culture petri dishes. It is recommended to transfer 20 mL/dish.
17. Add 1 mL collagenase type I to 20 mL of total pellet to favor tumor tissue dissociation.
18. Incubate for 20 min at 37°C.
19. Add 3 mL PBS 1X to the dish.
20. In the dish, mechanically disrupt tissue fragments by 10–15 up and down pipetting with a sterile 5 mL pipette, then with an 18 G needle in a 1 mL syringe till the tumor resuspension turns velvety.

Note: mechanical disruption should proceed smoothly; if difficult, dishes can be incubated at +37°C for additional 10 min to prolong collagenase activity; however, long incubation can result in reduced cell viability.

21. Collect tumor resuspension with a syringe and filter it through a sterile 100 μm cell strainer, collecting the eluate in a 50 mL conical tube. See troubleshooting 3.

Note: in the case of high tumor resuspension density, it's suggested to further dilute with PBS 1X in order to facilitate the filtration step.

22. Repeat the above step, passing tumor suspension through a sterile 70 μm cell strainer.

23. To recover as many cells as possible, wash dish and strainers once with 5 mL PBS 1X. At this point the resuspension should mostly contain single, dissociated cells, together with abundant debris, as you can appreciate under the microscope.
24. Centrifuge for 10 min at 270 *g*, RT (+22°C–25°C).
25. Discard supernatant by vacuum aspiration.

Note: if blood red cells lysis is incomplete (evident as a residual red coloration of supernatant), ACK treatment can be repeated once as describe above.

26. Pool together and resuspend cell pellets by adding the appropriate volume of GSC Medium; around 1 mL (final volume) of cell resuspension is required for each cell culture condition planned (e.g., for testing conditions depicted in Figure 2, resuspend the pellet in 8 mL). See troubleshooting 2.

Note: for biobanking and nucleic acid collection (both highly recommended) cell pellets have to be resuspended in additional 0.5–1 mL of medium for each sample to be collected (e.g. 8 mL for cell culture + 3–4 mL for vital frozen cells + 1–2 mL to be aliquoted for DNA and RNA extraction, see below, step-by-step method details, step 59). Moreover, additional 1 mL of medium has to be used for estimating the amount of starting viable tumor cells (see below, step-by-step method details, steps 39–55). Pellet resuspension in a final volume up to 15 mL usually does not result in excessive cell dilution as to affect GSC derivation.

27. Transfer the cell resuspension in dedicated 75 cm^2 flasks, each containing 10–15 mL of GSC standard medium without GFs (1 mL/flask; for testing conditions depicted in Figure 2, 8 mL are dispensed in 8 flasks).
28. Transfer flasks in the incubator at 37°C, 5% CO_2 and 20% O_2 concentration for 24 h.
29. After 24 h, collect each flask suspension in a dedicated 15 mL conical tube (e.g., for testing conditions depicted in Figure 2, 8 different conical tubes are required).
30. Centrifuge for 10 min at 120 *g*, RT (+22°C–25°C).
31. Discard supernatant by vacuum aspiration.
32. Resuspend each pellet with 10 mL of GSC standard medium with the desired concentration and combination of GFs.
33. Transfer cell resuspension in 75 cm^2 flasks and maintain in the incubator at 37°C in 5% CO_2 and 20% O_2 concentration.

Pause point: the protocol can be paused at this point if the user opts for freezing cell resuspension as vital material (see below, step-by-step method details, step 56).

Derivation, growth and propagation of GSC spheroid cultures (neurospheres)

Timing: on average 6–10 weeks

In this step, cells are kept in culture and monitored for the emergence of established neurospheres, which are then propagated by passaging. Forming neurospheres (Figure 4) are grown by medium addition or replacement. When neurospheres reach around 100 μm diameter or when the spheroid' core appears dark (indicating the presence of dying cells), and when their amount increase significantly, neurospheres should be passaged by dissociation and splitting in separate flasks, maintaining the same GF cocktail.

Note: during the first weeks, cultures contain not only tumor cells but also tumor-associated immune cells and normal nervous tissue cells that will be negatively selected by culture conditions. Thus, it is recommended to monitor cell cultures daily by microscopic observation and to change medium when it turns milky or dying cells are noticed.

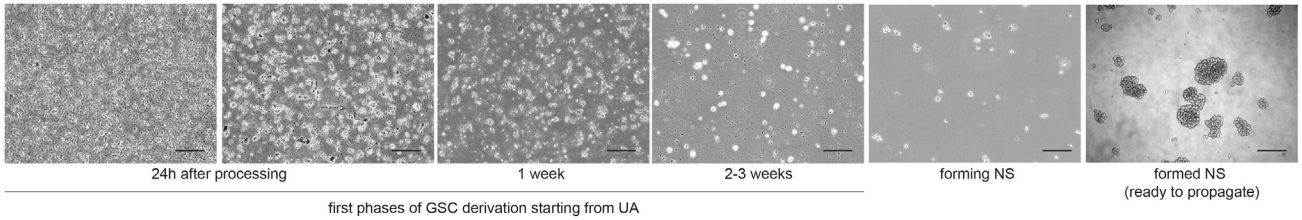

Figure 4. Neurosphere formation

Representative images of progressive neurosphere formation after cell seeding in selective media. Scale bar: 25 μm.

34. Once culture is free of non-tumor cells, the medium must be added or replaced at least once every 3–5 days depending on the acidification rate (shift in DMEM/F12 pH indicator) (Figure 5).

Note: formation of GSC floating spheroids (neurospheres), i.e. cell colonies derived from proliferation of a single GSC, is observed at variable times after cell culture start. If neurospheres do not reach passage 1 after 6 months, the culture is likely devoid of propagation potential and can be considered extinguished. Success rate, spheroid-formation timing, culture doubling time etc. depend on intrinsic GSC biological and genetic features (variable among different tumors) and extrinsic selective pressures (different GFs added to standard medium), and are therefore mostly unpredictable. Assiduous cell culture monitoring by microscopy is recommended.

35. For the addition of medium in growing neurospheres (Figure 5):
    a. Stand flasks upright in the sterile biological safety cabinet for 5 min as to allow forming spheroids to deposit at the flask bottom.
    b. Collect 3/10 mL of exhausted medium from the top by the use of a sterile 5 mL pipette.
    c. Add 3 mL of freshly prepared GSC Medium with GFs (at 3X concentration) avoiding pipetting.
    d. Transfer again flasks in the incubator at 37°C, 5% CO_2 and 20% O_2 concentration.
36. For renewal of medium in growing neurospheres (Figure 5). See troubleshooting 4:
    a. Collect cell resuspensions in 15 mL conical tubes (one tube/flask).
    b. Centrifuge for 10 min at 120 *g*, RT (+22°C–25°C).
    c. Discard supernatant by vacuum aspiration (it is suggested to leave around 0.5 mL of medium to avoid disturbing pellet).
    d. Carefully resuspend pellet in 10 mL of fresh GSC Medium with GFs. Carefully avoid repetitive and harsh pipetting to preserve integrity of forming spheroids.
    e. Transfer flasks in the incubator at 37°C, 5% CO_2 and 20% O_2 concentration.

⚠ CRITICAL: remember that GFs are stable at 37°C for no more than 2 weeks, thus it is absolutely required to change culture medium at least once every 10–15 days to maintain the applied selective pressure. On the other hand, avoid too frequent changes as to minimize manipulation of forming spheroids.

37. For neurosphere dissociation without passaging (Figure 6).
    a. Collect medium containing cells in 15 mL conical tubes (one tube/flask).
    b. Centrifuge for 10 min at 120 *g*, RT (+22°C–25°C).
    c. Discard supernatant by vacuum aspiration (it is suggested to leave around 0.2 mL of medium to avoid disturbing pellet).
    d. Mechanically disrupt spheroids by vigorous up and down pipetting for 100 times using a p200 pipette. Assess by eye the presence of residual spheroids and, in case, pipette for additional 50–100 times.

Optional: in case enzymatic methods are required for dissociating neurospheres, add 0.1 mL of Trypsin (or collagenase type I) v/v with PBS 1X (or pure in case of sticky spheroids), incubate

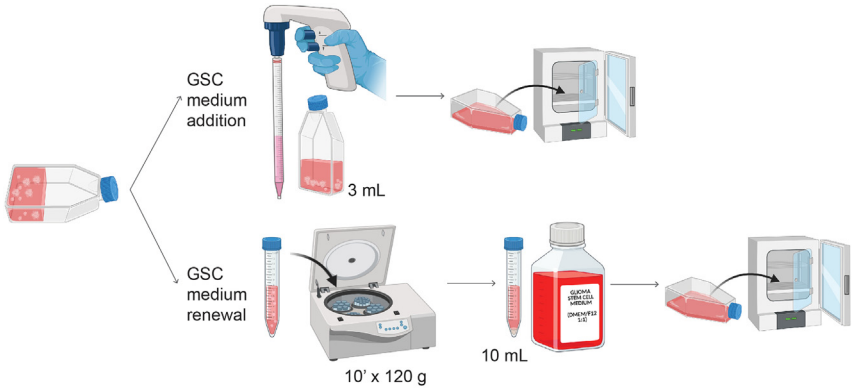

Figure 5. GSC selection and growth
Schematic procedure for medium addition and renewal in flasks containing forming GSC spheroids (neurospheres).

5 min at RT (+22°C–25°C) (or at 37°C in case of sticky spheroids), and mechanically disrupt spheroids by vigorous up and down pipetting for 100 times using a p200 pipette. Once dissociated, immediately wash with 5 mL PBS 1X, centrifuge for 10 min at 120 *g* and discard supernatant by vacuum aspiration. See troubleshooting 5.

e. Resuspend pellet in 10 mL of fresh GSC Medium with GFs.
f. Transfer flasks in the incubator at 37°C, 5% CO_2 and 20% O_2 concentration.

⚠ CRITICAL: As stated above, each culture displays different and unpredictable spheroid-formation times, so that careful evaluation of each culture is needed. Under passaging or over passaging spheroids increases cell death and slows down the proliferation rate.

38. For neurosphere passaging (Figure 6).
    a. Dissociate neurospheres as above.
    b. Perform cell counts (if possible also monitoring cell viability by using trypan blue or other vital staining). See troubleshooting 6, 7.
    c. Dispense 4–8 × 10^5 dissociated vital cells in 10 mL of fresh GSC standard medium with GFs.
    d. Track the number of passages (every 1:2 or higher split has to be considered as a +1 passage).
    e. Transfer flasks in the incubator at 37°C, 5% CO_2 and 20% O_2 concentration.

⚠ CRITICAL: it is suggested to freeze stocks at early passages as described below (see step-by-step method details, step 56); once formed, it is recommended to freeze non-dissociated spheroids by applying the same protocol. This will increase viability after thawing.

Note: periodical testing for mycoplasma contamination (see key resources table for suggested Mycoplasma detection kit) and cell identity is strictly recommended.

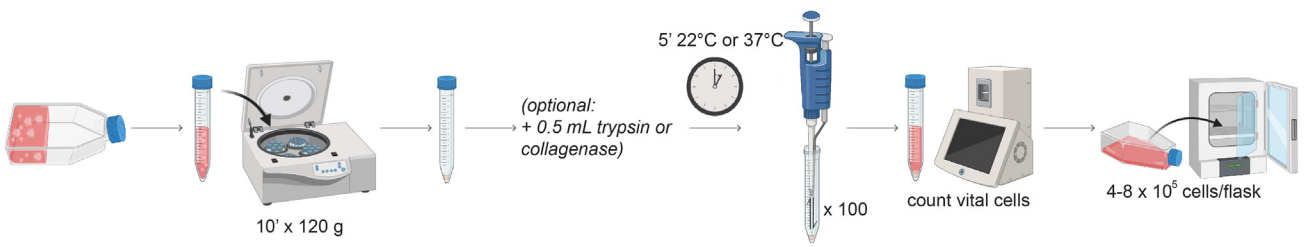

Figure 6. GSC propagation
Schematic procedure for neurosphere dissociation and splitting.

Assessment of the percentage of viable tumor cells in UA

Timing: 90 min

For further correlation analysis and for normalizing variant allele frequencies (VAF), gene copy number and transcriptomic data obtained in UA, it is recommended to estimate the percentage of viable tumor cells in freshly processed cell resuspensions by flow cytometry. CD56 (neural cell adhesion molecule 1, NCAM1) is suggested to identify tumor cells of neural or neuroendocrine origin,[1,17] while CD45 (protein tyrosine phosphatase receptor type C, PTPRC) is required to exclude blood derived leukocytes. GBM cells are thus identified as the $CD56^{pos}/CD45^{neg}$ subpopulation (Figure 7).

39. Transfer 1 mL of processed cell resuspension from step-by-step method details, step 26 in a 15 mL conical tube (to avoid pellet dispersion 50 mL tubes are not recommended).
40. Add 9 mL of PBS 1X to the cell resuspension.
41. Estimate total cell number by the use of an automated cell counter. See troubleshooting 6.
42. Centrifuge for 5 min at 150 *g*, RT (+22°C–25°C).
43. Discard supernatant by vacuum aspiration.
44. Resuspend the pellet at the optimal concentration of 2×10^6 cells/mL in PBS 1X with 1% BSA, in order to avoid non-specific antibody binding through Fc receptors.
45. Transfer 0.5 mL/tube of resuspended cells in four 5 mL polystyrene FACS tubes as follows (Figure 7A). See troubleshooting 8:
    a. 1 NEGATIVE CONTROL TUBE with unstained cells (no antibody will be added).

    Note: This tube is essential to (i) set voltages of the photomultiplier tube (PMT), or the gains of avalanche photodiodes (APDs); (ii) distinguish negative cells from electronic noise, allowing stained positive cells to be visualized; (iii) determine the level of autofluorescence; (iv) appropriately set negative gates.

    b. 1 COMPENSATION TUBE for single CD45 stained cells.
    c. 1 COMPENSATION TUBE for single CD56 stained cells.

    Note: These tubes are essential to reveal the spillover level of the fluorophore into the other detectors. Thus, a compensation matrix has to be applied to correct this effect and ensure that only specific signals are used.

    d. 1 SAMPLE TUBE with both CD45 and CD56 antibodies.
46. Add CD45 and/or CD56 antibodies to the tubes. Suggested titration for CD45 PE-CF594 and CD56 PE-Cy7 (see key resources table for details) is 1:20 (e.g., 25 μL of each antibody in 500 μL of PBS 1X-1% BSA and 10^6 cells).
47. Vortex tubes and incubate 20 min RT (+22°C–25°C), on a rocking platform, in the dark (required to avoid antibody bleaching).
48. Add 2 mL/tube of PBS 1X.
49. Centrifuge for 5 min at 150 *g*, RT (+22°C–25°C).

    Note: steps 48–49 are essential to minimize non-specific detection due to excess of antibodies.

50. Discard supernatant by decanting.
51. Resuspend all pellets in 300 μL of DAPI 0.2X solution for staining viable population ($DAPI^{neg}$).
52. Filter samples in Falcon Round-Bottom Polystyrene Test Tubes with Cell Strainer Snap Cap, 5 mL (or alternatively by CellTrics 50 μm).

Note: Assistance by an expert flow cytometrist may be required in the following steps.

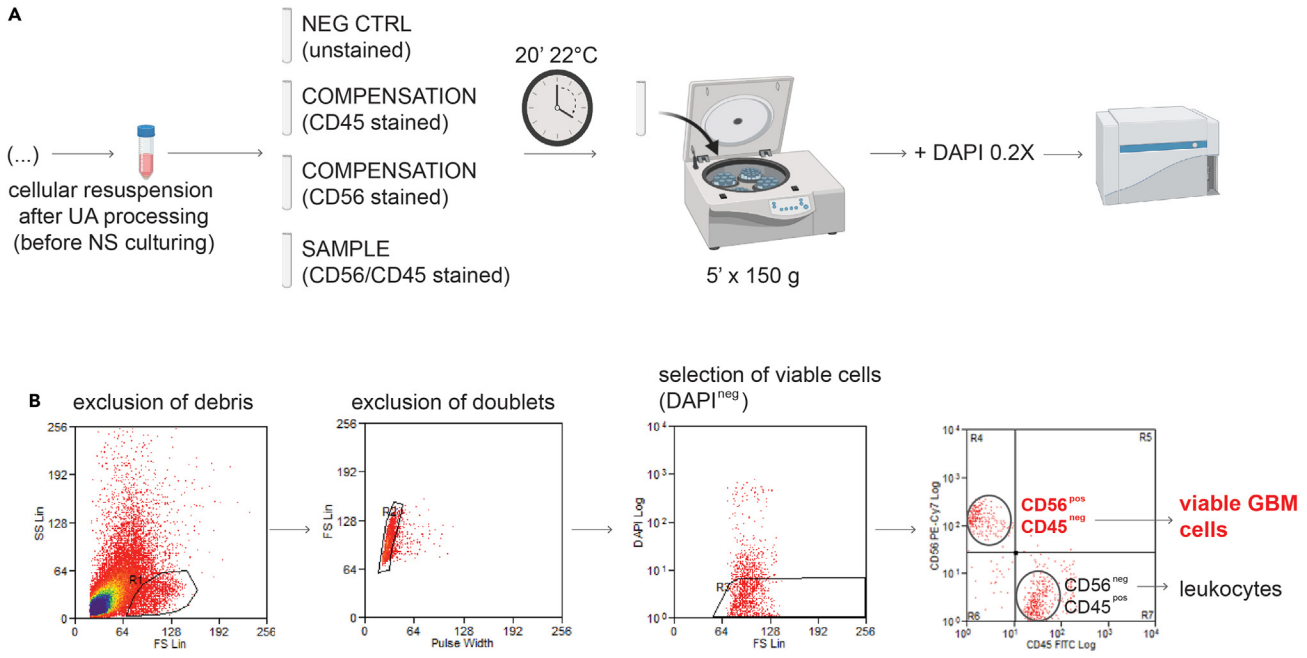

Figure 7. GBM cell quantification by flow cytometry

(A) Sample preparation for flow-cytometry evaluation of the $CD56^{pos}CD45^{neg}$ subpopulation (corresponding to GBM cells) in whole UA cell resuspension.

(B) Gate logic builder for flow cytometric analysis.

53. Immediately acquire samples by flow cytometry (using a Beckman Coulter CytoFLEX Flow Cytometer, collection of data is performed with the Beckman Coulter software CytExpert 2.4).

⚠ CRITICAL: a prolonged interval between filtering and flow cytometry can cause cell aggregation and hamper analysis.

54. Apply the following gate logic builder (Figure 7B):
    a. Set the first gate on the physical parameters Forward Scatter (FSC)/Side Scatter (SSC) to exclude debris;
    b. Within this population, exclude doublets and aggregates by evaluating the Pulse Width on a physical parameter (FSC or SSC);
    c. Select the viable population, which has not incorporated DAPI ($DAPI^{neg}$);
    d. Combination of these three gates defines the healthy single cells where CD45/CD56 expression should be analyzed;
55. Analyze data by Beckman Coulter Kaluza Flow Analysis Software 1.3 or equivalent.

Note: $CD45^{neg}/CD56^{pos}/DAPI^{neg}$ cells are bona fide viable GBM cells; $CD45^{pos}/CD56^{neg}$ identifies leukocytes and $CD45^{pos}/CD56^{pos}$ cells identify Natural Killer (NK) cells (Figure 7B).

GSC spheroid cultures freezing for further derivation

Timing: 15 min (for alternative freezing or thawing)

At the end of derivation protocol, a part of tumor cells resuspended in GSC Medium in step 26 (see step-by-step method details) can be frozen in vital condition for further culture derivation; in that case, frozen materials have to be carefully thawed for preserving cell viability (Figure 8).

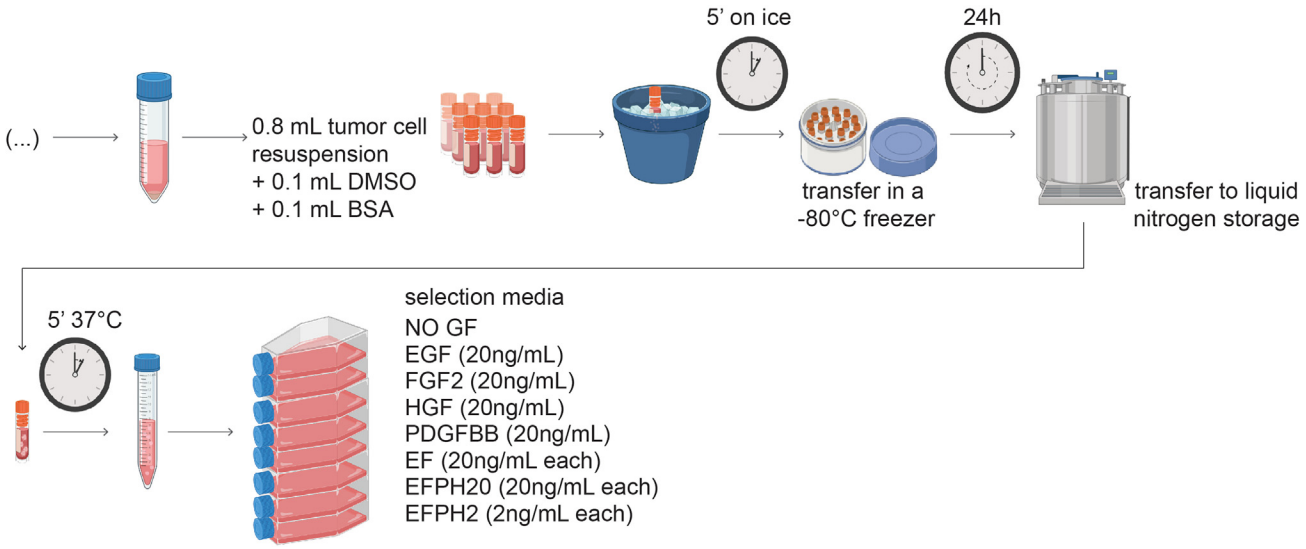

Figure 8. Neurosphere biobanking
Schematic procedure for vital cell freezing and thawing for further culture derivation.

56. For cell freezing (Figure 8).
    a. Aliquot 0.8 mL of tumor cell resuspension in 3–4 cryogenic vials.
    b. Add 0.1 mL of additional BSA.
    c. Add 0.1 mL of dimethyl sulfoxide (DMSO) to each cryogenic vial.
    d. Place vials on wet ice for no more than 5 min.
    e. Transfer cryogenic vials in an insulated box placed at −80°C freezer to ensure slow freezing of cells.
    f. After 24–48 h, transfer vials to liquid nitrogen storage.
57. For cell thawing (Figure 8).
    a. Rapidly thaw frozen cells in a 37°C water bath.
    b. Dilute cells slowly in pre-warmed GSC Medium without GFs.
    c. Centrifuge for 10 min at 120 *g*, RT (+22°C–25°C).
    d. Gently resuspend pellet in a 75 cm^2 flask containing 10–15 mL of pre-warmed GSC Medium without GFs.
    e. Transfer flasks in the incubator at 37°C, 5% CO_2 and 20% O_2 concentration for 24 h.
    f. Collect medium containing cells in 15 mL conical tubes.
    g. Centrifuge for 10 min at 120 *g*, RT (+22°C–25°C).
    h. Resuspend pellet as to obtain 1 mL of cell resuspension for each desired culture.
    i. Transfer in 75 cm^2 flasks containing different concentration and combinations of GFs and maintain in the incubator at 37°C in monitored CO_2 and 20% O_2 concentration.
    j. For the first weeks, follow the same procedure described for fresh material (see above, step-by-step method details, steps 34–38).

Note: frozen cells generally display lower derivation rate than freshly isolated cells; it is strongly recommended to derive cultures immediately after processing and to preserve part of the tumor resuspension for additional derivation procedure.

Biobanking of related samples for further genetic and molecular analysis

Timing: 1 h (day 1), 1 h (day 2). 24 h incubation required.

For biobanking (strongly recommended) part of the processed tumor resuspension is collected for histology (Figure 9) and DNA and RNA extraction (Figure 10).

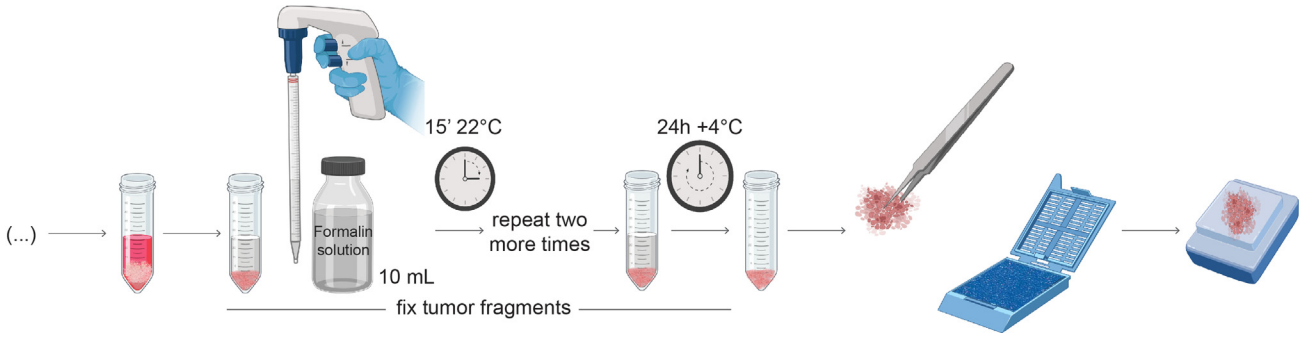

Figure 9. Biobanking of starting material: UA resuspension

Schematic procedure of FFPE sample preparation starting from UA resuspension. FFPE: formalin-fixed paraffin-embedded.

58. For inclusion of tumor fragments for histological analysis (FFPE tissues, Figure 9), one of the pellet obtained after the first UA centrifugation (see above, step-by-step method details, steps 1–3) is processed as follow:
    a. Add 10 mL of fixative (formalin or other fixative of choice).
    b. Incubate for 15 min, pelleting cells by gravity.

    ⚠ CRITICAL: it is recommended to avoid centrifugation to better preserve tissue histology.

    c. Discard supernatant via sterile pipette.
    d. Repeat twice till fixative and pellet are clean from blood.
    e. Add 10 mL of fixative.
    f. Incubate for 16–24 h at +4°C.
    g. Discard supernatant by decanting.
    h. With the help of sterile forceps, collect and gently put fixed tumor fragments into a biocassette with foam pads.
    i. Process fixed tumor fragments as the other histological samples.
59. For tumor DNA and RNA extraction (Figure 10).
    a. Aliquot 0.5 mL of tumor resuspension in 4/5 1.5 mL RNase-free Eppendorf tubes for DNA and RNA extraction (multiple small aliquots are suggested to prevent excessive freeze-thaw cycles).
    b. Centrifuge for 5 min at 900 *g*, RT (+22°C–25°C).
    c. Discard supernatant by vacuum aspiration.
    d. Store pellets at −80°C or proceed to gDNA and total RNA extraction according to kit manufacturer's instructions (for gDNA extraction, https://ita.promega.com/-/media/files/resources/protocols/technical-manuals/101/reliaprep-gdna-tissue-miniprep-system-protocol.pdf?rev=4a90105b1f5648c8b647907de054ccfa&la=en; for RNA extraction, https://ita.promega.com/-/media/files/resources/protocols/technical-manuals/101/maxwell-rsc-mirna-tissue-kit-protocol.pdf?rev=da4435cb40424d489e500cb9748386e6&sc_lang=en).

Note: Nucleic acid preservatives can be used at this stage following manufacturer instruction.

60. For normal DNA from PBMC extraction (Figure 11).
    a. Transfer blood in a 50 mL conical tube.
    b. Add 40 mL ACK solution for blood red cell lysis.
    c. Vigorously flip the tube 10–15 times or resuspend by pipetting with a 25 mL disposable pipette.
    d. Incubate for 15 min at RT (+22°C–25°C) in the dark.
    e. Centrifuge for 5 min at 750 *g*, RT (+22°C–25°C).
    f. Discard supernatant by vacuum aspiration.

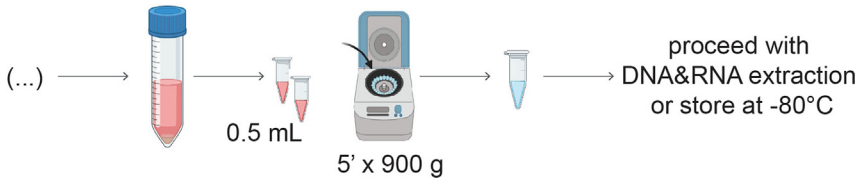

Figure 10. Biobanking of starting material: DNA and RNA
Schematic procedure for DNA and RNA sample collection from UA resuspension.

g. Resuspend pellet in the appropriate volume of PBS 1X (starting from 5 mL of peripheral blood use 3 mL of PBS).
h. Aliquot in appropriate number of Eppendorf tubes (1 mL/Eppendorf).
i. Centrifuge for 5 min at 900 *g*, RT (+22°C–25°C).
j. Discard supernatant by vacuum aspiration.
k. Store pellets at −80°C or proceed to PBMC gDNA extraction according to DNA kit manufacturer's instructions (for PBMC gDNA extraction, https://www.thermofisher.com/document-connect/document-connect.html?url=https://assets.thermofisher.com/TFS-Assets%2FLSG%2Fmanuals%2Fpurelink_genomic_mini_man.pdf).

Note: Nucleic acid preservatives can be used at this stage following manufacturer instruction.

EXPECTED OUTCOMES

The described protocol shows a derivation rate of around 60%, higher than that obtained with the standard protocol starting from a single biopsy[4]; this increase relies on the use of the ultrasonic aspirator, which allows for better preservation of cellular viability during both neurosurgery and the ensuing derivation procedures. Considering the suggested selective pressures, media containing the standard derivation factors EGF and FGF2 at 20 ng/mL (EF and EFPH20) have a comparable derivation rate. As discussed in the study by De Bacco et al.,[1] media containing EGF or FGF2 alone or the four selected GFs at a lower concentration (EFPH2) display a reduced derivation rate (~48%), but are important for preserving heterogeneous gene amplifications and transcriptional profiles. Finally, as shown, media without EGF and FGF2 (NO GF, H and P) display a significantly reduced derivation rate (~13%) and can select aggressive, cell-autonomous GSCs.[1] As stated above (step-by-step method details, steps 34–38), time to derivation is highly variable among GSC families. Family members selected by the richest media (EF or EFPH20) are expected to be established within 6 months; members selected by the other media could take longer times.

LIMITATIONS

The use of the ultrasonic aspirator in neurosurgery displays two limitations: (i) not all GBMs are eligible for surgical removal using this tool (i.e., those that are highly invasive), leading to a possible

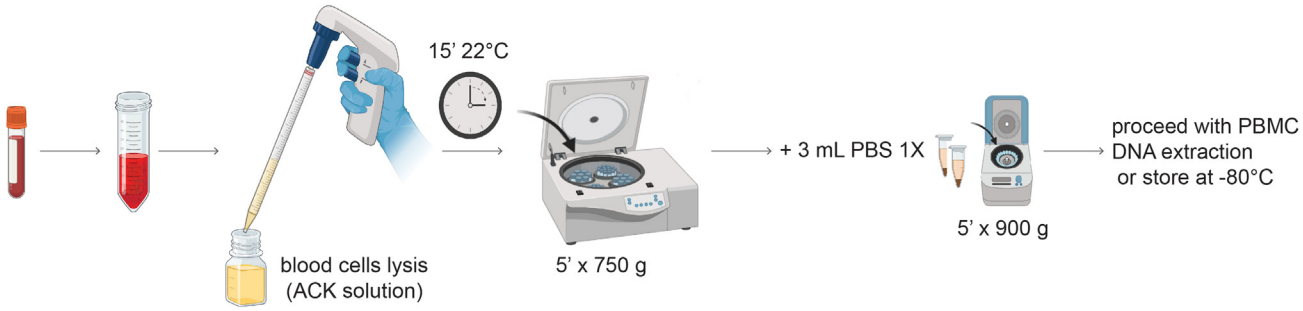

Figure 11. Biobanking of starting material: PBMCs
Schematic procedure for PBMC DNA collection starting from peripheral blood sampling. PBMC: peripheral blood mononuclear cells.

underrepresentation of some tumor typologies; (ii) it significantly increases neurosurgery costs. A possible solution has been described below (see troubleshooting, problem 1).

GFs and GF cocktails used were rationally selected, but do not completely recapitulate the GBM microenvironment; according to experimental requirements, other GFs can be included in the protocol described here.

For assessment of the percentage of viable tumor cells in UA, this protocol relies on the use of markers such as CD45 (to exclude leukocytes) and CD56 (to identify GBM cells). CD56 is known as a marker of cells neural and neuroendocrine origin and does not discriminate between GSCs and more pseudodifferentiated cells.

TROUBLESHOOTING

Problem 1

Reduced availability of GBMs removed by ultrasonic aspirator (step-by-step method details, step 1).

Potential solution

Utilizing the ultrasonic aspirator in neurosurgery may not be cost-effective. As an alternative, we successfully used the receptal of a conventional aspirator as well. It is recommended to change the receptal after cleaning the surgical field, so as to collect tumor cells in a new receptal and avoid excessive tumor cell dilution.

Problem 2

Reduced amount of cell resuspension at the end of the processing procedure (step-by-step method details, step 26).

Potential solution

Reduce the number of derivation conditions or use 25 cm^2 ultra-low attachment flasks instead of 75 cm^2.

Problem 3

Inefficient/difficult filtering procedure (step-by-step method details, step 21).

Potential solution

Avoid overloading the filters. If the filtering step remains difficult, dilute the tumor resuspension with PBS 1X and use more cell strainers (for processing a median UA receptal of around 500 mL, up to 8 cell strainers may be necessary, depending on the efficiency of tissue disruption and the amount of recovered cells).

Problem 4

After UA processing, some fibrous material not disrupted by collagenase treatment can remain in cell cultures. Moreover, tumor-associated immune cells and normal nervous tissue cells are expected to be negatively selected by culture conditions, so that dying cell products will rapidly accumulate in the supernatant, damaging viable tumor cells (step-by-step method details, step 36).

Potential solution

When medium removal and change is not sufficient to remove fibrous material and/or dying cells, two possible solutions are suggested.

- Repeat washes (twice/week) with PBS 1X as described in step-by-step method details, step 36. Centrifuge tubes 10 min at 120 *g*, RT (+22°C–25°C). Discard supernatant and replace with culture medium.

- Use of the Debris Removal Solution (Miltenyi Biotec, see key resources table for details) according to manufacturer's instruction.

Problem 5

During the use of Trypsin or collagenase type I for dissociating neurospheres, nucleic acids released by dying cells appear after centrifugation. This has to be considered a sign of cell culture suffering (step-by-step method details, step 37).

Potential solution

It is important to dissociate cells as to expand cultures and avoid hypoxia-induced cell death. If Trypsin or collagenase type I is absolutely required to dissociate cells, it is suggested to.

- Increase Trypsin or collagenase dilution up to 1:5 in PBS 1X.
- Add DNase I (see key resources table and manufacturer's instruction for details) to the collection tube before dissociating cells.

Problem 6

Viable cell counts yield low values when using an automated cell counter (step-by-step method details, steps 38 and 41).

Potential solution

Some cell counter may exclude aggregated viable cells leading to cell count underestimation. Especially at the beginning of neurosphere derivation, it is recommended to stain cells with trypan blue and manually perform cell counting.

Problem 7

Low percentage of viable cells during neurosphere propagation (step-by-step method details, step 38).

Potential solution

As stated above (see step-by-step method details, steps 34–38), initial neurospheres are fragile and require careful manipulation; both under passaging or over passaging can increase cell death. If during daily culture observation few cells look viable, it is suggested avoid cell passaging (thus wait and see), and change the medium every 2 weeks. It is discouraged to transfer growing cells in a smaller 25 cm^2 flask, as it is possible to lose cells during centrifugation step.

Problem 8

During viability assessment by flow cytometry, it can be useful to monitor the expression of surface markers of interest. However, collagenase type I treatment can significantly reduce cell surface epitopes (step-by-step method details, steps 39–55).

Potential solution

Analysis of specific surface markers by flow cytometry can be performed few days after UA processing. In that case, it is suggested to prepare additional dedicated flasks. Flow cytometric analysis can be performed as described (see step-by-step method details, steps 39–55).

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Prof. Carla Boccaccio, carla.boccaccio@ircc.it.

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate or analyze any datasets and did not use any code.

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AUTHOR CONTRIBUTIONS

Conceptualization, F.D.B. and F.O.; investigation and methodology, F.D.B., F.O., M.P., and E.C.; writing, F.D.B. and C.B.; supervision, F.D.B., F.O., and C.B.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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