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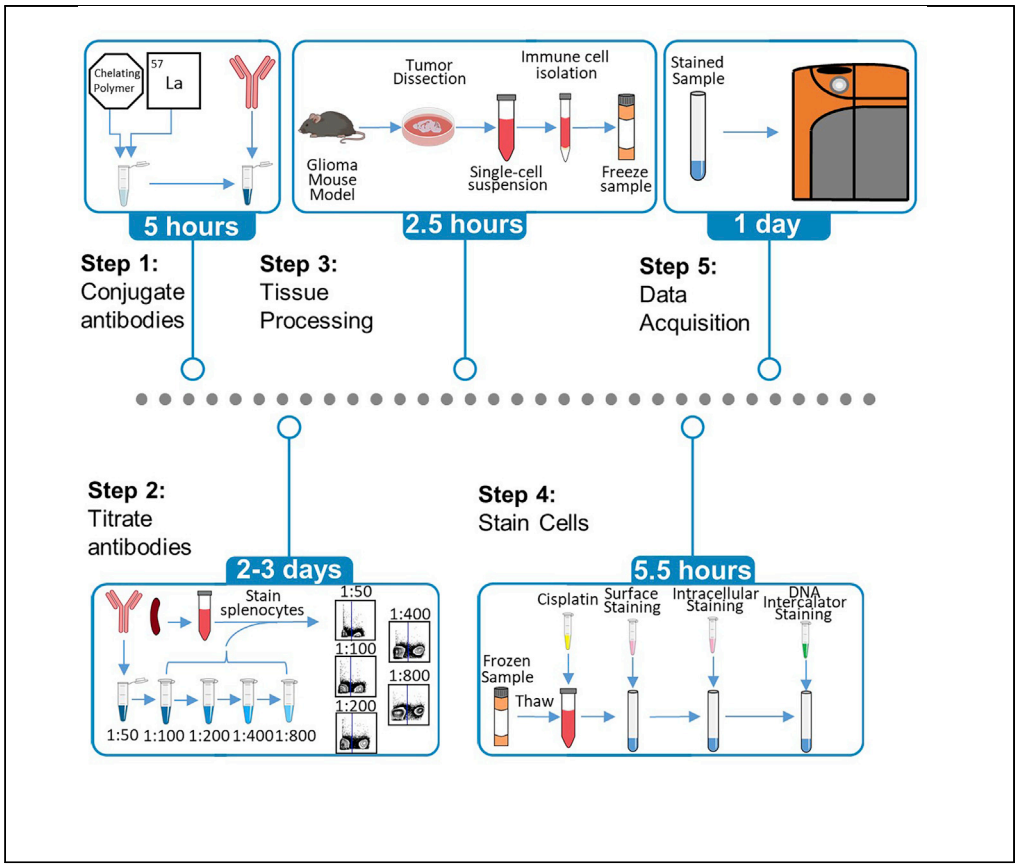
Murine brain tumor microenvironment immunophenotyping using mass cytometry



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Highlights

Mass cytometry protocol to enable profiling of the immune cells in mouse glioma models

Antibody titrations to determine optimal dilutions for analysis of immune cells

Mouse brain tumor tissue collection and processing to generate a single cell suspension

Analytical workflow to identify viable, single cells and apply clustering algorithms

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Murine brain tumor microenvironment immunophenotyping using mass cytometry

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SUMMARY

Here, we present a mass cytometry protocol optimized to examine the phenotype of immune cells within the mouse glioma microenvironment, using a Sleeping Beauty transposon-mediated mouse glioma model. We describe antibody conjugation and titrations for analysis of immune cells. We then detail mouse brain tumor tissue collection and processing, staining, followed by data acquisition, analysis, and gating strategy. This protocol can be applied to any brain tumor-harboring mouse model.

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

BEFORE YOU BEGIN

Mass cytometry is a robust tool, which utilizes principles of mass spectroscopy and flow cytometry to perform the simultaneous detection of over 35 proteins within each single cell. Since mass cytometry detects proteins on the same cells, this prevents confounding variables, such as technical variability generated by repeating the experiment or using different samples to examine multiple flow cytometry panels. Here, we describe a mass cytometry-based protocol optimized to profile immune cells infiltrating glioma tumors that are generated using genetically engineered mouse models (GEMMs). These GEMMs were developed *de novo* using the Sleeping Beauty (SB) transposon system as described previously (Calinescu et al., 2015; Garcia-Fabiani et al., 2020; Núñez et al., 2019). This protocol can also be applied to profile immune cells from any brain tumor-harboring mouse model (Alghamri et al., 2021).

Note: The panel is generated based on the desired phenotypic markers of immune cells.

Institutional permissions

All studies were approved by and in compliance with the institutional animal care and use committee (IACUC) of the University of Michigan.

Conjugating the antibodies to metal isotopes

Timing: 5 h

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Although a large library of antibodies targeting common markers are available for purchase already conjugated to lanthanide metals, some targets lack commercially available pre-conjugated antibodies. Thus, purified antibodies need to be purchased and conjugated prior to use. Here, we describe the protocol to conjugate antibodies when pre-conjugated antibodies are not commercially available. This protocol is adapted from the Maxpar® X8 Antibody Labeling Kit protocol from the Maxpar® Antibody Labeling User Guide. This protocol was optimized to conjugate 100 μg of the unlabeled antibody. The X8 Polymer was selected due to the larger number of metal isotopes available for use relative to the MCP9 polymer. If the quantity of the antibody is different, all volumes and concentrations should be adjusted accordingly.

⚠ CRITICAL: This protocol is specific to the X8 polymer and is not applicable to the MCP9 polymer.

⚠ CRITICAL: Only filtered pipette tips should be used for the entire protocol to prevent potential metal contamination. (See limitations section).

1. Combine the polymer with the lanthanide indicated by the panel (See Table 1. "[a]" identified antibodies need to be conjugated).
   a. Spin the Maxpar® X8 polymer tube for 10 s in a mini-centrifuge to pull polymer to the bottom of the tube.
   b. Resuspend polymer in 95 μL of L-Buffer.

   Note: The L-Buffer is a part of the Maxpar® X8 Antibody Labeling Kits specified in the key resources table. This buffer is used in this protocol without any further modification.

   Note: The Maxpar® X8 polymer tubes are reagents from the Maxpar® X8 Antibody Labeling Kits specified in the key resources table.

   c. Add 5 μL of the 50 mM lanthanide metal solution to the tube for a final concentration of 2.5 mM in 100 μL.
   d. Mix thoroughly with a pipette and incubate solution in a 37°C water bath for 30–40 min.
2. Antibody reduction.
   a. Get a 50 kDa filter and label it with the antibody intended to be conjugated.
   b. Place filter within flow-through tube.
   c. Add 100 μg of the stock antibody solution to the 50 kDa filter and adjust the volume up to 400 μL with R-Buffer.

   Note: The R-Buffer is a part of the Maxpar® X8 Antibody Labeling Kits specified in the key resources table. This buffer is used in this protocol without any further modification.

   d. Centrifuge at 12,000 × *g* for 10 min at 25°C in a microcentrifuge.
   e. Discard the flow-through.
   f. Using R-Buffer, dilute 0.5 M TCEP [tris(2-carboxyethyl)phosphine] stock to make 100 μL of 4 mM TCEP per antibody.

   Note: The 4 mM TCEP solution should be freshly prepared before use.

   g. Add 100 μL of 4 mM TCEP to the filter and pipette to thoroughly mix the TCEP with the antibody.
   h. Incubate in a 37°C water bath for 30 min.

   ⚠ CRITICAL: Do not exceed 30 min for this incubation step.

Table 1. Antibody master mix

Target	Label	Clone	Source	Catalog number
CD11a[a]	143Nd	H155-78	Biolegend	Cat# 153102
CD48[a]	147Sm	HM48-1	Biolegend	Cat# 103433
CD16/32[a]	148Nd	93	Biolegend	Cat# 101335
Ly-6B[a]	149Sm	7/4	Abcam	Cat# ab53457
CD103[a]	151Eu	QA17A24	Biolegend	Cat# 121402
CXCR2[a]	155Gd	SA044G4	Biolegend	Cat# 149302
Ly-6A/E (Sca-1)[a]	158Gd	D7	Biolegend	Cat# 108135
CD90.2/Thy-1.2[a]	161Dy	30-H12	Biolegend	Cat# 105333
CD162[a]	163Dy	4RA10	BD Bioscience	Cat# 557787
CD43[a]	173Yb	S11	Biolegend	Cat# 143202
CD170/siglec-F[a]	170Er	S17007L	Biolegend	Cat# 155502
Ly-6G	141Pr	1A8	Fluidigm	SKU# 3141008B
CD11c	142Nd	N418	Fluidigm	SKU# 3142003B
CD115	144Nd	AFS98	Fluidigm	SKU# 3144012B
CD4	145Nd	RM4-5	Fluidigm	SKU# 3145002B
F4/80	146Nd	BM8	Fluidigm	SKU# 3146008B
CD24	150Nd	M1/69	Fluidigm	SKU# 3150009B
CD3e	152Sm	145-2C11	Fluidigm	SKU# 3152004B
CD274 (PD-L1)	153Eu	10F.9G2	Fluidigm	SKU# 3153016B
TER-119 (Glycophorin A)	154Sm	TER-119	Fluidigm	SKU# 3154005B
CD14	156Gd	Sa14-2	Fluidigm	SKU# 3156009B
CD184 (CXCR4)	159Tb	L276F12	Fluidigm	SKU# 3159030B
CD45R (B220)	160Gd	RA3-6B2	Fluidigm	SKU# 3160012B
Ly-6C	162Dy	HK1.4	Fluidigm	SKU# 3162014B
CD62L (L-selectin)	164Dy	MEL-14	Fluidigm	SKU# 3164003B
CD161 (NK1.1)	165Ho	PK136	Fluidigm	SKU# 3165018B
CD117 (c-Kit)	166Er	2B8	Fluidigm	SKU# 3166004B
CD335 (NKp46)	167Er	29A1.4	Fluidigm	SKU# 3167008B
CD8a	168Er	53-6.7	Fluidigm	SKU# 3168003B
CD206 (MMR)	169Tm	C068C2	Fluidigm	SKU# 3169021B
CD44	171Yb	IM7	Fluidigm	SKU# 3171003B
CD11b (Mac-1)	172Yb	M1/70	Fluidigm	SKU# 3172012B
Ly-6G/C (Gr-1)	174Yb	RB6-8C5	Fluidigm	SKU# 3174008B
CD38	175Lu	90	Fluidigm	SKU# 3175014B
FceR1a	176Yb	Mar-1	Fluidigm	SKU# 3176006B
I-A/I-E	209Bi	M5/114.15.2	Fluidigm	SKU# 3209006B
CD45	89Y	30-F11	Fluidigm	SKU# 3089005B

This table lists the metal conjugated antibodies used for the immunophenotyping in this protocol. The metals listed in the table are Neodymium (Nd), Samarium (Sm), Europium (Eu), Gadolinium (Gd), Dysprosium (Dy), Ytterbium (Yb), Erbium (Er), Praseodymium (Pr), Terbium (Tb), Holmium (Ho), Thulium (Tm), Lutetium (Lu), Bismuth (Bi), and Yttrium (Y).

[a]Indicates the antibodies that are not pre-conjugated, so these must be conjugated in-house from purified antibodies.

3. Upon completion of the 30 min antibody incubation, purify the partially reduced antibody.
   a. Add 300 μL of C-Buffer to the 50 kDa filter to wash.
   b. Centrifuge at 12,000 × *g* at 25°C for 10 min in a microcentrifuge and discard the flow-through.
   c. Add 400 μL of C-Buffer to the 50 kDa filter.
   d. Wait 15–20 min to allow antibody and polymer prep timing to align in an upcoming step.
   e. Centrifuge the reduced antibody at 12,000 × *g* at 25°C for 10 min in a microcentrifuge.
4. Perform the polymer wash.
   a. Add 200 μL of L-Buffer and the 100 μL polymer mixture to a 3 kDa filter.
   b. Centrifuge at 12,000 × *g* at 25°C for 25 min in a microcentrifuge and discard the flow-through.

c. Add 400 μL of C-Buffer and centrifuge at 12,000 × *g* at 25°C for 30 min in a microcentrifuge.

Note: The C-Buffer is a part of the Maxpar® X8 Antibody Labeling Kits specified in the key resources table. This buffer is used in this protocol without any further modification.

Note: To save time, step 4 can be performed simultaneously with steps 2 and 3.

5. Conjugate the antibody.
   a. Retrieve the 3 kDa and the 50 kDa filters from the microcentrifuges and discard the flow-throughs.
   b. To reach a final volume of approximately 80 μL, add 60 μL C-Buffer to the residual volume in the 3 kDa filter and resuspend the polymer by rinsing the filter walls.
   c. Transfer the approximately 80 μL polymer solution from the 3 kDa filter to corresponding antibody solution in the 50 kDa filter.

Note: Accounting for the residual volume in the 50 kDa filter, the final volume should now be approximately 100 μL.

   d. Rinse the walls of the 50 kDa filter and mix the solution gently by pipetting.
   e. Incubate at 37°C in a water bath for 90 min.
6. Wash conjugated antibody.
   a. Add 200 μL of W-Buffer to the 100 μL conjugated antibody mixture within the 50 kDa filter and mix gently by pipetting.

Note: The W-Buffer is a part of the Maxpar® X8 Antibody Labeling Kits specified in the key resources table. This buffer is used in this protocol without any further modification.

   b. Centrifuge at 12,000 × *g* at 25°C for 10 min in a microcentrifuge and discard the flow-through.
   c. Repeat steps 6a and 6b three more times.
7. Determine yield.
   a. Add 80 μL of W-Buffer to the 50 kDa filter to resuspend the conjugated antibody to a final volume of 100 μL.
   b. Rinse the walls of the filter and gently mix the W-Buffer and conjugated antibody by pipetting approximately five timesusing a 200 μL pipette tip.
   c. Using W-Buffer as the blank solution, quantify yield using the NanoDrop™ by measuring absorbance at 280 nm.

Note: In addition to the absorbance, the NanoDrop™ will return a concentration in ng/μL. Given that the total solution should be in 100 μL, calculate the total mass of conjugated antibody. Expected yield is about 60%, which can be calculated by dividing the mass of conjugated antibody by the 100 μg that were initially added in step 2c. (See Equation 1).

$$Concentration\ \frac{ng}{\mu L} \cdot 100\ \mu L \cdot \frac{1\ \mu g}{1000\ ng} = Mass\ \mu g \qquad \text{(Equation 1)}$$

Equation 1: Calculating the mass of conjugated antibody yielded from the protocol. This equation uses the concentration given by the NanoDrop™ reading.

   d. Calculate the volume of Antibody Stabilizer PBS needed for a final antibody concentration of 0.5 mg/mL. (See Equation 2).

$$Mass\ \mu g \cdot \frac{1\ mg}{1000\ \mu g} \cdot \frac{1\ mL}{0.5\ mg} = Volume\ mL \qquad \text{(Equation 2)}$$

Equation 2: Calculating the volume of Antibody Stabilizer PBS needed to achieve a final concentration of 0.5 mg/mL of the conjugated antibody. This equation uses the mass calculated in Equation 1.

8. Store conjugated antibody in its final solution.
   a. Centrifuge 50 kDa filter at 12,000 × *g* at 25°C for 10 min in a microcentrifuge.
   b. Add the calculated volume of Antibody Stabilizer PBS supplemented with 0.05% sodium azide.

   Note: Use approximately **20 µL less** than calculated to account for the residual volume.

   c. Invert the 50 kDa filter into a new collection tube and centrifuge at 1,000 × *g* at 25°C for 2 min in a microcentrifuge.
   d. Label, seal with parafilm, and store the tubes with conjugated antibodies at 4°C.

Titration of antibodies and assessing signal spillover

Timing: 2–3 days

Optimal generation of an antibody panel for mass cytometry requires antibody titrations and checking for signal spillover. The data from the titration experiment is analyzed to identify the optimal concentration, at which, there is a good separation between antibody-labeled positive and negative populations. The spillover test is used to confirm that there is minimal overlap between antibody channels, as this can lead to inaccurate results and false conclusions.

9. Prepare the surface antibody tubes for the spillover test.
   a. Label spillover tubes for the spillover test ("Panel A 1:50", "Panel B 1:50", "Panel C 1:50"), which correlate with the antibodies to be added to them (see Table 2).
   b. In the spillover tube labeled "Panel A", add 36 µL of Maxpar® Cell Staining Buffer and 1 µL of each antibody indicated in the column.
   c. In the spillover tube labeled "Panel B", add 39 µL of Maxpar® Cell Staining Buffer and 1 µL of each antibody indicated in the column except CD16/32 and CD206.
   d. In the spillover tube labeled "Panel C", add 40 µL of Maxpar® Cell Staining Buffer and 1 µL of each antibody indicated in the column.
10. Prepare the surface antibody tubes for the titration test.
   a. Label titration tubes for serial dilutions ("Surface 1:50", "Surface 1:100", "Surface 1:200", "Surface 1:400", "Surface 1:800").
   b. In the "Surface 1:50" tube, add 30 µL of Maxpar® Cell Staining Buffer and 2 µL of each antibody indicated in the column except the highlighted CD16/32 and CD206. Mix well.
   c. Add 50 µL of Maxpar® Cell Staining Buffer to the four other titration tubes.
   d. Perform a serial dilution by adding and mixing 50 µL of the 1:50 tube to the 1:100 tube; then transfer 50 µL from the 1:100 tube to the 1:200 tube, and so on until the 1:800 tube is left with a final volume of 100 µL of surface antibody mix (See Figure 1).
11. Prepare the intracellular antibody tube for the spillover test.
   a. Label a spillover tube "Panel B Intracellular".
   b. To this tube at 98 µL Maxpar® Perm S Buffer and 2 µL of the CD206 antibody.
12. Prepare the intracellular antibody tube for the titration test.
   a. Label titration tubes for serial dilutions ("Intracellular 1:50", "Intracellular 1:100", "Intracellular 1:200", "Intracellular 1:400", "Intracellular 1:800").
   b. In the "Intracellular 1:50" tube, add 196 µL of Maxpar® Perm S Buffer and 4 µL of the CD206 antibody. Mix well.
   c. Add 100 µL of Maxpar® Perm S Buffer to the four other titration tubes.

Table 2. Antibody tubes to detect signal spillover

Tube: Metal	Panel A	Panel B	Panel C
141Pr	Ly-6G		
142Nd		CD11c	
143Nd	CD11a		
144Nd		CD115	
145Nd	CD4		
146Nd			F4/80
147Sm	CD48		
148Nd		CD16/32	
149Sm			Ly-6B
150Nd	CD24		
151Eu			CD34
152Sm		CD3e	
153Eu	CD274 (PD-L1)		
154Sm			TER-119 (Glycophorin A)
155Gd	CXCR2		
156Gd		CD14	
158Gd			Ly-6A/E (Sca-1)
159Tb		CD184 (CXCR4)	
160Gd	CD45R (B220)		
161Dy		CD90.2/Thy-1.2	
162Dy	Ly-6C		
163Dy			CD162
164Dy	CD62L (L-selectin)		
165Ho		CD161 (NK1.1)	
166Er			CD117 (ckit)
167Er		CD335 (NKp46)	
168Er			CD8a
169Tm		CD206 (MMR)	
170Er	CD127		
171Yb			CD44
172Yb	CD11b (Mac-1)		
173Yb		CD43	
174Yb	Ly-6G/C (Gr-1)		
175Lu			CD38
176Yb		FceR1a	
209Bi		I-A/I-E	
89Y	CD45		

The table outlines the metal conjugated antibodies to be added to each tube during the test for assessing signal spillover. The CD16/32 and CD206 (MMR) antibodies are stained at times seperate from the rest of the antibodies. The CD16/32 antibody will be used during the Fc block staining step of the other two tubes. The CD206 antibody is used during the intracellular staining step.

d. Perform serial dilutions by adding and mixing 100 µL of the 1:50 tube to the 1:100 tube; then transfer 100 µL from the 1:100 tube to the 1:200 tube, and so on until the 1:800 tube is left with a final volume of 200 µL of intracellular antibody mix.

13. Prepare the Fc receptor blocking solutions for the spillover tubes.
    a. Label 2 tubes "Panel A&C Fc Block" and "Panel B Fc Block".

Note: The Panel A and Panel C tubes will be receiving a normal Fc Block solution (consisting of anti-CD16/32), so this antibody will be prepared in the same tube in step 13b. The Panel B tube will be receiving the lanthanide-labeled antibody targeting CD16/32 to examine this

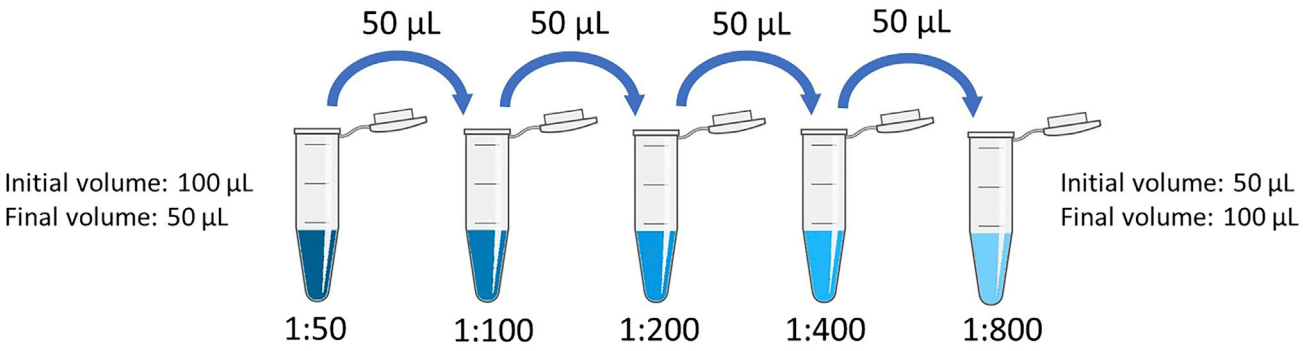

Figure 1. Representation of a serial dilution

The serial dilution step allows one to make the different antibody solutions required for the titration. The tube on the far left depicts the initial solution, where the antibody is in a 1:50 dilution from the stock. The subsequent steps involve pipetting 50 µL from one tube to the next to create the further dilutions. Finally, the tube on the far right contains the most diluted solution.

molecule on the cells, since this molecule is a target in the panel, so it will be prepared separately in step 13c.

b. In the "Panel A&C Fc Block" tube, add 2 µL purified anti-CD16/32 antibody (1 mg/mL) to 98 µL of Maxpar® Cell Staining Buffer to make the 1:50 solution at 0.02 mg/mL.
c. In the "Panel B Fc Block", add 1 µL of the lanthanide conjugated anti-CD16/32 antibody (0.5 mg/mL) to 49 µL of Maxpar® Cell Staining Buffer to make the 1:50 solution at 0.01 mg/mL.

14. Prepare the Fc receptor blocking solutions for the titration tubes.
    a. Label titration tubes for Fc receptor blocking: "Fc Block 1:50", "Fc Block 1:100", "Fc Block 1:200", "Fc Block 1:400", "Fc Block 1:800".
    b. In the 1:50 tube, add 98 µL of Maxpar® Cell Staining Buffer and 2 µL of the lanthanide-conjugated anti-CD16/32 antibody. Mix well.
    c. Add 50 µL of Maxpar® Cell Staining Buffer to the four other Fc receptor blocking titration tubes.
    d. Perform serial dilutions by adding and mixing 50 µL of the 1:50 tube to the 1:100 tube; then transfer 50 µL from the 1:100 tube to the 1:200 tube, and so on until the 1:800 tube is left with a final volume of 100 µL of the Fc receptor blocking solution.
15. Prepare the media solutions (Refer to "materials and equipment" section).
16. Prepare a cell suspension of naïve wild type mouse splenocytes in a concentration of 3×10^6 cells/mL of media.
17. Add 1 mL of splenocyte solution into 8 polypropylene tubes. Discard the rest.
18. Label these tubes as the spillover tubes (Panel A-C) and titration tubes (Titration 1:50–1:800).
19. Spin down the splenocytes at 300 × *g* for 5 min at 4°C.
20. Resuspend the cells at 2×10^7 cells/mL of media.
21. Follow the "cisplatin (cell viability) staining" section protocol below.
22. Centrifuge the cells at 300 × *g* for 5 min at 4°C and discard the supernatant.
23. Resuspend the pellets in 50 µL of the Fc receptor blocking solution which corresponds to each tube.
24. Incubate at 25°C for 10 min.
25. Wash the cells.
    a. Add 2 mL of Maxpar® Cell Staining Buffer.
    b. Centrifuge at 300 × *g* at 4°C for 5 min.
    c. Discard supernatant.
26. Gently pipette to resuspend the pellet in 50 µL of the surface antibody mix which corresponds to each tube (see Table 3).

Table 3. Staining solutions added to each tube

Cell solution tube	Surface antibody	Intracellular antibody	Fc block
Panel A	Panel A	No Antibody	Panel A&C Fc Block
Panel B	Panel B	Panel B Intracellular	Panel B Fc Block
Panel C	Panel C	No Antibody	Panel A&C Fc Block
Titration 1:50	Surface 1:50	Intracellular 1:50	Fc Block 1:50
Titration 1:100	Surface 1:100	Intracellular 1:100	Fc Block 1:100
Titration 1:200	Surface 1:200	Intracellular 1:200	Fc Block 1:200
Titration 1:400	Surface 1:400	Intracellular 1:400	Fc Block 1:400
Titration 1:800	Surface 1:800	Intracellular 1:800	Fc Block 1:800

27. Incubate for 30 min total at 25°C, gently vortexing the tubes at the 15 min halfway point.
28. Wash the cells as done is step 25.
29. Repeat the wash one additional time.
30. Follow the "fixing and intracellular antibody staining" section protocol below using the intracellular antibody mix which corresponds for each tube (see Table 3).

Note: Since spillover tubes for Panel A and Panel C do not have the stains for intracellular antibodies, add Maxpar® Perm S Buffer with no antibodies in place of intracellular antibody stain.

31. Follow the "fresh fix and DNA intercalator staining" section protocol below.

Pause point: The cells can be left up to 2 days at 4°C until acquisition.

32. Follow the "sample acquisition on CyTOF® instrument" protocol below.
33. Follow the "normalization" section protocols below.
34. Using Cytobank, as described in the "expected outcomes" section, gate for the cell populations using the sample antibodies for each concentration.
35. Assess spillover by comparing the antibody positive cells in samples stained with either "Panel A", "Panel B", or "Panel C" antibody mixes (see Table 2). There is no spillover if the cells stained with each antibody panel are only positive for the antibodies they were stained for. If samples are identified as positive for antibodies that were not added in that panel's antibody mix, there is most likely spillover. In this case, you would need to use a lower concentration of antibodies that are of the same element and adjacent atomic weight within that panel.

Note: For example, there would be spillover if a sample stained with "Panel B" antibody mix has cells that are positive for 143Nd, since the 143Nd-labeled antibody is not present in "Panel B" (see Table 2). Thus, you would need to use a lower concentration of the 142Nd-labeled and 144Nd-labeled antibodies in the main experiment to minimize their spillover into the 143Nd channel, since the 142Nd-labeled and 144Nd-labeled antibodies in "Panel B" are spilling over into the 143Nd channel.

36. Compare the ability of the antibodies to bind and identify the positive cell population using the different antibody dilutions (See Figure 2). The lowest antibody concentration that allows for clearly discerning the positive and negative populations of the antibody-targeted molecule should be selected and used for the main experiment.

⚠ CRITICAL: Give close attention to choosing the optimal dilution for each antibody. This determines the antibody dilutions to use for the remainder of the protocol.

Processing the tissues into the single cell suspension

Timing: 2.5 h (this time is for processing 6 mouse samples)

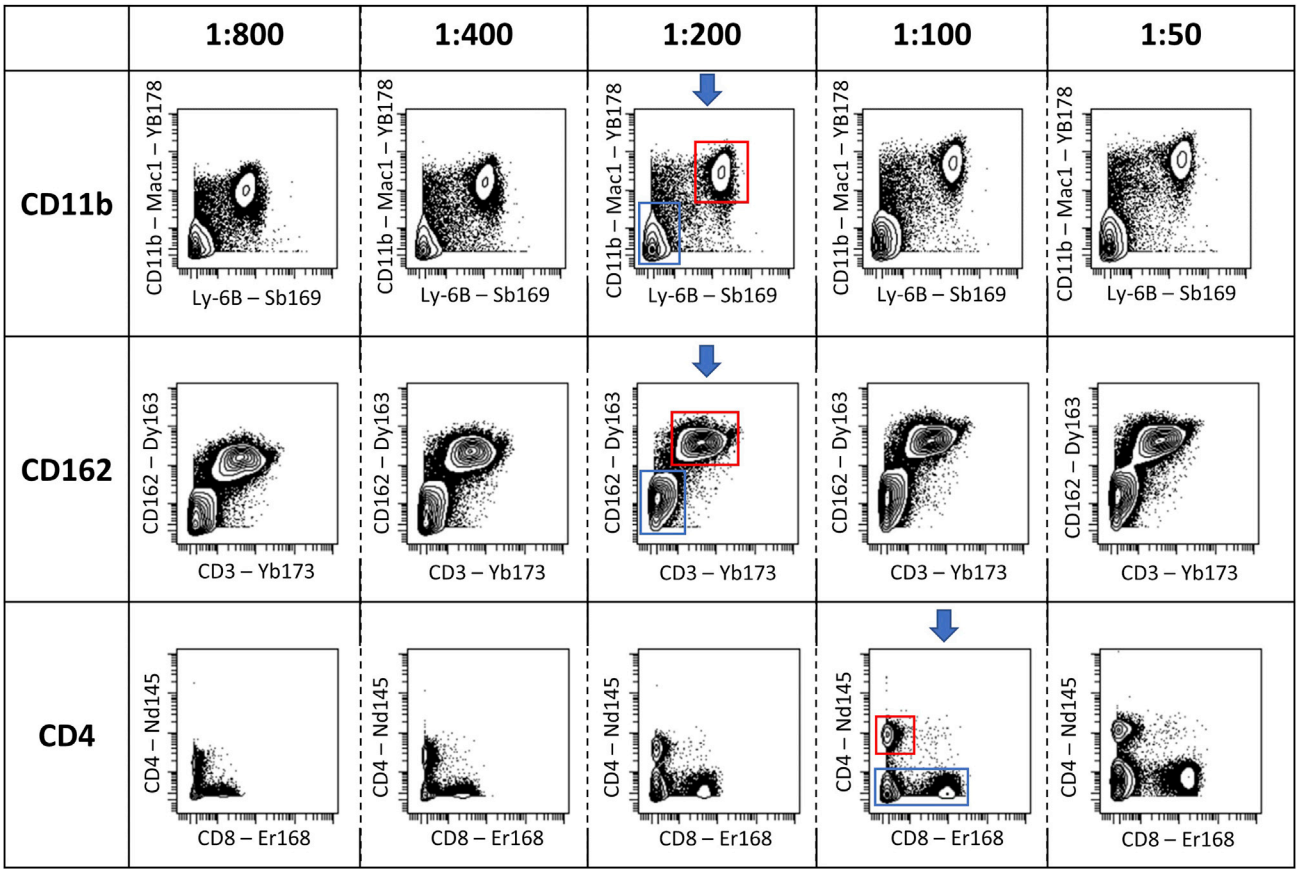

Figure 2. Example selection of optimal concentration of antibodies from titration data

The titration step is used to determine the best concentration of antibody for use in the staining process for the main experiment. This CD11b, CD162, and CD4 titration data is an example of the gates generated from these antibodies at different concentrations. The blue arrow indicates it as the selected concentration for further use in identifying the positive cell populations. The red and blue boxes in the selected graphs identify the positive and negative cell populations, respectively.

This step details the process of generating a single cell suspension from the glioma-harboring GEMM and isolating the immune cells for staining.

37. Prepare materials.
    a. Per sample label: one 50 mL conical tube; two 15 mL conical tubes; one 70 μm cell strainer and one cryovial.
    b. Place the cell strainer into the 50 mL conical tube.
    c. Wet the cell strainer by adding 2 mL of media.
    d. Insert the conical tubes into an ice bucket.
38. Euthanize mouse and begin tissue collection.
    a. Inside a fume hood, add 1 mL isoflurane to a cotton pad.
    b. Place the isoflurane-soaked cotton pad to the bottom of the Nalgene™ Polypropylene Desiccator chamber and cover that area with the desiccator plate. quickly cover the desiccator to prevent vaporized isoflurane from escaping the desiccator chamber.
    c. Place the mouse into the chamber and cover the chamber.
    d. Once mouse stops all movement, wait for 20 more seconds.
    e. Confirm the mouse is euthanized by toe pinch and perform decapitation.

Note: For isoflurane euthanasia, any chamber that allows for administration of highly concentrated, vaporized isoflurane without the mouse coming into direct contact with the liquid

isoflurane can be used in place of the Nalgene™ Polypropylene Desiccator. Additionally, alternative forms of euthanasia, such as a cervical dislocation, may be used in place of isoflurane overdose as long as they do not elicit alterations in the cell characteristics.

39. Process the tumor to generate a single cell suspension.
    a. Dissect the brain and place it onto a petri dish with enough media to cover the surface of the dish.
    b. Dissect the tumor mass under a stereo-microscope and place it on the 70 micron strainer.

Note: The GEMM tumors used in this protocol expressed GFP and were dissected based on fluorescence. Although not optimal, the dissection of tumors without a reporter can be conducted by location, appearance, and texture. Upon examining the area of the brain where the tumor cells were implanted, features such as changes in color and texture vary between healthy brain and tumors. These features can be used to dissect the murine brain tumors lacking reporters.

    c. Dissociate the tumor by applying mechanical pressure with the end of a syringe plunger to push the tumor through the strainer.
    d. Rinse the strainer four times using 10 mL of media, making sure to keep the tube on ice.
40. Isolate the immune cells.
    a. Prepare the stock isotonic Percoll (SIP) and 70% Percoll solutions.

Note: Refer to "materials and equipment" section for instructions on how to prepare the SIP and media solutions.

    b. Centrifuge the tumor cells at 300 × *g* at 4°C for 5 min.
    c. Discard the supernatant and resuspend the pellet in 1 mL of media.
    d. Add the 1 mL cell suspension to 6 mL of media in a 15 mL conical tube labeled "Tumor with Percoll".
    e. Add 3 mL of the SIP solution and mix well.
    f. Slowly insert a 1 mL serological pipet filled with the 70% Percoll solution along the side of the tube until it reaches the bottom.
    g. Slowly layer 1 mL of 70% Percoll at the bottom of the tube.

⚠ CRITICAL: Exercise caution to very slowly add the 70% Percoll, pipetting too quickly will disturb the gradient and mix the layers.

    h. Centrifuge the tube with the gradient at 800 × *g* for 20 min at 25°C.

⚠ CRITICAL: This centrifugation step must be completed with the brakes off to prevent disrupting the gradient.

    i. During centrifugation from the previous step, add 10 mL of media to a 15 mL conical tube labeled "Tumor after Percoll".
    j. Remove the tube from the centrifuge and use a transfer pipette to discard the top layers (containing debris, tumor cells, SIP-media solution) until it approaches the central white-cloudy immune cell layer located between the pink and clear layers (see Figure 3).

⚠ CRITICAL: Be careful not to disrupt the gradient when moving the tube.

    k. Use another transfer pipette to remove the immune cell layer completely and resuspend it in the labeled "Tumor after Percoll" tube.
    l. Centrifuge the tube at 300 × *g* for 5 min at 4°C.
    m. Discard the supernatant and resuspend in 1 mL of freezing media.
    n. Transfer to a labeled cryovial.

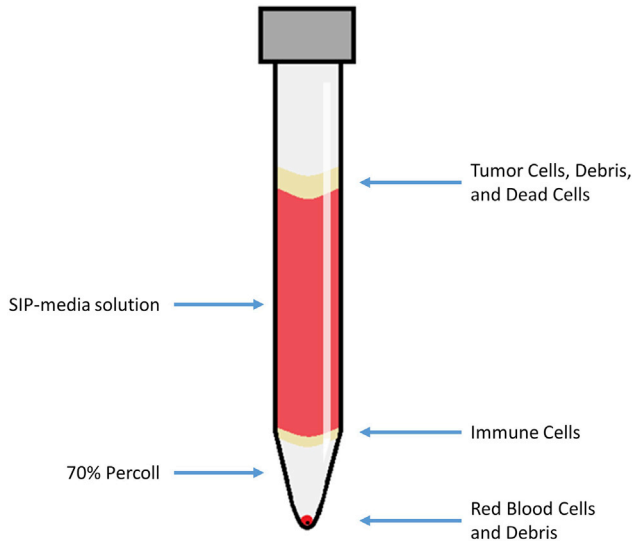

Figure 3. Completed percoll gradient

From top to bottom, the tube will contain a white layer (tumor cells, debris, and dead cells), a pink layer (30% SIP media solution), a white-cloudy layer (immune cells), a clear layer (70% Percoll solution), and a dark red layer (red blood cells and debris).

o. Immediately place the cryovial in a –20°C freezer for one hour before transferring to a −80°C freezer for 12–18 h.

Note: It is recommended to stain and fix samples for mass cytometry on the same day rather than freezing, if possible.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-Mouse CD45 (30-F11)-89Y—100 Tests	Fluidigm	SKU# 3089005B
Anti-Mouse Ly-6G (1A8)-141Pr—100 Tests	Fluidigm	SKU# 3141008B
Anti-Mouse CD11c (N418)-142Nd—100 Tests	Fluidigm	SKU# 3142003B
Anti-Mouse CD115 (AFS98)-144Nd—100 Tests	Fluidigm	SKU# 3144012B
Anti-Mouse CD4 (RM4-5)-145Nd—100 Tests	Fluidigm	SKU# 3145002B
Anti-Mouse F4/80 (BM8)-146Nd—100 Tests	Fluidigm	SKU# 3146008B
Anti-Mouse CD24 (M1/69)-150Nd—100 Tests	Fluidigm	SKU# 3150009B
Anti-Mouse CD3e (145-2C11)-152Sm—100 Tests	Fluidigm	SKU# 3152004B
Anti-Mouse CD274/PD-L1 (10F.9G2)-153Eu—100 Tests	Fluidigm	SKU# 3153016B
Anti-Mouse TER-119 (TER-119)-154Sm—100 Tests	Fluidigm	SKU# 3154005B
Anti-Mouse CD14 (Sa14-2)-156Gd—100 Tests	Fluidigm	SKU# 3156009B
Anti-Mouse CD184/CXCR4 (L276F12)-159Tb—100 Tests	Fluidigm	SKU# 3159030B
Anti-Mouse CD45R/B220 (RA3-6B2)-160Gd—100 Tests	Fluidigm	SKU# 3160012B
Anti-Mouse Ly-6C (HK1.4)-162Dy—100 Tests	Fluidigm	SKU# 3162014B
Anti-Mouse CD62L (MEL-14)-164Dy—100 Tests	Fluidigm	SKU# 3164003B
Anti-Mouse NK1.1 (PK136)-165Ho—100 Tests	Fluidigm	SKU# 3165018B
Anti-Mouse CD117/c-kit (2B8)-166Er—100 Tests	Fluidigm	SKU# 3166004B
Anti-Mouse Nkp46 (29A1.4)-167Er—100 Tests	Fluidigm	SKU# 3167008B
Anti-Mouse CD8a (53-6.7)-168Er—100 Tests	Fluidigm	SKU# 3168003B
Anti-Mouse CD206/MMR (C068C2)-169Tm—100 Tests	Fluidigm	SKU# 3169021B
Anti-Human/Mouse CD44 (IM7)-171Yb—100 Tests	Fluidigm	SKU# 3171003B

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Anti-Mouse CD11b (M1/70)-172Yb—100 Tests	Fluidigm	SKU# 3172012B
Anti-Mouse Ly-6G/C/Gr-1 (RB6-8C5)-174Yb—100 Tests	Fluidigm	SKU# 3174008B
Anti-Mouse CD38 (90)-175Lu—100 Tests	Fluidigm	SKU# 3175014B
Anti-Mouse FceR1a (MAR-1)-176Yb—100 Tests	Fluidigm	SKU# 3176006B
Anti-Mouse I-A/I-E (M5/114.15.2)-209Bi—100 Tests	Fluidigm	SKU# 3209006B
BD Pharmingen™ Purified NA/LE Rat Anti-Mouse CD162	BD Bioscience	Cat# 557787
Purified anti-mouse CD43 Antibody	BioLegend	Cat# 143202
Purified anti-mouse CD170 (Siglec-F) Antibody	BioLegend	Cat# 155502
Purified anti-mouse CD11a Antibody	BioLegend	Cat# 153102
Purified anti-mouse CD48 Antibody	BioLegend	Cat# 103433
Purified anti-mouse CD16/32 Antibody	BioLegend	Cat# 101335
Purified anti-mouse CD103 Antibody	BioLegend	Cat# 121402
Purified anti-mouse Sca-1 Antibody	BioLegend	Cat# 108135
Purified anti-mouse CD90.2/Thy1.2 Antibody	BioLegend	Cat# 105333
Purified anti-mouse CD182 (CXCR2) Antibody	BioLegend	Cat# 149302
Anti-Neutrophil antibody [7/4]	Abcam	Cat# ab53457
Chemicals, peptides, and recombinant proteins		
Bond-Breaker™ TCEP Solution, Neutral pH	Thermo Scientific	Cat# 77720
Isoflurane	Fluriso™	Cat# 501017
DMEM, high glucose, HEPES	Gibco	Cat# 12430054
Fetal Bovine Serum, qualified, USDA-approved (FBS)	Gibco	Cat# 10437028
DPBS, no calcium, no magnesium	Gibco	Cat# 14190250
RBC Lysis Buffer (10×)	BioLegend	Cat# 420301
Dimethyl Sulfoxide (DMSO)	Fisher Scientific	CAS: 67-68-5
HBSS (1×), no calcium, no magnesium, no phenol red	Gibco	Cat# 14175095
HBSS (10×), no calcium, no magnesium, no phenol red	Gibco	Cat# 14185052
Cytiva Percoll Centrifugation Media (Percoll)	Fisher Scientific	Cat# 45-001-747
Maxpar® Cell Staining Buffer	Fluidigm	Cat# 201068
Maxpar® Perm-S Buffer	Fluidigm	Cat# 201066
Maxpar® Fix I Buffer (5×)	Fluidigm	Cat# 201065
Maxpar® Fix and Perm Buffer	Fluidigm	Cat# 201067
Maxpar® PBS	Fluidigm	Cat# 201058
Maxpar® Water	Fluidigm	Cat# 201069
EQ™ Four Element Calibration Beads	Fluidigm	Cat# 201078
Cell-ID™ Intercalator–Ir [125 μM]	Fluidigm	Cat# 201192A
Cell-ID™ Cisplatin	Fluidigm	Cat# 201064
Trypan Blue Solution, 0.4%	Gibco	Cat# 15250061
Electron Microscopy 16% Paraformaldehyde Aqueous Solution, EM Grade, Bottle 100 mL	Fisher Scientific	Cat# 50-980-488
PBS Antibody Stabilizer	Boca Scientific Inc.	Cat# 131000
Sodium azide, 99%, extra pure, Thermo Scientific™	Fisher Scientific	Cat# AC190381000
Critical commercial assays		
Maxpar® X8 Antibody Labeling Kit, 143Nd—4 Rxn	Fluidigm	SKU 201143A
Maxpar® X8 Antibody Labeling Kit, 147Sm—4 Rxn	Fluidigm	SKU 201147A
Maxpar® X8 Antibody Labeling Kit, 148Nd—4 Rxn	Fluidigm	SKU 201148A
Maxpar® X8 Antibody Labeling Kit, 149Sm—4 Rxn	Fluidigm	SKU 201149A
Maxpar® X8 Antibody Labeling Kit, 151Eu—4 Rxn	Fluidigm	SKU 201151A
Maxpar® X8 Antibody Labeling Kit, 155Gd—4 Rxn	Fluidigm	SKU 201155A
Maxpar® X8 Antibody Labeling Kit, 158Gd—4 Rxn	Fluidigm	SKU 201158A
Maxpar® X8 Antibody Labeling Kit, 161Dy—4 Rxn	Fluidigm	SKU 201161A
Maxpar® X8 Antibody Labeling Kit, 163Dy—4 Rxn	Fluidigm	SKU 201163A
Maxpar® X8 Antibody Labeling Kit, 170Er—4 Rxn	Fluidigm	SKU 201170A
Maxpar® X8 Antibody Labeling Kit, 173Yb—4 Rxn	Fluidigm	SKU 201173A
Maxpar® Nuclear Antigen Staining Buffer Set—120 Tests	Fluidigm	SKU 201063

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Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Experimental models: Organisms/strains		
Sleeping Beauty-Mediated Mouse Glioma C57BL/6 Model (male/female) (upon showing symptoms of tumor development; 2–4 months old)	Developed in-house	Calinescu et al. (2015); Garcia-Fabiani et al. (2020)
Software and algorithms		
CyTOF® Software v7.0	Fluidigm	https://go.fluidigm.com/cytofsw/v7
Cytobank	Cytobank Inc.	https://premium.cytobank.org/cytobank/
Other		
50 mL Centrifuge tube Polypropylene, Conical Bottom	Alkali Scientific Inc.	Cat# CW 5603
15 mL Centrifuge tube Polypropylene, Conical Bottom	Alkali Scientific Inc.	Cat# CW 5601
Fisherbrand™ 70 µm Sterile Cell Strainers	Fisher Scientific	Cat# 22-363-548
Fisherbrand™ 40 µm Sterile Cell Strainers	Fisher Scientific	Cat# 22-363-547
Fisherbrand™ Disposable Graduated Transfer Pipettes	Fisher Scientific	Cat# 13-711-9AM
Parafilm™ M Wrapping Film	Fisher Scientific	Cat# S3-594-6
Falcon™ Round-Bottom Polypropylene Test Tubes with Cap	Fisher Scientific	Cat# 14-959-11A
Fisherbrand™ Premium Microcentrifuge Tubes: 1.5 mL	Fisher Scientific	Cat# 05-408-129
1.8 Self-Standing Cryovial Internal Thread Q7350	Alkali Scientific Inc.	Cat# VCI18-C
Covidien™ Monoject™ 1 mL Tuberculin Syringe	Fisher Scientific	Cat# 22-257-154
Fisherbrand™ Petri Dishes round 100 mm × 15 mm round	Fisher Scientific	Cat# FB0875713
1,000 µL Reach Ergonomic Filter Pipet Tips	Genesee Scientific	Cat #: 24-430
10 µL Reach Ergonomic Filter Pipet Tips	Genesee Scientific	Cat #: 24-401
200 µL Ergonomic Filter Pipet Tips	Genesee Scientific	Cat #: 24-412
20 µL Ergonomic Filter Pipet Tips	Genesee Scientific	24-404
Nalgene™ Autoclavable Polypropylene Desiccator	Fisher Scientific	5315-0150PK
Allegra™ 6R Refrigerated Benchtop Centrifuge	Beckman Coulter	N/A
Fisherbrand™ Mini-Centrifuge 100–240 V, 50/60 Hz Universal Plug, Gray	Fisher Scientific	12-006-901
Fisherbrand™ accuSpin™ Micro 17R Microcentrifuge	Fisher Scientific	S39740
VWR® Standard Heavy-Duty Vortex Mixer	VWR	97043-562
Thermo Scientific™ Precision™ General-Purpose Water Bath	Fisher Scientific	15-474-22
Hausser Scientific Bright Line™ Counting Chamber (Hemacytometer)	Fisher Scientific	02-671-51A
Thermo Scientific™ NanoDrop™ OneC Microvolume UV-Vis Spectrophotometer	Fisher Scientific	027420P21H2
Olympus Stereomicroscope System SZX16	Olympus	N/A
Helios™, A CyTOF® System	Fluidigm	N/A

Note: The antibody dilutions vary based on the several factors such as the efficiency of the conjugation. Thus, it is optimal to always do the antibody titration when using a new batch of antibodies or using it on a different tissue type (See "titration of antibodies and assessing signal spillover" in the "before you begin" section). Step 35 of "Before you Begin" and Figure 2 outline choosing the best dilution used for the rest of the protocol.

MATERIALS AND EQUIPMENT

Freezing Media		
Reagent	Final concentration	Amount
FBS	90%	9 mL
DMSO	10%	1 mL
Total	**N/A**	**10 mL**

Store at 4°C up to 3 weeks.

Media		
Reagent	Final concentration	Amount
DMEM	90%	450 mL
FBS	10%	50 mL
Total	**N/A**	**500 mL**
Store at 4°C up to 3 weeks.		

Serum-free media		
Reagent	Final concentration	Amount
DMEM	100%	500 mL
Total	**N/A**	**500 mL**
Store at 4°C up to 3 weeks.		

Stock Isotonic Percoll (SIP)		
Reagent	Final concentration	Amount
Percoll	90%	27 mL
HBSS (10×), no calcium, no magnesium, no phenol red	10%	3 mL
Total	**N/A**	**30 mL**
Prepare the day of experiment and leave at 25°C.		

70% Percoll		
Reagent	Final concentration	Amount
SIP	70%	5.6 mL
HBSS (1×), no calcium, no magnesium, no phenol red	30%	2.4 mL
Total	**N/A**	**8 mL**
Prepare the day of experiment and leave at 25°C.		

STEP-BY-STEP METHOD DETAILS

Thaw and prepare samples for staining

Timing: 20 min

Here, frozen samples are used to generate a single-cell suspension for staining. If the cells were not previously frozen, steps 3–5 should be skipped.

1. Warm media and serum-free media to 37°C using a water bath.

   Note: The media recipes are in the "materials and equipment" section.

2. Prepare a beaker with bleach for liquid waste.

   Note: The amount of bleach should be enough so that the end volume has at least 10% bleach.

3. Label 15 mL conical tubes for the samples, which will be added.
4. Add 9 mL of media into the labeled 15 mL conical tubes.
5. Thaw the samples and quench the freezing media.
   a. Place the cryovials into the 37°C water bath for about a min or until about 80% thawed.
   b. Transfer the thawed cell suspensions into the labels 15 mL conical tubes.

⚠ CRITICAL: Once thawed, frozen cell must be transfer quickly to media to minimize toxic exposure to DMSO.

6. Centrifuge the cell suspensions for 5 min at 300 × *g*.
7. Discard the supernatant and resuspend in 1 mL of medium.
8. Count the cells using a hemacytometer.
9. Centrifuge for 5 min at 300 × *g* and discard supernatant.
10. Resuspend the cells at 2 × 10^7 cells/mL of warm serum-free media.

Cisplatin (cell viability) staining

Timing: 30 min

Cisplatin is used for identifying dead cells during data analysis.

11. Prepare 10 uM (2×) Cell-ID™ Cisplatin working solution.
    a. Thaw Cell-ID™ Cisplatin stock aliquot to 25°C.
    b. Add 2 μL of 5 mM (1,000×) Cell-ID™ Cisplatin stock to 998 μL of warm serum-free media.

Note: The volumes listed for the preparation of the 10 uM (2×) Cell-ID™ Cisplatin working solution are there as an example. The volume necessary will vary based on the volume of the sample cell suspension. It is recommended to prepare approximately 10% more 10 uM (2×) Cell-ID™ Cisplatin working solution than the volume of the sample cell suspension to account for error.

12. Add an equal volume of the 10 μM Cell-ID™ Cisplatin working solution to the cell suspension solution making a final Cell-ID™ Cisplatin concentration of 5 μM (1×). Pipette to mix well.
13. Incubate for 5 min at 25°C.
14. Quench by adding warmed media at 5× the volume of the stained cells.
15. Centrifuge at 300 × *g* for 5 min. Discard the supernatant. Resuspend the pellet in 4 mL of Maxpar® Cell Staining Buffer.
16. Count the cells using a hemacytometer.
17. Centrifuge at 300 × *g* for 5 min. Discard the supernatant.
18. Resuspend the cells at a concentration of 1–3 million cells in 50 μL of Maxpar® Cell Staining Buffer.
19. Transfer the cells to 5 mL polypropylene tubes.

Fc receptor blocking and surface antibody staining

Timing: 1 h 45 min

Here, Fc receptors are blocked to prevent non-specific binding of receptors to the constant domain (Fc region) of the antibodies. The antibodies are then added to bind and label the targeted cell surface molecules.

20. Centrifuge the cells at 300 × *g* for 5 min. Discard the supernatant.
21. 50 μL of Fc Block is prepared per sample by diluting the lanthanide-conjugated anti-CD16/32 antibody at the appropriate concentration determined by the titration.

Note: The Fc Block solution can be prepared during the previous centrifugation step to save time.

22. Resuspend the pellet in 50 μL of Fc Block (anti-CD16/32 antibody solution).

23. Incubate at 25°C for 10 min.
24. To prepare the surface antibody master mix, add the surface antibodies shown in Table 1 (in this case, all antibodies except anti-CD206 and anti-Gr-1) to Maxpar® Cell Staining Buffer to make 50 μL of antibody solution per sample.

    Note: The volume of each antibody used for the master mix is based on the concentration determined by the titration (refer to the "titration of antibodies and assessing signal spillover" section).

    Note: CD206 is stained during the intracellular staining process. Anti-Gr-1 recognizes both Ly6G and Ly6C at different binding sites than either clone of the Ly6G and Ly6C antibodies used in this panel. However, to avoid any potential steric hindrance, it is recommended to do sequential staining for anti-Ly6G and anti-Ly6C, followed by anti-Gr-1.

25. Add 50 μL of surface antibody master mix to the Fc Block-cell suspension. Gently pipette to mix.
26. Incubate for 30 min total at 25°C, gently vortexing the tubes at the 15 min halfway point.
27. Prepare anti-Gr-1 antibody at 2× the recommended concentration determined by the titration.
28. Add 50 μL of anti-Gr-1 and incubate for an additional 30 min at 25°C, gently vortexing the tubes at the 15 min halfway point.
29. Wash the cells.
    a. Add 2 mL of Cell Staining Buffer.
    b. Centrifuge at 300 × *g* for 5 min.
    c. Discard supernatant.
30. Repeat the wash for a total of 2 washes.

Fixing and intracellular antibody staining

Timing: 2 h

The cells are fixed and permeabilized to allow for entry of the intracellular antigens staining antibodies, which then bind to and label the targeted intracellular antigens.

31. Gently vortex to resuspend the cell pellet in 1 mL of 1× Maxpar® Fix I Buffer.
32. Incubate for 30 min at 25°C to fix the cells.
33. During incubation, make 100 μL intercellular antibody master mix per sample by adding the intracellular antibodies to Maxpar® Perm-S Buffer. In this case, anti-CD206 is the only antibody used for intracellular labeling.

    Note: The volume of each antibody used for the master mix is based on the concentration determined by the titration (refer to the "titration of antibodies and assessing signal spillover" section).

    Note: If the intracellular target is a transcription factor or an intranuclear protein, it is recommended to perform the permeabilization using the Maxpar® Nuclear Antigen Staining Buffer Set (Fluidigm, Cat# 201063).

34. Wash the cells.
    a. Add 2 mL of Maxpar® Perm-S Buffer.
    b. Centrifuge at 800 × *g* for 5 min.
    c. Discard supernatant.
35. Repeat the wash for a total of 2 washes.
36. Gently resuspend the pellet in 100 μL of intracellular antibody stain.
37. Incubate for 30 min at 25°C.

38. Wash the cells two more times as done in step 34.

Fresh fix and DNA intercalator staining

Timing: 45 min, left overnight (12–18 h)

In this step, the cells are stained with DNA intercalator, which allows for downstream identification of cell singlets. As DNA intercalator binds to nucleic acids, and cell-cell doublets have more DNA, doublets have a stronger DNA intercalator signal than singlets.

39. Prepare 1 mL of 1.6% paraformaldehyde (PFA) per sample from the 16% Paraformaldehyde Aqueous Solution with Maxpar® PBS.

Note: The 1.6% paraformaldehyde solution should be prepared fresh before use.Resuspend the cell pellet in 1 mL of 1.6% PFA.

40. Incubate for 20 min at 25°C.

Note: Fixation is a very important step to allow entry of the DNA intercalator into the nucleus. Fixation also preserves the cells from the hypotonic solution (deionized H_2O) used during data acquisition.

41. Centrifuge at 800 × *g* for 5 min. Discard supernatant.
42. Gently vortex to resuspend the pellet in the residual solution.
43. Prepare 1 mL intercalation solution per sample by diluting 125 uM Cell-ID™ Intercalator (1,000×) stock solution to 125 nM (1×) with Maxpar® Fix and Perm Buffer.

Note: This solution can be prepared during the centrifugation in step 41 to save time.

44. Add 1 mL of the 1× intercalation solution and gently vortex to mix.
45. Incubate overnight (12–18 h) at 4°C to label the nucleated cells.

Pause point: The cells can be left up to 2 days at 4°C until acquisition.

Sample acquisition on CyTOF® instrument

Timing: 1 day (3–4 h per sample)

It is important to run the samples along with Fluidigm's EQ™ Four Element Calibration Beads, which allow for changes during sample acquisition to be monitored. The steps for sample acquisition and monitoring are as follow:

46. Wash the cells.
    a. Add 3 mL of Maxpar® Cell Staining Buffer.
    b. Centrifuge at 800 × g for 5 min.
    c. Discard supernatant.
47. Resuspend the cells in 1 mL of Milli-Q water and count the cells.
48. Add an additional 3 mL to wash the cells and centrifuge at 800 × g for 5 min.
49. Resuspend the pelleted cells with Milli-Q water containing 0.1× EQ™ Four Element Calibration Beads (diluted from 1× stock bottle) according to cell count to achieve 8–10 × 10^5 cells/mL.
50. Filter cells with a 40 μm cell strainer prior to introduction into the Helios™, a CyTOF® System.

Note: Changes in sample acquisition can happen due to variations in instrument performance. Because of these variations in instrument performance, quality control of the instrument before and during sample acquisition is essential.

Note: This protocol focuses on the preparation and staining of cell suspensions along with the analysis of mass cytometry data. The process of acquiring the sample data is beyond the scope of this protocol. As such, samples should be submitted to a mass cytometry core specifically trained to run the samples for data acquisition.

Normalization

Timing: 1 h

Data normalization is crucial to minimize technical variation. This process can be performed using the Fluidigm CyTOF® software, which uses a standard "Bead passport" with standardized target values for the calibration beads. Using this tool, a bead's signal is detected and recorded throughout the sample acquisition, and the median intensity is calculated within a user-defined time interval. By assuming a linear rate of signal decay, the rate constant of signal decay in each interval is calculated against the global mean of signal intensity. Every event within the specified interval is multiplied by the rate constant.

51. Using the CyTOF® software, select the raw FCS file, choose the "Process" tab, and select FCS processing.
52. Select the corresponding bead passport to the EQ™ Four Element Calibration Beads lot number used in data acquisition.
53. Select the time interval that the normalization factor will be applied to.

Note: By default, the software will use a 100 s interval.

Note: Another method of normalization was developed by the Nolan lab to mitigate the impact of signal drift on the data.

Concatenation (optional)

Timing: 1 h

Sample acquisition may need to be run in various rounds because of the volume of the sample or a change in system performance during acquisition, such as a clog or unexpected shutdown. This results in multiple FCS files for a single sample which can then be concatenated into a single file.

54. In the CyTOF® Software, select the FCS processing tab. Then select the raw FCS files to concatenate.
55. Click on the "concatenate" tab. The concatenated FCS file will be generated and ready for export.

⚠ CRITICAL: Make sure that the original data box is checked.

⚠ CRITICAL: Route the name and the directory of the new concatenated file. The normalization process will take place automatically once you concatenate.

Note: Instead of the CyTOF® software, concatenation may be performed using Cytobank. However, data must be normalized before using this tool.

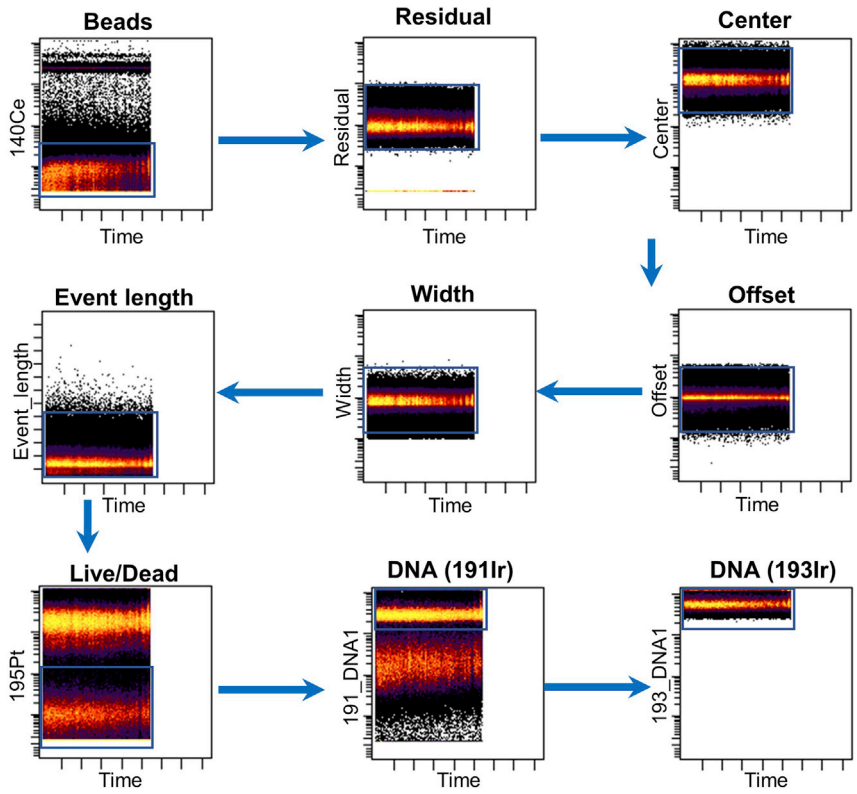

Figure 4. Recommended gating strategy using the using Gaussian parameters for data cleanup

EXPECTED OUTCOMES

Due to the large number of antibodies used to run the mass cytometry panel, data analysis can be complex. This is particularly true in situations that require annotation of subpopulations that express a previously unidentified marker. However, mass cytometry analysis is facilitated by a number of publicly available analysis tools that rely mainly on dimensionality reduction. Several publications examine these tools in higher detail, which are not within the scope of this protocol (Kimball et al., 2018; Liu et al., 2019; Matos et al., 2017; Weber and Robinson, 2016). Here, we will discuss some commonly used advanced analytical tools available in Cytobank in the context of the questions they were designed to answer. We will also discuss the advantages and disadvantages of each analysis method and the ideal situation in which to run each one of them, which depends heavily on the biological question being asked.

QUANTIFICATION AND STATISTICAL ANALYSIS

A standard mass cytometry analysis workflow consists of the following steps: (1) Identification of different cell subsets using manual or automated gating detection, (2) Applying a clustering algorithm to detect the abundance of a specific cell subset and/or differential biomarker expression in cluster subsets across different conditions, and (3) visualization of cell clusters using dimensionality reduction.

Data visualization and gating

The first step of mass cytometry data analysis is performing data cleanup. This consists of the removal of unwanted events, dead cells, debris, normalization beads, true aggregates, and coincident ion clouds from raw data. The most common approach is to use the Gaussian distribution-based models available on Fluidigm Helios™ and CyTOF® instruments. The four Gaussian

parameters, residual, offset, center, and width, can be used to describe the shape of the distribution of pushes in a pulse, where a push is each instance of ions being sent to the detector and an individual pulse is composed of pushes integrated together to form an event (see Figure 4) (Lee and Rahman, 2019). These parameters are valuable for detecting well-formed pulses, eliminating coincident positive ion clouds, and to further exclude cell doublets. An example of using Gaussian-based parameter for single cell gating is illustrated in Figure 4.

To perform data cleanup:

1. Gate out the EQ™ Four Element Calibration Beads by plotting 140Ce against Time.

   Note: 140Ce is tagged to the EQ™ Four Element Calibration Beads. 195Pt is from the Cell-ID™ Cisplatin. 191Ir and 193Ir are from the Cell-ID™ Intercalator.

2. Gate on the four Gaussian parameters sequentially (i.e., residual, center, offset, width) to eliminate unwanted non-Gaussian pulses as shown in Figure 4.
3. Select the ideal metal ion content and ion cloud size by plotting Event Length vs Time.
4. Gate on the live cells by plotting 195Pt vs time.
5. Remove doublets by applying two DNA gates (i.e., 191Ir, and 193Ir) vs Time. In both plots, the doublets will be the cloud with higher DNA content.

Automated gating

After data cleanup, the next step is to identify the different cell populations. This can be done using supervised or unsupervised gating to identify populations of interest. Below we will compare the advantages and the disadvantages of each method.

Unsupervised gating

This approach is done via clustering methods which place cell populations with similar phenotypes together in a low dimensional space. This allows the discovery of unknown cell populations from high dimension data analysis in a large dataset by comparing two groups (such as, control vs treated). A variety of advanced approaches are available to perform such analysis. For example, FlowSOM, SPADE, and Phonograph are tools used to perform high dimensional data analysis based on automated clustering. A variety of user-defined parameters, such as k-mean value, can determine the resolution and subculturing of immune cell populations. It is important to set these values to represent the expected subpopulations and avoid over clustering.

Supervised gating

This approach is useful when the experimental expectations and the cell type compositions in the samples are known. It is useful to discover and validate novel biomarkers by comparing two populations of interest in distinct groups through high-dimensional analysis. FlowDensity and OpenCyto are useful tools to perform automated gating using user-defined gates. The main advantage of using these tools is that the threshold for identifying the positive population is adjusted in a data-dependent manner for each sample rather than manually gating each sample, which can be subjective.

Cell subset detection (clustering)

Many clustering algorithms for mass cytometry have been published in recent years. However, the differences in their accuracy and sensitivity when identifying different cell populations is still a point of debate. An ideal algorithm for analyzing mass cytometry data should not only detect all major immune cell populations but also detects rare cell populations. Below, we discuss some commonly used clustering algorithms in Cytobank.

t-SNE: Clustering on dimensionality reduction channels (e.g., viSNE) is a more practical approach for samples consisting of heterogeneous cell subsets (high dimensional data). These include samples

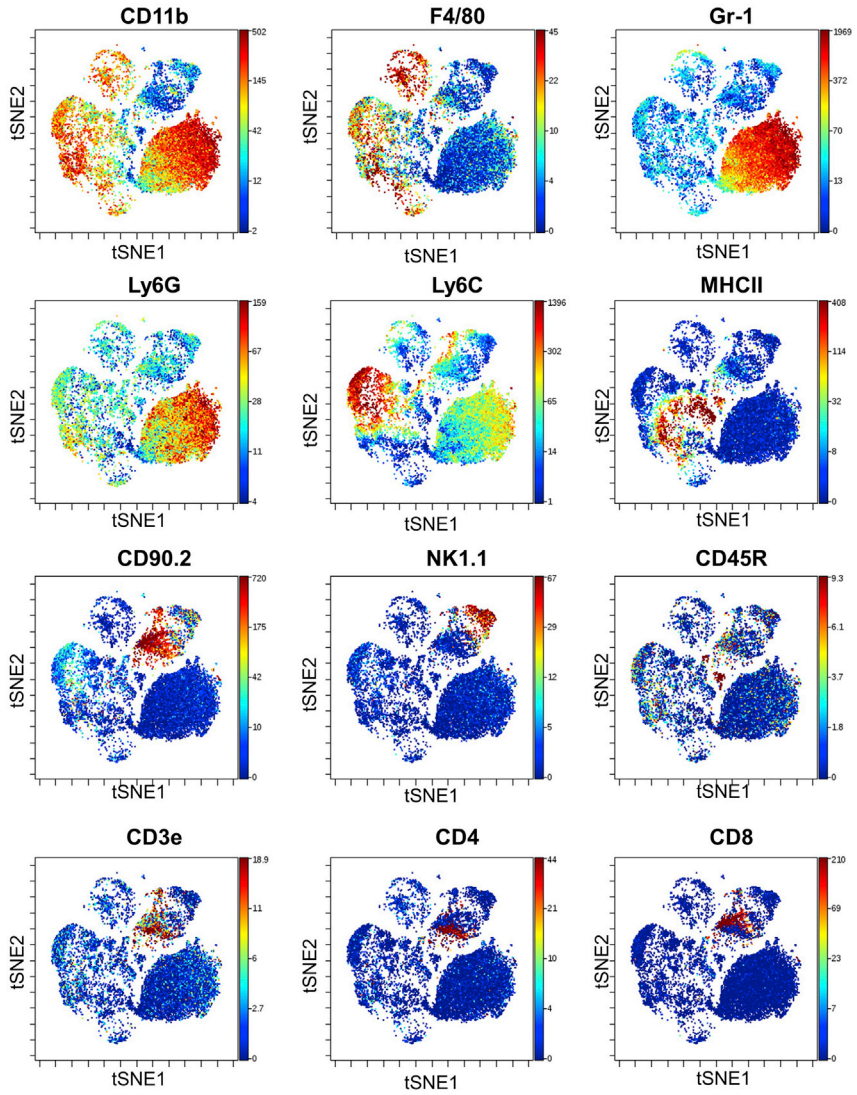

Figure 5. t-distributed stochastic neighbor embedding (t-SNE) clustering of immune cells infiltrating glioma tumor microenvironment

tSNE-based clustering represents an excellent identification and visualization of mass cytometry data. Each panel represent feature plot of expression of lineage specific marker.

containing cells with different and/or variable biomarker expression. In these situations, it is important to do t-SNE dimensionality reduction before applying any clustering algorithm such as SPADE or FlowSOM. However, because each dataset is different, it is difficult to determine when this approach is needed. Therefore, data must be explored in depth to determine the expression pattern and the heterogeneity of the sample. An example of using the t-SNE clustering approach to identify different immune cells infiltrating a GBM tumor microenvironment is shown in Figure 5.

Spanning-tree progression analysis for density-normalized events (SPADE): SPADE is one of the most widely used algorithms for immune cell mass cytometry data analysis. The main advantages of SPADE are that it overcomes computational challenges while lowering the risk of missing rare populations by performing density-dependent downsampling before hierarchical clustering. Density-dependent downsampling is an algorithm that extracts events to normalize the cell density. This allows for smaller cell populations not be overshadowed by larger cell populations. Utilizing

hierarchical clustering enables SPADE to capture the continuity of phenotypes in the hematopoietic system.

FlowSOM: FlowSOM reveals how all markers behave on all cells by utilizing Self-Organizing Maps (SOMs) to shorten analysis time and improve clustering quality. The main advantage of this algorithm is that it is quick and eliminates the need for downsampling. In addition, it avoids the need to define the number of clusters in the output, which is a major drawback of SPADE which can introduce bias to the analysis.

LIMITATIONS

Be wary of environmental metal contamination. Reagents from other companies and certain dish cleaning detergents can add unwanted metal contamination. Additionally, this technique should be conducted in an area without traces of metals in the air, such as those generated when using flints and Bunsen burners.

This protocol has the preparation of the antibody mixes on the day of the main staining experiment. To shorten the experiment time, the antibody mixes can be made the day prior and stored at 4°C in the dark.

Tissues can be harvested on the day of the main staining experiment to reduce loss of cell viability at the cost of a lengthier main experiment day.

TROUBLESHOOTING

Problem 1

When conjugating the purified antibodies to lanthanide metal labels, it is difficult to manage the timing of the different incubation periods due to only having one centrifuge, especially when the antibody reduction requires an exactly 30 min incubation (see step 2 in the "conjugating the antibodies to metal isotopes" section).

Potential solution

While the protocol may recommend that the polymer and antibody be processed at the same time, the polymer washes can be delayed until after the antibody reduction and purification are complete. The antibody reduction must be completed with the incubation timings given to prevent undesirable conjugations. On the other hand, the polymer wash can be delayed without consequence.

Problem 2

Since samples will be resuspended in water just prior to acquisition, cells are prone to degradation and debris formation (see step 49).

Potential solution

Proper fixation will protect cells from the hypotonic environment of the water before data acquisition.

Problem 3

Detector voltage may vary during sample acquisition, which may create high variability between samples (see step 50).

Potential solution

As part of the quality control for each sample, it is important to check the intensity of the beads in each sample. The best way to do that is by checking the median signal intensity of Eu153 on the beads and comparing it to the reference beads' intensity value. If the signal drops more than 25% in any sample, stop the acquisition, and recalibrate the detector voltage before running the sample again. Also, the coefficient of variance (CV) of the beads is reflective of the amount of error during

data acquisition. High CVs are indicative of a reduced ability to accurately detect minor changes in protein expression levels between cells.

Problem 4

During sample acquisition, oxidation of metal conjugated antibodies may occur, resulting in a 16 dalton (Da) increase in the mass channel of that isotope (for example Nd 146 oxides will be recorded as Dy162). This can be a source of cross reactivity between the metal isotopes and should be considered during the panel design (see step 50).

Potential solution

EQ™ Four Element Calibration Beads can be used to monitor alteration due to oxide formation during sample acquisition by calculating the ratio between the dual counts of Ce140 and Gd156. Also, Fluidigm recommends tuning the instrument using CyTOF® Tuning Solution (Fluidigm, SKU, 201072) to a ratio threshold of 3%. Due to the trade-off nature between oxidation and signal intensity, increasing signal intensity during tuning typically indicates a higher oxidation ratio.

Problem 5

Cell signal intensity may decrease during acquisition because of instrument performance or sample degradation.

Potential solution

While instrument performance can be tracked using EQ™ Four Element Calibration Beads, cell-specific degradation cannot. First, identify the Ce140+ DNA− EQ™ Four Element Calibration Beads population and the Ce140−DNA+ cell population. Next, plot time vs. a measured parameter (e.g., Eu153 for beads or CD45 for cells) and calculate the slope for each. A slight negative slope is generally expected, reflecting gradual loss of instrument sensitivity over time. A steeper slope for both cells and beads indicates a more rapid loss of instrument sensitivity over the period of acquisition, while a steeper slope for measured cell parameters relative to the measured bead parameters can indicate sample degradation. This sample degradation is indicative of poor sample fixation.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Maria G. Castro, mariacas@med.umich.edu.

Materials availability

This study involved tumor tissue from SB glioma mouse models. The protocol for generation of this model is as described in Garcia-Fabiani et al. (2020).

Data and code availability

No datasets or code was generated or analyzed during this study.

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AUTHOR CONTRIBUTIONS

B.L.M., R.T., and M.S.A. performed the experiments. B.L.M., M.S.A., and R.T. prepared the figures. B.L.M., M.S.A., and R.T. wrote the manuscript under the supervision of M.G.C. and P.R.L. M.G.C. and P.R.L. provided the funding. All authors read and edited the final version of this manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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