STAR Protocols

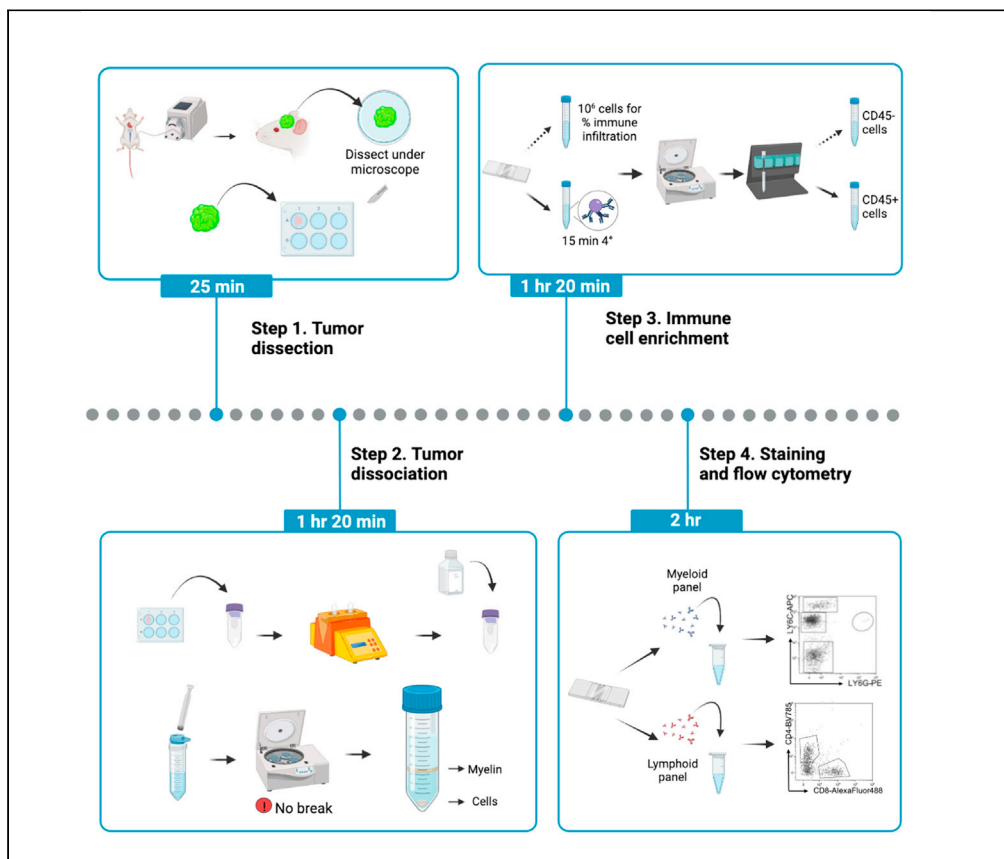**Protocol**

Isolation and characterization of the immune cell fraction from murine brain tumor microenvironment

Ignacio Mastandrea,
Divsha Sher, Prerna
Magod, Dinorah
Friedmann-
Morvinski

ignacio.mastandrea@
gmail.com (I.M.)
dino@tauex.tau.ac.il
(D.F.-M.)

Highlights

Magnetic-bead-
enrichment-based
protocol to isolate
immune cells from
brain tumors

Optimized protocol
to achieve high yields
of viable cells

Companion protocol
for characterization of
immune
subpopulations by
FACS

Applicable for all
kinds of brain tumor
animal models

The immune fraction of the tumor microenvironment has been proven to play a fundamental role in glioblastoma progression and therapeutic response. Here, we present a detailed magnetic-bead-enrichment-based protocol to isolate and analyze the composition of this fraction from mouse brain tumors. The protocol is optimized to achieve high yields of viable immune cells. We also detail characterization of the immune subtypes by FACS analysis. Our procedure is applicable for either lentiviral-induced tumors or transplant models in syngeneic immunocompetent mice.

STAR Protocols

Protocol

Isolation and characterization of the immune cell fraction from murine brain tumor microenvironment

Ignacio Mastandrea,[1,3,*] Divsha Sher,[1,3] Prerna Magod,[1] and Dinorah Friedmann-Morvinski[1,2,4,*]

[1]The School of Neurobiology, Biochemistry and Biophysics, The George S. Wise Faculty of Life Sciences, Tel Aviv University, Tel Aviv 6997801, Israel

[2]Sagol School of Neuroscience, Tel Aviv University, Tel Aviv 6997801, Israel

[3]Technical contact

[4]Lead contact

*Correspondence: ignacio.mastandrea@gmail.com (I.M.), dino@tauex.tau.ac.il (D.F.-M.)
https://doi.org/10.1016/j.xpro.2021.101106

SUMMARY

The immune fraction of the tumor microenvironment has been proven to play a fundamental role in glioblastoma progression and therapeutic response. Here, we present a detailed magnetic-bead-enrichment-based protocol to isolate and analyze the composition of this fraction from mouse brain tumors. The protocol is optimized to achieve high yields of viable immune cells. We also detail characterization of the immune subtypes by FACS analysis. Our procedure is applicable for either lentiviral-induced tumors or transplant models in syngeneic immunocompetent mice.

For complete details on the use and execution of this protocol, please refer to Magod et al. (2021).

BEFORE YOU BEGIN

Initiation of brain tumors

ⓘ Timing: ∼2 months

This protocol requires tumor tissue derived from mouse brain tumor models. Ethical approvals are required prior to starting this procedure. Animal experiments described in this protocol were approved by the animal care and use committee (IACUC) of Tel Aviv University (approval protocol no. 04-19-074 and 04-16-073) and conducted in accordance with NIH guidelines.

For a detailed protocol of lentiviral models of Brain Cancer, including design and cloning of lentivectors expressing oncogenes and shRNAs, production of lentiviral vectors and stereotaxic injection into the brain, refer to Friedmann-Morvinski and Singer (2013).

Preparation of myelin buffer

ⓘ Timing: 30 min

Refer to materials and equipment for buffer recipes.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rat Anti-mouse CD45-FITC (Clone I3/2.3) Working dilution – 1:200	BioLegend	Cat# 147709; RRID: AB_2563541
Rat Anti-mouse CD11b-BV785 (Clone M1/70) Working dilution – 1:20	BioLegend	Cat#101243; RRID: AB_2561373
Armenian hamster Anti-mouse CD11c-BV605 (Clone N418) Working dilution – 1:20	BioLegend	Cat# 117333; RRID:AB_11204262
Rat Anti-mouse Ly-6C-APC (Clone HK1.4) Working dilution – 1:100	BioLegend	Cat# 128015; RRID:AB_1732087
Rat Anti-mouse Ly-6G-PE (Clone 1A8) Working dilution – 1:100	BioLegend	Cat#127607; RRID: AB_11204262
Rat Anti-mouse CD3-APC (Clone 17A2) Working dilution – 1:100	BioLegend	Cat# 100236; RRID: AB_2561455
Rat Anti-mouse CD4-BV785(Clone GK1.5) Working dilution – 1:100	BioLegend	Cat#100453; RRID: AB_2565843
Rat Anti-mouse CD8-FITC (Clone 53-6.7) Working dilution – 1:200	BioLegend	Cat#100726; RRID: AB_312744
Rat TruStainFcX™-Fc blocker CD16/32 (Clone 93) Working dilution – 1:100	BioLegend	Cat#101320; RRID: AB_1574975
Chemicals, peptides, and recombinant proteins		
H_2NaO_4P- Sodium phosphate monobasic monohydrate	Fisher BioReagents	Cat#7558-80-7
NaCl	Fisher BioReagents	Cat#7647-14-5
KCl	Fisher BioReagents	Cat#7447-40-7
Glucose	Formedium LTD	Cat#GLU02
HNa_2O_4P- Sodium phosphate dibasic dihydrate	Fisher BioReagents	Cat#7558-79-4
FBS- Fetal bovine serum	Biological Industries	Cat#04-007-1A
PBS	Biological Industries	Cat#02-023-1A
EDTA	Fisher Chemical	Cat#6381-92-6
Percoll™	GE Healthcare	Cat#17-0891-02
CLORKETAM® 1000 - Ketamine (as Hydrochloride) 1g/10mL	Vetoquinol	Cat#LA01250
SEDAXYLAN–Xylazine (as Hydrochloride) 20mg/mL	Phibro Israel	Cat#6031-1-01/04
Critical commercial assays		
Neural Dissociation Kit	Miltenyi Biotec	Cat# 130-092-628
CD45MicroBeads	Miltenyi Biotec	Cat#130-052-301
Experimental models: organisms/strains		
B6.Cg-Tg(Syn1-cre)671Jxm/J mice	The Jackson Laboratory	Cat# JAX:003966; RRID:IMSR_JAX:003966
Recombinant DNA		
pTomo-HRas-shp53	Friedmann-Morvinski et al. (2012)	N/A
pTomo-PDGFB-shp53	Magod et al. (2021)	N/A
pTomo-mock	Marumoto et al. (2009)	RRID:Addgene_26291
pMDLg/pRRE	Addgene	RRID:Addgene_12251
pRSV-Rev	Addgene	RRID:Addgene_12253
pCMV-VSV-G	Addgene	RRID:Addgene_8454
Software and algorithms		
Kaluza software v2.1	Beckman Coulter	https://www.beckman.com/flow-cytometry/software/kaluza RRID: SCR_016182
GraphPad Prism Version 8.2.1	GraphPad Software, La Jolla, California USA	https://www.graphpad.com:443/; RRID: SCR_002798

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Other		
Cell Strainer – 70μm pore size	SPL Life Sciences	Cat# 93070
Fixable Viability Dye eFluor™ 450	eBioscience	Cat# 65-0863-14
gentleMACS™Dissociator	Miltenyi Biotec	Cat#130-093-235
MACS™MultiStand	Miltenyi Biotec	Cat#130-042-303
MiniMACS™ Separator	Miltenyi Biotec	Cat#130-042-102
MS Columns	Miltenyi Biotec	Cat#130-042-201
Water bath or incubator adjusted to 37°C	N/A	N/A
Multipurpose- and Micro-centrifuge	N/A	N/A
Attune NxT Flow Cytometer	Thermo Fisher Scientific	N/A
Perfusion pump: Miniature Peristaltic Pump BQ50-1J	Longer	N/A

MATERIALS AND EQUIPMENT
Buffers

Myelin gradient buffer		
Reagent	Final concentration	Amount
H_2NaO_4P	6.5 mM	0.78 g
NaCl	14 mM	8 g
KCl	5.3 mM	0.4 g
Glucose	11.10 mM	2 g
HNa_2O_4P	~25 mM	Until pH is adjusted to 7.4 (~3.56g)
ddH_2O	N/A	Adjust to 1 L
Total	N/A	1 L

Note: Dissolve the H_2NaO_4P (Sodium phosphate monobasic monohydrate) in 900 mL double distilled H_2O. Add NaCl, KCl and Glucose. Adjust pH to 7.4 with HNa_2O_4P (Sodium phosphate dibasic dihydrate). Adjust volume to 1 L and filter sterilize. **Keep refrigerated at 4°C for up to 3 months**.

FACS buffer		
Reagent	Final concentration	Amount
FBS	2% (v/v)	1 mL
EDTA	2 mM	2 mL (from 50mM solution)
PBS	N/A	47 mL
Total	N/A	50 mL

Note: Keep refrigerated at 4°C up to 1 week.

Equipment and reagent alternatives

- GentleMACS™Dissociator (MiltenyiBiotec, Cat#130-093-235) can be replaced simply by a set of 3 glass Pasteur Pipettes in steps 3d, 3f, 3i. The Pipette's diameter must be adjusted manually and used as further detailed in the protocol.
- In the key resources table, we recommend using the listed antibodies. However, different antibodies of choice can replace the ones we mention. It is of vital importance to perform optimization and antibody titration for each panel in case of using antibodies with different properties (vendor, fluorophore, clonality).

- The Attune NxT Flow Cytometer was used to perform acquisition of fluorescence. Any other flow cytometer that allows fluorescent acquisition at the required optical paths can be used. Please, note that the settings are specific for the brand and model of the flow cytometer and optimization of the procedure always needs to be performed.
- A Miniature Peristaltic Pump BQ50-1J was used to perform the trans-cardiac perfusion. Any other perfusion will do the work.

STEP-BY-STEP METHOD DETAILS
Tumor dissection and dissociation

⏱ Timing: 1 h 45 min

This step describes our approach to dissect and dissociate brain tumor tissue. The expected outcome after dissociation is a high yield of mixed cell subpopulations that comprise the tumor stroma and the tumor microenvironment (TME). The applications for the resultant cells (either with or without enrichment for a particular cell subpopulation) that we mostly use are: staining for FACS analysis, RNA extraction, and cell culture.

Note: Sterile conditions during the protocol depend on the final procedure to be executed with the cells. However, we do advise working in a biological hood, in order to keep all the reagents sterile for further use and applications.

1. Anesthetize mice at the desired time point.

   Note: We usually use a Ketamine (100mg/mL)/Xylazine(200mg/mL) combination by preparing the mix of 3:1 ratio, giving a dose of 70 μL per 10g of mouse.

   Note: The desired time point depends on the researcher's questions, hypothesis and model. In Magod et al. 3 time points were used: 2, 5 and 8 weeks. The last one corresponds with the endpoint of our R53 model, by then the mice are already showing symptoms (hunched back, difficulty to walk, loss of body weight) and are close to death.

   Note: We usually work with mice aged 7–8 weeks at the time of the injection. No gender preferences were considered in this study. Mice species are detailed in key resources table.

2. Dissect brain tumors.
   a. Perfuse the mice with sterile ice-cold PBS.
   b. Isolate the brain and transfer it to sterile 10cm transparent plate.
   c. Dissect the brain tumors under fluorescent microscope.

   Note: Tumor tissue is easily identifiable in our model as GFP+ (Figure 1). For similar approach in the dissection a model with a fluorescent reporter is needed. The transfer vector used in our system contains HRasV12 oncogene, a hairpin targeting p53, and a GFP reporter (Figure 2) (Friedmann-Morvinski et al., 2012).

   d. Transfer the tumor to a well of a 6-well plate containing cold HBSS−/−.

   Note: Keep plate with tumors on ice until further use.

3. Dissociate tumor tissue using a Neural Dissociation Kit (Miltenyi Biotec) according to manufacturer's instructions (https://www.miltenyibiotec.com/US-en/products/neural-tissue-dissociation-kits.html#gref).

Note: For this step, we use a gentleMACS™Dissociator (MiltenyiBiotec, Cat#130-093-235)

Note: This protocol describes the amounts necessary for 1 mouse. Increase the amounts by the number of mice to be processed.

a. Prepare enzyme 1 mix (1900μL buffer x + 50μL enzyme P) according to manufacturer's instructions and warm up in C tubes for 15 min.
b. Aspirate the HBSS−/− and cut the tumor into small pieces (2–3mm).
c. Add enzyme 1 mix on top of chopped dissected tissue, then transfer it back to C tube. Cut end of P1000 tip to transfer chopped tissue easily.
d. Run "m_brain_01" protocol on gentleMACS™ tissue dissociator (pre-programmed by manufacturer).
e. Incubate at 37°C for 15 min.
f. Run "m_brain_02" protocol on gentleMACS™ tissue dissociator (pre-programmed by manufacturer).
g. Prepare enzyme A mix according to manufacturer's instructions (20μL buffer Y + 10μL enzyme A). Then add the mix to the C tube containing tissue homogenate.
h. Incubate at 37°C for 10 min.
i. Run "m_brain_03" protocol on gentleMACS™ tissue dissociator (pre-programmed by manufacturer).
j. Add 3mL of cold HBSS +/+ into C tube in order to stop reaction.
k. Transfer the content from the C tube into a Falcon tube through a 70μm cell strainer.
l. Add 3mL of HBSS+/+ into C tube to wash and repeat step j.
m. Grind the remaining tissue pieces against the mesh. Use the rubber tip of a syringe and add HBSS +/+ to help (Usually 1 mL before you start grinding and 1mL once you finish) (Figure 3 and Methods video S1).

Note: For a better myelin separation we recommend performing all the further spin steps at 4°C.

n. Spin the Falcon tube at 300*g* for 10min.
o. Prepare Percoll gradient mix.

| Reagent	Final concentration	Amount
Myelin gradient buffer	N/A	19 mL
Percoll™	22%	5.5 mL
NaCl (1.5M)	35.9 mM	600 μL

p. Resuspend the pellet in percoll gradient mix (25mL). Add on top 5mL of cold PBS SLOWLY!!! To create the gradient (Figure 4A)
q. Spin for 20 min at 950*g*. NO BREAK.

⚠ CRITICAL: In this step it is critical to work in a no break regime in the centrifuge, for both acceleration and break. Gradient and further myelin layer formed in centrifugation are quite fragile.

r. Aspirate the myelin layer first, then the rest of the supernatant (both PBS and Percoll layers) without disturbing the pellet (Figure 4B).

Note: We recommend to vacuum the myelin layer first given it is not very compact and may get carried on as the rest of the supernatant is removed.

s. Resuspend in 0.5% BSA in 1✕ PBS. 10mL for each brain.
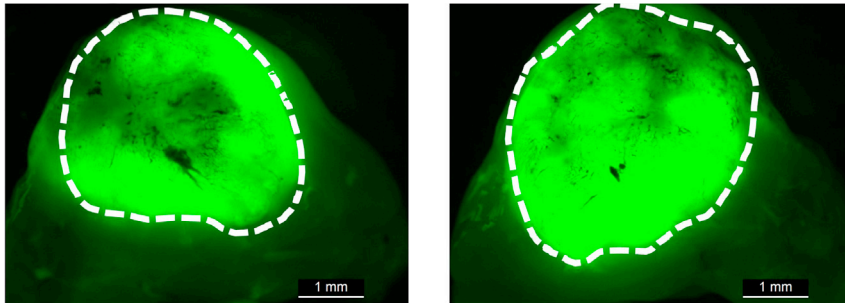
Figure 1. Brain from tumor-bearing mice under the epifluorescence microscope
GFP expression denotes tumor area and is used as guideline for the tumor dissection.

Note: We advise to continue with the rest of the protocol as soon as possible. If needed, cells can be kept on ice. However, both time and temperature of storage have effects on cell viability, as seen in Figure 5.

Alternatives: This protocol can also be achieved without a gentleMACS™ tissue dissociator. Prepare a set of 3 glass Pasteur pipettes by changing their diameter using fire. These pipettes will be used to break down the tissue homogenates by pipetting several times, mocking the action of the different protocols from the gentleMACS™ tissue dissociator. Pipettes should be used in decreasing diameter order as you move forward in the steps. Special caution to bubble formation while resuspending the homogenate. While this is a good alternative, the use of the gentleMACS™ tissue dissociator is preferred. Higher yields are possible and the protocol is more accurate and less time consuming. Bordt et al. (2020) give a detailed and very illustrative instruction on how to use shear force of successively smaller Pasteur pipets for tissue dissociation.

Immune cell-subpopulation enrichment and staining for FACS analysis

⏱ Timing: 3 h 30 min

In this part of the protocol we describe how to isolate the immune cell population from the whole-tumor cell suspension obtained in the previous step. Here we also describe our staining protocol for further TME composition analysis.

Note: Sterile conditions are not required since cells will be stained for FACS analysis.

4. Proceed to immune cell enrichment using CD45 murine MicroBeads (MiltenyiBiotec, Cat#130-052-301). Follow manufacturer's instructions according to the MS Columns protocol (https://www.miltenyibiotec.com/US-en/products/ms-columns.html#gref).

   Note: This protocol describes procedure for one sample. Increase the amounts by the number of samples to be processed.

   a. Count the cells and divide them according to the column's capacity. Additionally, take 1 million cells to stain with CD45 to estimate % of total CD45+ cells in the tumor.

   ⚠ CRITICAL: Take into consideration column capacity, which is 10 million magnetically labeled cells from up to 20 million total cells. For the amount of CD45+ percentage we

expect in our type of samples we advise to take up to 20 million live cells from the whole-tumor cell suspension.

b. Transfer the cells to 15mL Falcon tubes.

Note: From this step all the spin steps could be done at room temperature (RT; 15°C–25°C).

c. Spin the cells at 300*g* for 10 min
d. Resuspend each pellet in 90μL of 0.5% BSA in PBS. Add 10μL of CD45microbeads.
e. Incubate 15 min at 4°C.
f. Wash with 1mL of 0.5% BSA, then centrifuge sample for 10min at 300*g*.
g. Aspirate the supernatant and then resuspend pellet in 500μL of 0.5% BSA.
h. Set up the MS columns against the magnet stand and a falcon tube beneath it to collect CD45- cell fractions.
i. Equilibrate the MS columns with 500μL of 0.5% BSA.

⚠ CRITICAL: Whenever using the MS columns avoid formation of air bubbles. Also, always wait until the column is empty before proceeding to add any more liquid.

⚠ CRITICAL: Clumps should be removed before adding cell suspension. Any type of filter should work.

j. Filter the cell suspension through 70μm mesh.

Note: In order to remove clumps and aggregates, we first wet the mesh of a FACS' cup tube with 0.5% BSA (250uL should work). Then we run the whole-tumor-cell suspension through it.

k. Add the filtered cell suspension to the column.
l. Wash the column 3 times with 500μL of 0.5% BSA. Collect eluate in 15mL Falcon tube.

Note: The eluate contains tumor cells and non-immune cell populations from the TME such as endothelial cells (Rousso-Nooriet al., 2021) and astrocytes. Any of the further described procedures can be done with the CD45- fraction.

m. Take the MS Column out of the magnetic stand. Quickly add 1mL of 0.5% BSA into it and immediately flush out retained fraction into a new Falcon tube labeled CD45+.

Note: The eluate contains the enriched fraction of the immune compartment of the TME.

5. Stain the enriched cells for flow cytometry analysis in order to determinate TME's myeloid and lymphoid composition.
   a. Count the cells and divide them according to the amount of antibody panels in use. Make sure to keep some cells for unstained or other necessary controls.

   Note: In this protocol, we work with two panels. One built to analyze the myeloid immune TME composition and another one to analyze the lymphoid immune TME component. We use from 0.5 to 1 million cells per panel.

   b. Spin the cells down in Eppendorf centrifuge for 5 min at 400*g*.
   c. Stain with eflour-405 Live/Dead Fixable dye (eBioscience) for live-cell population.
      i. Resuspend the cell pellet in 100 μL of FACS buffer.
      ii. Add 1μL of eflour-405 Live/Dead Fixable dye per million cells.
      iii. Incubate at 4°C for 30 min.
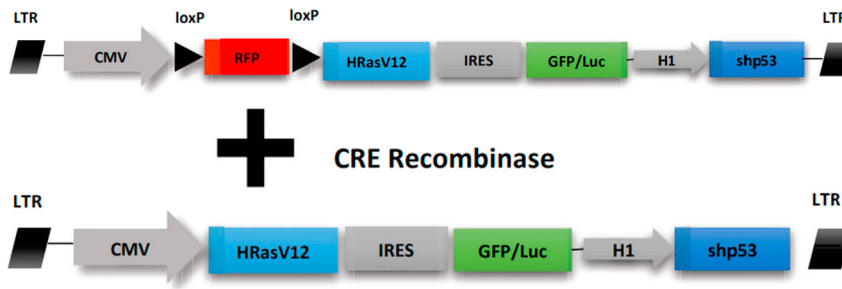
Figure 2. Schematic representation of the lentiviral vector used as an example in this protocol
The schemes show the pTomo lentiviral vector constructs with HRasV12 (Friedmann-Morvinski et al., 2012) in both their "off" state and the activated state after the CRE-recombinase processing.

    iv. Add 1mL of FACS buffer to wash and spin the samples down for 5 min at 400*g*.
  d. Incubate with TruStainFcXTM-Fc blocker CD16/32 (Biolegend).
    i. Resuspend the cell pellet in 100 μL of FACS buffer.
    ii. Add 1μL of Fc blocker per million cells.
    iii. Incubate at room temperature for 15 min.
  e. Stain the samples for 30 min at 4°C using different combinations of anti-mouse antibodies listed in key resources table according to the table below.

| Marker	Fluorophore	Company	Final dilution	Volume (for 1 million cells in 100μL)
*CD45	FITC	BioLegend	1:200	0.5 μL
*CD11b	BV785	BioLegend	1:20	5 μL
*CD11c	BV605	BioLegend	1:20	5 μL
*LY6G	PE	BioLegend	1:100	1 μL
*LY6C	APC	BioLegend	1:100	1 μL
**CD3	APC	BioLegend	1:100	1 μL
**CD4	BV785	BioLegend	1:100	1 μL
**CD8	Alexa Fluor-488	BioLegend	1:200	0.5 μL

*Myeloid Panel
**Lymphoid Panel

  f. Add 1mL of FACS buffer to wash and spin the samples down for 5 min at 400*g*.
  g. Resuspend in 300 μL of FACS buffer.
  h. Read each sample on Attune NxT Flow Cytometer.
  i. Perform analysis using Kaluza software v2.1 and determine the composition of the TME according to our gating strategy (Figure 6).

EXPECTED OUTCOMES

Following this protocol, we expect to obtain a highly enriched cell population of immune (CD45+) cells from mouse brain tumors.

The amount of CD45+ cells present in the tumors depend on several factors, including the type of model you are working with and the time point chosen for the TME analysis.

For our R53 model, where the percentage of CD45+ cells at the endpoint average is 20%–25% (Figure 7A), we usually get between 4 to 5 million highly enriched (percentage of purity ~98%, (Figure 7B) immune cells.
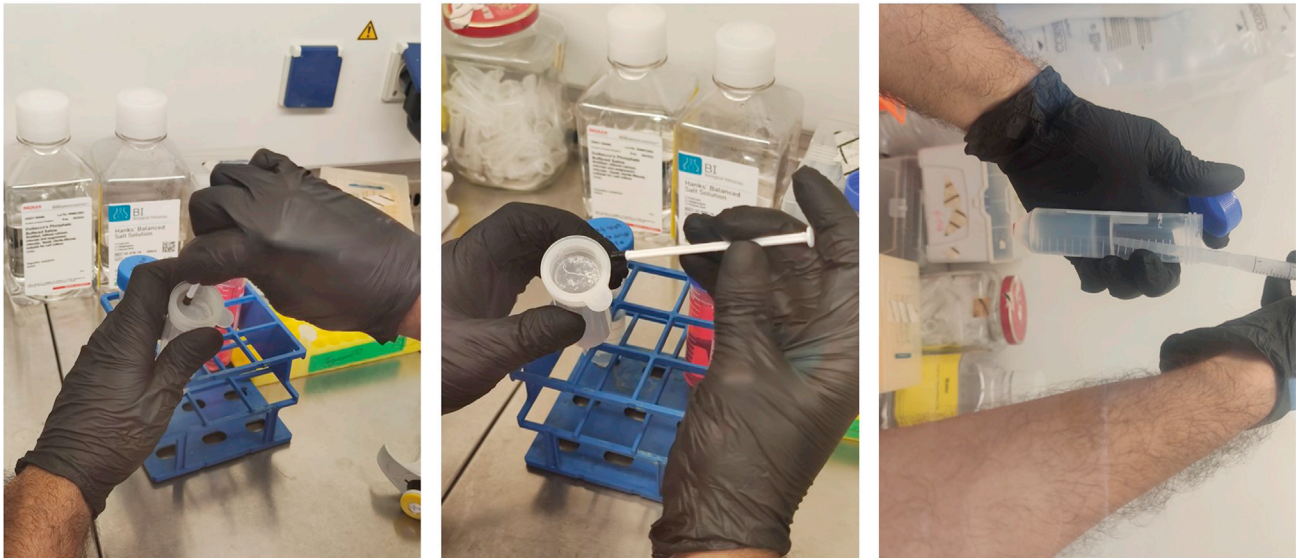
Figure 3. Use of plunger rubber tip to finish grinding digested tissue against the mesh

We developed gating strategies for analysis of myeloid and lymphoid cell composition in the TME, which can be visualized in (Figure 6).

Composition of TME varies within each model, with the time point being considered and with experimental conditions. Here, we merely illustrate results at the endpoint of our R53 model (Figure 8). Examples of said TME variations can be seen in Magod et al.(2021).

LIMITATIONS

This protocol was optimized to work with brain tissue and brain tumors of any kind. While the antibody panel was used to study the immune composition of other organs such as spleen and bone marrow, the dissociation and enrichment part of the protocol are restricted to brain tissue. Working with any other organs will require optimization of a new protocol.
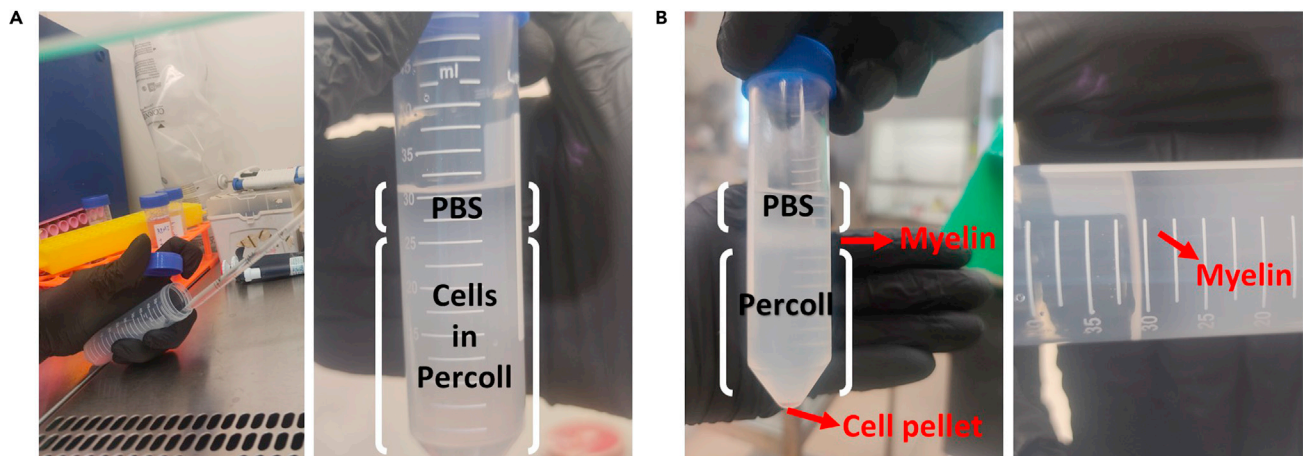
Figure 4. Myelin separation
(A) Layering of PBS on top of Percoll cell-suspension.
(B) Myelin layer is clearly visible and easily removable after 20 min spin.
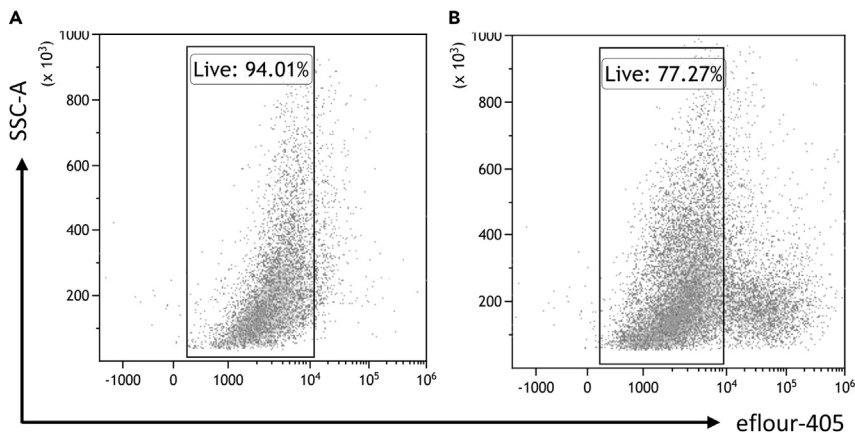
Figure 5. Effect of time and temperature on cell viability
(A) Cell viability following our protocol recommendations for staining right after enrichment and keeping cells on ice.
(B) Cell viability after 4 h at room temperature.

The panels were designed to distinguish most of the myeloid cell types in brain TME. For these cells, very reliable results are obtained and there is room for even further characterization by adding other antibodies that can work in the panel configuration. However, given the infiltration of cells from the lymphoid lineage is so poor, we are limited to deep characterization. Enrichment with CD3 magnetic beads and further pooling of the cell fractions obtained could be an alternative for a deeper lymphoid-related characterization.

Additionally, this protocol is time consuming and the number of samples processed at once depends on the expertise of the researcher. The estimated time describes in the protocol is based on the processing of 2 samples at once by one person.

TROUBLESHOOTING
Problem 1
Mice showing no symptoms of bearing brain tumors (before you begin–initiation of brain tumors) after the expected time period (for H-RasV12-shp53 the latency is 8–10 weeks).

Potential solution
This means that very few or no cells were infected at all after injecting the virus. One possibility is that the needle was clogged and no virus was actually injected. Another problem could have been the virus titer was too low given a mistake or problem in the titer protocol. A solution to confirm if the cells were successfully infected, a representative mouse from the group of injected mice can be euthanized 7 days after the injection and the brain sections can be analyzed for the presence of GFP-positive cells (or reporter present in the lentiviral vector). If no cells are infected we recommend checking if the needle is clean and working correctly, if that is the case we would proceed to make new virus from the starting point.

Problem 2
Low amounts of CD45 population in early time points (end of step 4).

Potential solution
This problem is most likely due to low infiltration of immune cells into the small tumors present at early time points. In this case we recommend pooling CD45+ fractions from several animals in order to reach the necessary cell amounts to do the staining.
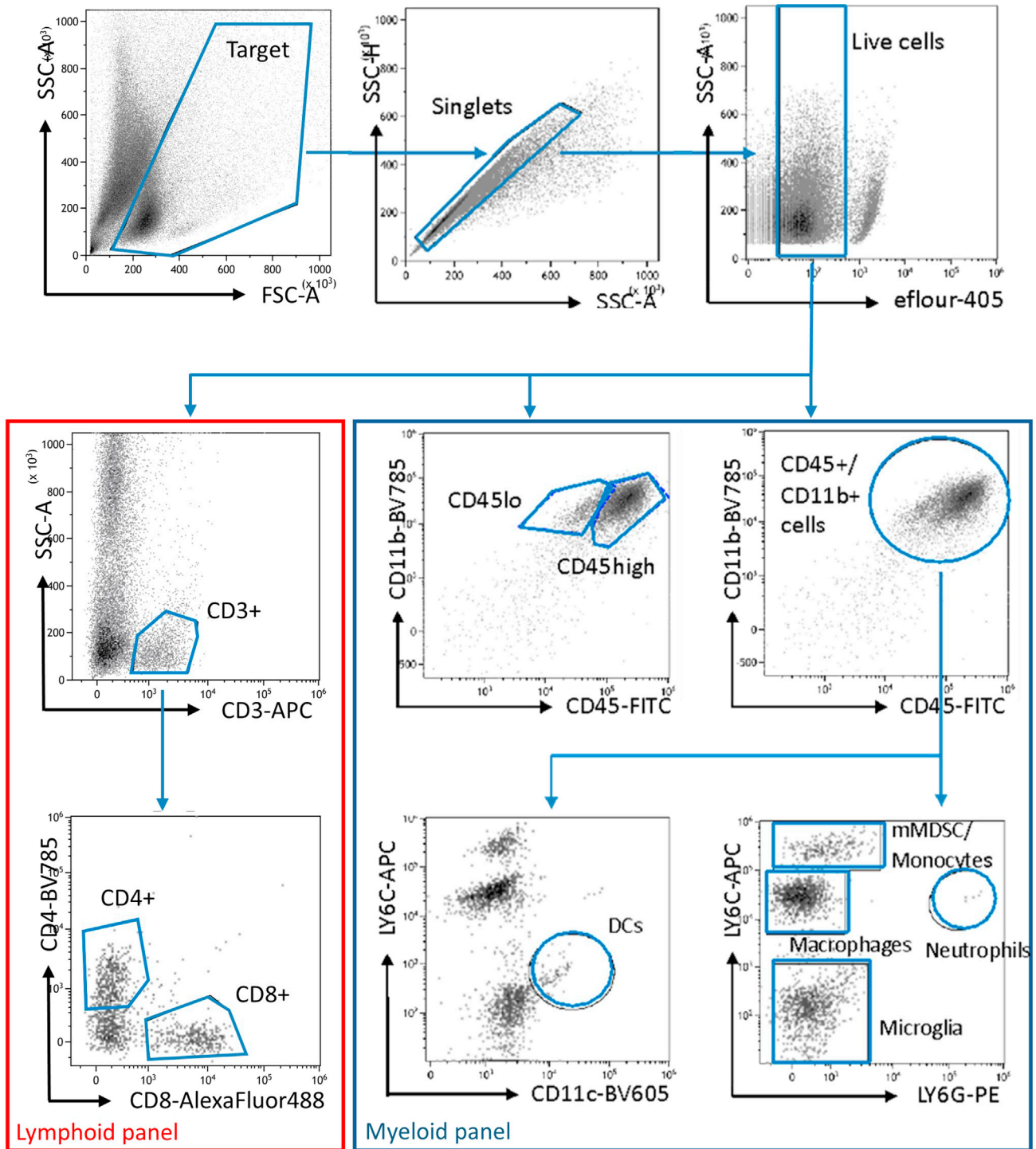
Figure 6. Gating strategies for flow cytometry analysis for both panels

In the myeloid panel, some of the gating is happening in parallel from the same parental population, indicated by appearing next to each other. Subsequent gating is separated by an arrow and appears under the parental population.

Problem 3

Low purity (i.e., <90%) of immune cells after enrichment (step 4).
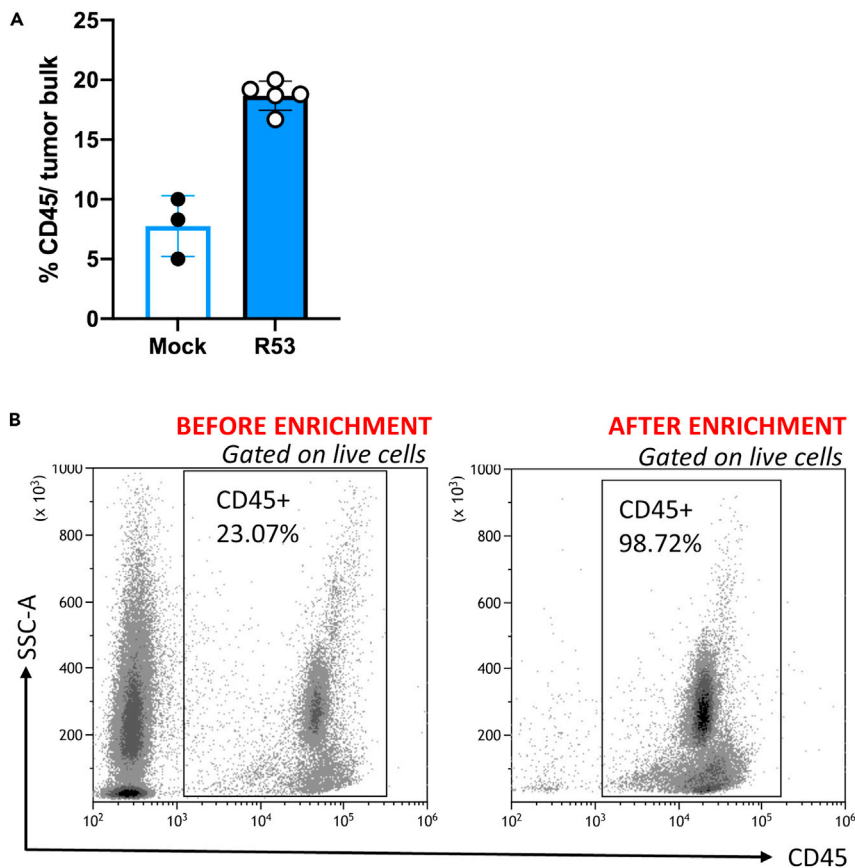
Figure 7. CD45 population analysis
(A) Increased CD45 infiltration in tumors, compared to normal brain. Analysis was performed using tumor-dissociate before CD45 enrichment.
(B) CD45 enriched fraction showed high purity after staining and flow cytometer analysis. Data are represented as mean ±SEM.

Potential solution

There are several reasons why this could be happening, and usually they affect both the yield and purity of the immune fraction. First, if the cell count is underestimated (refer to step 4c in the step-by-step method details section), the number of cells added to the column may be over its capacity and may result in clogging. Second, excessive number of clumps may not only clog the column but also may bind CD45MicroBeads in a non-specific way. If there is still clumps after filtering through a 70 μm, a 40 μm filter could be used. Third, non-specific binding could have happened if the step 4e was performed for too long at a too high temperature. It is critical to be careful about temperature and time in this step.

Problem 4

Low viability of isolated cells (steps 4 and 5).

Potential solution

The timing is vital to keep high cell viability. Substantial delays (>3h) in sample processing can result in a diminished cell viability. Be cautious with temperature, pH and composition of buffers and work-solutions. Any big deviation, such as time taken for start staining and cell storage conditions (Figure 5), will affect cell viability.
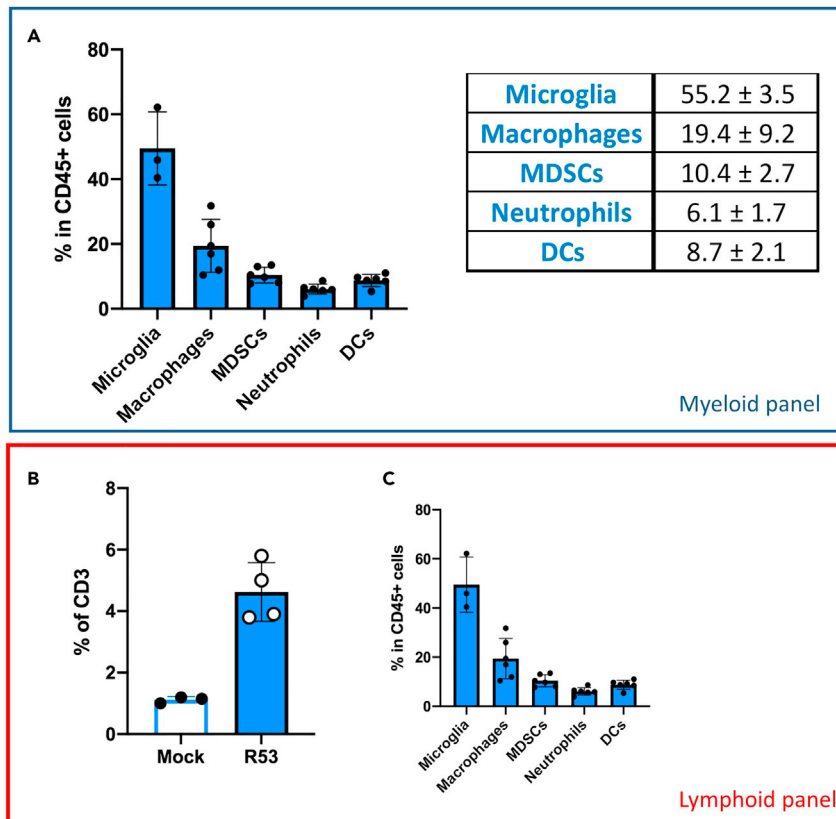
Figure 8. Example of immune TME composition analysis for the R53model

(A) Presents the results obtained using the myeloid panel and gating strategies described in the protocol.

(B and C) Presents the results obtained for the Lymphoid panel. (B) shows differences in T-cell infiltration compared to a control. % of CD3 was obtained from live cell population. (C) shows within the tumor, T-cell phenotypes. Data are represented as mean ± SEM.

Problem 5

No staining (step 5).

Potential solution

Problems with the staining could have several origins. We do recommend before working with the antibody panel to use single stains to find the fitting antibody dilution and the appropriate photomultiplier tubes (PMT) voltage in the flow cytometer that gives the clearest separation. If there is any problem with the antibodies they should be detected at this stage. In that case using a new clone or a new antibody from other company is suggested. Another reason for lacking of staining could be related to epitope damage by digestion enzymes used on the protocol (step 3). A potential solution to this problem should involve changing the enzyme in use.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, DinorahFriedmann-Morvinski (dino@tauex.tau.ac.il)

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate/analyze datasets/code.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2021.101106.

ACKNOWLEDGMENTS

AUTHOR CONTRIBUTIONS

D.F.-M. and P.M. designed the research. I.M., D.S., and P.M. performed the experiments. I.M., D.S., P.M., and D.F.-M. analyzed the results. I.M., D.S., and D.F.-M. wrote the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

Bordt, E.A., Block, C.L., Petrozziello, T., Sadri-Vakili, G., Smith, C.J., Edlow, A.G., and Bilbo, S.D. (2020). Isolation of microglia from mouse or human tissue. STAR Protoc. *1*, 100035.

Friedmann-Morvinski, D., and Singer, O. (2013). Overexpression models: lentiviralmodeling of brain cancer. Curr.Protoc. Mouse Biol. *3*, 121–139.

Friedmann-Morvinski, D., Bushong, E.A., Ke, E., Soda, Y., Marumoto, T., Singer, O., Ellisman, M.H., and Verma, I.M. (2012). Dedifferentiation of neurons and astrocytes by oncogenes can induce gliomas in mice. Science *338*, 1080–1084.

Magod, P., Mastandrea, I., Rousso-Noori, L., Agemy, L., Shapira, G., Shomron, N., and Friedmann-Morvinski, D. (2021). Exploring the longitudinal glioma microenvironment landscape uncovers reprogrammed pro-tumorigenic neutrophils in the bone marrow. Cell Rep. *36*, 109480.

Marumoto, T., Tashiro, A., Friedmann-Morvinski, D., Scadeng, M., Soda, Y., Gage, F.H., and Verma, I.M. (2009). Development of a novel mouse glioma model using lentiviral vectors. Nat. Med. *15*, 110–116.

Rousso-Noori, L., Mastandrea, I., Talmor, S., Waks, T., Globerson Levin, A., Haugas, M., Teesalu, T., Alvarez-Vallina, L., Eshhar, Z., and Friedmann-Morvinski, D. (2021). P32-specific CAR T cells with dual antitumor and antiangiogenic therapeutic potential in gliomas. Nat.Commun. *12*, 3515.