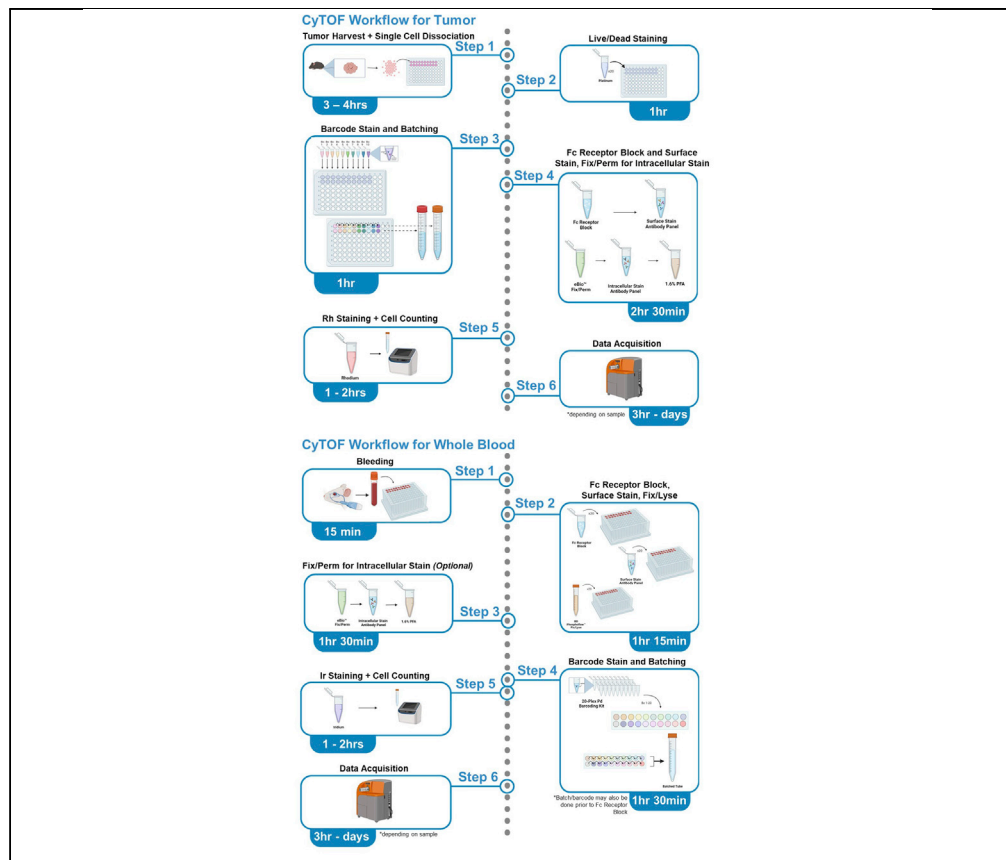


Protocol

CyTOF protocol for immune monitoring of solid tumors from mouse models



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Highlights

Facilitating discovery through high-parameter immune profiling in tumor mouse models

Design surface and intracellular antibody panels specific to experimental needs

Immune and non-immune live-cell barcoding scheme to enable multiplexing of samples

Mass cytometry (cytometry by time-of-flight; CyTOF) for mouse tumors and whole blood

Techniques for robust immune profiling of mouse tumor and blood are key to understanding immunological responses in mouse models of cancer. Here, we describe mass cytometry (cytometry by time-of-flight) procedures to facilitate high-parameter profiling of low-volume survival blood samples and end-of-study tumor samples. We employ live-cell barcoding systems to mark all cells from each tumor and blood to improve cost-effectiveness and minimize batch effects.

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

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Protocol

CyTOF protocol for immune monitoring of solid tumors from mouse models

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SUMMARY

Techniques for robust immune profiling of mouse tumor and blood are key to understanding immunological responses in mouse models of cancer. Here, we describe mass cytometry (cytometry by time-of-flight) procedures to facilitate high-parameter profiling of low-volume survival blood samples and end-of-study tumor samples. We employ live-cell barcoding systems to mark all cells from each tumor and blood to improve cost-effectiveness and minimize batch effects. For complete details on the use and execution of this protocol, please refer to Charmsaz et al. (2021).¹

BEFORE YOU BEGIN

The preparation for immune profiling of tumors and survival blood samples in mouse models of cancer requires the following major steps.

First, the protocol requires the development of adequate tumor samples and a working dissociation protocol for single-cell analysis. We also describe an example protocol for survival whole blood collection. The subsequent sections review the strategies for designing the barcoding schema and the panel of antibodies, including a representative protocol for custom conjugation of antibodies. We then provide the step-by-step protocol to carry out the staining of tumor and blood samples as well as the expected results and possible pitfalls for those procedures.

This protocol includes instructions for profiling tumors and survival blood samples as independent procedures. To perform CyTOF for tumors, follow steps 1–31, 35–68 in ‘before you begin’ and steps 1–38 in ‘step-by-step method details.’ To perform CyTOF for whole blood, follow steps 1–2, 32–57, 69–75 in ‘before you begin’ and steps 39–74 in ‘step-by-step method details.’

Institutional permissions

The female C57BL/6J 6–8 week old mice used in this protocol were purchased from Jackson Laboratories. The mice were fed a standard diet without fasting prior to beginning the experiment and all interventions took place during the light cycle. Mice were maintained according to the guidelines of the Institutional Animal Care and Use Committee.

Establish experimental mouse model

© Timing: 30 days



1. Set up an experimental MC38 subcutaneous tumor mouse model for a tumor harvest.
 - a. Culture and expand [MC38](#) for subcutaneous injections.
 - i. Thaw a vial of cryopreserved MC38 for 1 min in a 37°C water bath.
 - ii. Add 1 mL pre-warmed complete DMEM media (Recipes found in '[materials and equipment](#)') to thawed vial.

Note: Complete media refers to media enriched with supplements as indicated in '[materials and equipment](#).' Incomplete media does not include these additives.

- iii. Transfer to a 15 mL tube, top off tube with an additional 10 mL of pre-warmed complete DMEM.
 - iv. Centrifuge at 500 g for 5 min, aspirate supernatant, and resuspend pellet in 10 mL complete DMEM.
 - v. Transfer cell solution to T175 flask, add 25 mL of complete DMEM, incubate in a 5% CO₂ incubator at 37°C.
 - b. Grow cells until optimal cell density is achieved.
 - i. A minimum of 2–3 cell passages prior to subcutaneous injection is recommended.
 - c. Inject 2.5×10^5 cells of MC38 in 100 μ L per mouse subcutaneously with a 26-gauge needle into the right thigh of mouse.
 - i. Tumors are roughly 200–400 mm³ in size by day 14 and 800–1,200 mm³ by day 21.

Note: Prior to day 14, tumors will be on the smaller side for yielding sufficient cell numbers for analysis. For more robust analysis at earlier timepoints, pooling of multiple tumors may be required.

2. Monitor mouse/mice until harvest timepoint (e.g., 21 days in this protocol, see below for details).

Sample collection 1: Example protocol for single-cell dissociation of mouse tumors

⌚ Timing: 3–4 h depending on sample size

3. Utilize a gentleMACS™ Octo Dissociator to process 8 samples at a time (Miltenyi) to obtain tumor infiltrating leukocytes (TIL). Access to multiple gentleMACS™ Octo Dissociators facilitates throughput.

Note: Alternative protocols to singly dissociate tumor samples^{2,3} will be compatible with downstream staining procedures. Cell subtype yield and composition may differ from the expected results shown below.

Note: For studies that require tumor retrieval and CyTOF staining of samples on different days, see [problem 1](#).

4. Set up the laminar-flow hood for tumor mincing and processing:
 - a. Per sample:
 - i. Razor blade.
 - ii. Petri dish.
 - iii. gentleMACS™ C Tube.
 - iv. 50 mL conical tube.
 - v. 15 mL conical tube.
 - vi. 15 mL conical tube (for Percoll® step).
 - vii. MACS® SmartStrainers (70 μ m).
5. Prepare enzyme mix of a mouse Tumor Dissociation Kit (Miltenyi): additional details are available in the [manufacturer's protocol](#).

- a. Reagents may be reconstituted ahead of time and stored.
 - i. Store as frozen aliquots sufficient for 5 samples each (calculating for 5.1 samples): mix 510 μ L Enzyme D + 255 μ L Enzyme R + 63.75 μ L Enzyme A, then store the aliquots in -20°C – avoid repeated freeze thaw.
 - ii. Aliquot 11.985 mL of incomplete RPMI separately in 4°C ; do not mix with enzyme mix until the day of the tumor dissociation.
 - b. Prepare the working enzyme mix.
 - i. Mix 828.75 μ L of enzyme mix into 11.985 mL of incomplete RPMI (as aliquoted for every 5.1 samples) on the day of tumor dissociation.
6. Prepare approximately 40% Percoll® and 80% Percoll® in 1 \times PBS on day of TIL protocol – enough for 4–6 mL per sample for both 40% and 80% Percoll®.
- a. Make 1 \times Percoll® solution by adding 5 mL of 10 \times PBS into 45 mL of Percoll®.
 - b. Make 40 mL of ~40% Percoll® from the 1 \times Percoll® solution by adding 16 mL of 1 \times Percoll® into 24 mL of 1 \times PBS.
 - c. Make 40 mL of ~80% Percoll® from the 1 \times Percoll® solution by adding 32 mL of Percoll® into 8 mL of 1 \times PBS.

Note: Percoll® results are temperature-sensitive. This protocol stores and uses Percoll® at 25°C .

7. Harvest tumors from mice.
- a. To harvest tumors, bring a 6-well plate with 3 mL/well of incomplete RPMI on ice, 70% ethanol in spray bottle, scissors, and forceps into a laminar-flow hood. Keep plates at 4°C .
 - b. Isolate the tumor as much as possible, trim off any excess tissue from the site of tumor growth, and then place the tumor in each well containing cold incomplete RPMI until all of the tumors have been harvested.

Note: Harvesting small cohorts of tumors, e.g., <10–15, are typically completed within a span of 30–60 min depending on the user's experience.

8. Mince tumor to ~2–4 mm pieces with a razor blade in petri dishes.
9. Transfer minced tumors to a gentleMACS™ C tube.
10. Add 2.5 mL enzyme mix to gentleMACS™ C tube with tumor. Close cap tightly.
11. Place tubes in the gentleMACS™ Octo Dissociator and add Heaters on top of each gentleMACS™ C tube.
12. Start gentleMACS™ Octo Dissociator with "37C_m_TDK_1".
13. Prepare and place a 70 μ m Smartstrainers on each 50 mL tube per sample.
14. Add 5 mL of incomplete RPMI to each 50 mL tube.
15. (Optional) surface staining control:
 - a. Mash spleen from healthy mouse through MACS® SmartStrainers (70 μ m).
 - b. Transfer splenocytes to 50 mL tube.
 - c. Rinse with 20 mL of incomplete RPMI.
 - d. Spin at 500 g for 5 min at 25°C .
16. Quench enzyme mix by pipetting 2.5 mL of incomplete RPMI through the septum-sealed opening in the cap of the gentleMACS™ C tube.
17. Transfer 5 mL in gentleMACS™ C tube to the sample's respective 50 mL tube through MACS® SmartStrainers (70 μ m).
18. Spin at 500 g for 5 min at 4°C .
19. Aspirate supernatant.
20. Resuspend in 4–6 mL–40% Percoll®.
21. Transfer whole volume of 50 mL tubes to respective 15 mL tubes.
22. Slowly pipette to underlay 15 mL tubes with 4–6 mL–80% Percoll®.

Note: Carefully take out pipette tip just before dispensing the entirety of ~80% Percoll® volume to avoid creating bubbles; Percoll® layers are prone to mixing readily.

23. Spin at 1,400 g with no brakes for 25 min at 25°C.
24. Gently aspirate off the topmost layer containing debris.
25. Pipette gray cell layer containing leukocytes with a p1000 or 16-gauge needle/syringe into new 50 mL tubes.

Note: For non-distinct leukocytic layers, see [problem 3](#).

26. Dilute out the Percoll® in 50 mL tubes with 10 mL of incomplete RPMI.
27. Spin at 500 g for 5 min at 25°C.
28. Aspirate supernatant.
29. Resuspend in appropriate volume of incomplete RPMI to count cells.
30. Count cells using trypan blue stain.
31. Plate cells in a 96-well U-bottom plate.
 - a. Recommend plating 1.5×10^6 cells per well.
 - b. Cells are now ready to proceed to staining steps.

Sample collection 2: Example protocol for survival blood collection

⌚ Timing: 15 min

32. Prepare a 5 mm lancet and a Microvette K3EDTA blood collection tube.
33. Poke lancet through the cheek of the mouse to facial vein and collect up to 100 μ L of blood.

Note: Many institutional guidelines for survival bleeding recommend no more than 150 μ L per week for mice weighing approximately 25 g.

34. Shake the collection tube vigorously to ensure thorough mixing of the anticoagulant (EDTA).

Key notes in barcoding schema and antibody panel designs

When designing panels, it is critical to validate and optimize each antibody prior to staining. Each antibody clone should be tested on known positive and negative control samples and titrated across multiple concentrations. For antibodies that have been custom conjugated with isotopically enriched metals, each new conjugated lot should be tested. Channels ranging 89Y-116Cd are of relatively lower intensity but are useful for robust antigens such as CD45. Thus, they are often allocated for barcoding of samples. In contrast, channels near 160 have higher mass signal and will be better suited for detecting low-intensity markers. As a general guide, we recommend testing new antibodies spaced out at least by 3–4 channels and the “M+16” channel open (e.g., to test new conjugation of 144, keeping 142, 143, 145, 146, and 160 open will help evaluate impurities and oxidation signals). It is also important to note that the optimal signal from a given antibody may vary depending on the tissue of interest. Thus, we also recommend testing the panel in its entirety using a sample from a similar tissue type to evaluate its performance if possible. Once the panel has been finalized, the panel can be used to profile experimental samples. See other published protocols also for guidelines on antibody optimization.⁴

Barcoding each individual sample with a unique combination of metals enables the multiplexing of several samples into batches, which then significantly reduces the workflow burden and reagent usage. Upon staining batched samples in aggregate and acquiring the resulting data, samples can be debarcoded by publicly available software for automated demultiplexing or gating on biaxial plots.

Barcoding of samples based on a live-cell staining strategy, e.g., the use of CD45 antibodies, requires live/dead cell incubation, barcoding incubation, several additional washing steps, and transfer steps that ultimately lead to some sample loss. For tumors, the current barcoding scheme utilizes CD45 as the pan-immune cell marker and CD29 or CD98 as a non-immune or tumor cell marker. Each cancer model of question should be verified for its expression of CD29 and/or CD98, two potential markers validated for barcoding. Each marker should be conjugated to metals 112–116. For example, if CD98 and CD45 were chosen as barcoding markers, a total of 10 metal-conjugates would be used to construct the whole barcoding scheme (CD98 with 112–116 and CD45 with 112–116). These antigens have been selected and validated as barcoding markers for mouse cancer cell lines, i.e., non-immune cells.¹ For blood samples, the use of CD45 alone should be sufficient for barcoding all circulating hematopoietic cells. Additionally, for barcoding of samples with low starting numbers of cells, e.g., samples from survival bleeding, we favor proceeding immediately to surface staining of individual samples followed by a fix/lyse step. Subsequently, a 20-plex barcoding system based on Pd isotopes may be performed (Standard BioTools, formerly Fluidigm) to combine samples into batches prior to any additional intracellular staining and data collection. See other published manuscripts for guidelines on live-cell barcoding strategies.¹

Another key consideration is designating the proper isotope channels for the Cell-ID™ and live-dead stains in accordance with the study's planned panel of antibodies and barcodes. The most optimal choice of commonly used live-dead stains (102–110Pd, 194/198Pt) or Cell-ID™ metals (103Rh or 191/193Ir) will be impacted by the barcoding and antibody panels being used (see sections below and [problem 4](#) for additional discussion).

Finally, when the goal is to evaluate highly time-sensitive and condition-specific markers, e.g., phospho-epitopes, fixation prior to staining may be favored. In such cases, the staining quality of the antibodies in the desired panel will need to be tested in that specific condition, e.g., post-fixation.

Custom conjugation of antibodies

⌚ Timing: 5 h

Note: The protocol below utilizes X8 polymers and enables the conjugation of 89Y, 113In, 115In, 141Pr-175Lu, 209Bi using a buffer and enriched metal sets that are commercially available (Standard BioTools, formerly Fluidigm). Successful conjugation of Cd isotopes can be achieved using MCP polymer conjugation kits which are also available commercially. More information on the respective kits is available from the [manufacturer](#).

Note: Pipette thoroughly, especially in earlier steps to ensure proper mixture of the metal to the polymer and antibody to the buffer.

35. Prepare the metal-polymer and purified antibody simultaneously.
 - a. To prepare the metal-polymer:
 - i. Spin thawed polymer in minicentrifuge for 10 s.
 - ii. Resuspend in 95 μ L of L buffer.
 - iii. Vortex and spin the polymer tube in minicentrifuge.
 - iv. Add 5 μ L of metal (50 mM) to polymer tube to obtain 2.5 mM concentration.
 - v. Incubate in a 37°C water bath for 30 min.
 - vi. Add 200 μ L of L buffer into a 3 kDa filter tube.
 - vii. Add 100 μ L of the metal-polymer into the 3 kDa filter tube.
 - viii. Spin at 12,000 g for 25 min at 25°C.
 - ix. Discard flow-through of 3 kDa filter tube.

- x. Add 400 μL of C buffer into 3 kDa filter tube.
- xi. Spin at 12,000 g for 30 min at 25°C.
- b. To prepare the purified antibody:
 - i. Add 100 μg of purified antibody to the 50 kDa filter tube up to a maximum volume of 300 μL .
 - ii. Add R buffer to make a total volume of 400 μL in the 50 kDa filter tube.
 - iii. Spin 50 kDa filter tube at 12,000 g for 10 min at 25°C.
 - iv. Discard flow through.
 - v. Add 100 μL of 4 mM TCEP (diluted from 0.5 M TCEP into R buffer) into 50 kDa filter tube.
 - vi. Incubate 50 kDa filter tube in a 37°C water bath for 30 min.
 - vii. Add 300 μL of C buffer into 50 kDa filter tube.
 - viii. Spin 50 kDa filter tube at 12,000 g for 10 min at 25°C.
 - ix. Discard flow-through.
 - x. Repeat steps vii.–ix.
- 36. Resuspend 3 kDa filter tube with 60 μL of C buffer.
- 37. Transfer by pipetting whole volume of 3 kDa filter tube into 50 kDa filter tube.
- 38. Wrap top of the 50 kDa filter tube with parafilm to aid as an additional sealant.
- 39. Incubate in a 37°C water bath for 90 min.
- 40. Wash 50 kDa tube with 200 μL of W buffer.
- 41. Spin 50 kDa tube at 12,000 g for 10 min at 25°C.
- 42. Discard flow through.
- 43. Wash 50 kDa tube with 400 μL of W buffer.
- 44. Spin 50 kDa tube at 12,000 g for 10 min at 25°C.
- 45. Discard flow through.
- 46. Repeat steps 43–45 two more times.
- 47. Resuspend 50 kDa tube in 80 μL of W buffer and quantify antibody concentration by Nanodrop with measurement mode configured for “Protein A280” (protein type “IgG”).
- 48. Add enough stabilization buffer to bring the antibody concentration to 0.5 mg/mL.
 - a. To make stabilization buffer, dissolve 0.05(w/v) of Sodium azide in PBS Antibody Stabilizer (Boca Scientific Inc.).
 - b. Account for residual volume in 50 kDa filter tube by subtracting 20 μL from the amount of stabilization buffer needed.
- 49. Invert 50 kDa filter into a new collection tube.
- 50. Spin at 1,000 g for 2 min at 25°C.
- 51. Transfer entire volume into a LoBind Eppendorf Tube.
- 52. Store at 4°C.

Note: Custom conjugated antibodies in 4°C storage have been tested for stability up to one year.

Selecting a CyTOF barcoding scheme and planning antibody panels

⌚ **Timing:** 1–2 h—must be done in advance of the tumor dissociation

- 53. Calculate the number of cells to plate per well from each sample: typically, 1–3 million cells per sample are plated into each well.

Note: If a larger percentage of cell death is predicted, plating additional cells will help to offset the excess needed to achieve a sufficient number of evaluable cell events. Similarly, if the tumor type of interest is known to be immune-restricted, higher number of cells would need to be analyzed to sufficiently profile the immune compartment.

Note: It is critical to estimate a proper total cell count needed for CyTOF staining, as this will determine the ability to detect rarer cell subsets.

54. Design a plating scheme.
 - a. When multiplexing samples into batches, each batch should be designed such that relatively equal numbers of samples from all experimental groups are represented in each tube (Table 1). This will minimize potential batch effects in downstream analysis.
 - b. For global immune profiling, the minimum recommended cell plating density is 1.5×10^6 cells/well. As noted above, this number should be optimized depending on the expected immune cell content such as CD45⁺ cells or viable percentage of total cells of the tumor model.
55. Select a barcoding scheme.
 - a. Use a five-choose-three barcoding scheme for up to ten barcode combinations (Table 2).

Note: Choosing three metals, i.e., as opposed to two metals in a five-choose-two scheme which still provides ten unique combinations, allows for discrimination of incidental doublets.

- b. For tumors, select a pan-immune cell marker and a non-immune or tumor cell marker. For additional notes and guidance, please refer to 'key notes in barcoding schema and antibody panel designs.'
 - c. For blood samples, use a palladium-based barcoding kit as a pan-immune, low-volume survival blood barcoding scheme. For additional notes and guidance, please refer to 'key notes in barcoding schema and antibody panel designs.'
 - d. Use approximately approximately 0.25 μg of each conjugated barcoding antibody for every 1–3 million cells.
56. Plan an antibody panel for surface staining.
 - a. Acquire and prepare antibodies.
 - i. When available from a commercial catalog, pre-conjugated antibodies may be ordered. Otherwise, purified antibodies can be ordered, custom conjugated, tested, then validated prior to staining use.
 - ii. Test at least 3–5 different concentrations of a newly conjugated antibody on both positive and negative controls before experimental staining.

Note: See above for a representative protocol for antibody conjugation. See other protocols for optimal titration workflows.⁴

- b. Create a surface stain antibody cocktail.
 - i. Calculate the staining volume per tube: The target staining volume is 100 μL for every $1-3 \times 10^6$ cells.

Note: For a final staining volume of 500 μL , 250 μL will be Fc block staining and 250 μL will be surface staining.

Note: Antibody dilutions should be calculated off of the final staining volume. Calculate 5% extra for each antibody volume and Maxpar® CSB.

Pipette each antibody into Maxpar® CSB to make the surface stain antibody cocktail.

- ii. Example calculations for 10 tumors (1.5×10^6 per tumor; 1.5×10^7 cells in one 10-plex batch) – this batch is stained in 500 μL of total staining volume.

Note: PDL1 (Clone: 10F.9G2) has been discontinued from the time of this representative run. An alternative clone of MIH5 (Cat: 3153031B, [Link](#)) may be used.

Table 1. An example plating scheme for 20 samples from 4 experimental groups with 5 replicates each group (A, B, C, D)

Bc1	Bc2	Bc3	Bc4	Bc5	Bc6	Bc7	Bc8	Bc9	Bc10	
A1	A3	A5	B2	B4	C1	C3	C5	D2	D4	= Tube 1
A2	A4	B1	B3	B5	C2	C4	D1	D3	D5	= Tube 2

Note: Antibodies marked with (c) indicate custom conjugated antibodies. Titrations for custom conjugations must be tested and validated based on manufacturer's recommendations before experimental use.

57. Plan an antibody panel for intracellular staining (*if applicable*).
 - a. See above for "Acquire and prepare antibodies" for surface staining antibody panel. Same principles apply.
 - b. Create an intracellular stain antibody cocktail.
 - i. Calculate the staining volume per tube: The target staining volume is 60 μL for every $1\text{--}3 \times 10^6$ cells. Calculate 5% extra for each antibody volume and perm buffer.
 - ii. Pipette each antibody into Perm Buffer to make the intracellular stain antibody cocktail.
 - iii. Example for 1.5×10^7 cells per tube – 300 μL intracellular staining volume.

Reagent preparation for CyTOF staining of tumors

⌚ Timing: 1–2 h

Note: Antibody cocktails have been optimized for storage at -20°C up to one week before use; however, fresh cocktails are recommended. Other reagents should also be prepared on the day of use.

58. Prepare 200 μL 2 mM EDTA in PBS per well.
59. Prepare Cell-ID™ Cisplatin solution for live/dead: Dilute 20 μM Cell-ID™ Cisplatin in PBS at 1:500 and make enough for 50 $\mu\text{L}/\text{well}$.

Note: Cell-ID™ Cisplatin may need titration to optimize live/dead discrimination.

60. Prepare complete RPMI media for 150 $\mu\text{L}/\text{well}$: 445 mL of RPMI, 50 mL of Fetal Bovine Serum (1:10 dilution), 5 mL of $1\times$ Pen Strep (1:100 dilution), sterile-filtered, keep in 4°C .
61. Prepare barcode stain, enough for 75 $\mu\text{L}/\text{well}$.
 - a. E.g., For 20 samples, there are 10 barcodes with 1 barcode needed for 2 wells each.
 - i. Make 175 μL of each barcode (x2.5 wells). Dilute barcodes accordingly – for CD29, CD98, and CD45 based barcoding, there are 6 antibodies added per barcode in Maxpar® CSB.¹ Vortex, spin down in minicentrifuge for 5 s, and keep in 4°C .
62. Prepare Fc block solution for 150 $\mu\text{L}/\text{tube}$: 2 μL of stock Purified Rat Anti-Mouse CD16/CD32 Fc block diluted in Maxpar® CSB should be used for every 3×10^6 cells.
63. Prepare Surface Stain Antibody Cocktail: for example calculations of a representative surface stain antibody cocktail, refer to [Table 3](#) as shown above.

Table 2. Representative five-choose-three barcode generation scheme for 10 barcodes (Bc1~10)

	Bc1	Bc2	Bc3	Bc4	Bc5	Bc6	Bc7	Bc8	Bc9	Bc10
112	X	X	X	X	X	X				
113	X	X	X				X	X	X	
114	X			X	X		X	X		X
115		X		X		X	X		X	X
116			X		X	X		X	X	X

Table 3. Representative surface staining panel design—Mouse immune panel

Mass	Marker	Clone	Dilution	Source	Volume (μL)	Volume*1.05 (μL)
141Pr	Ly6G	1A8	1:50	Standard BioTools	10.00	10.5
142Nd	CD11c	N418	1:100	Standard BioTools	5.00	5.25
145Nd	CD69	H1.2F3	1:100	Standard BioTools	5.00	5.25
146Nd	F4/80	BM8	1:100	Standard BioTools	5.00	5.25
148Nd	CD11b	M1/70	1:100	Standard BioTools	5.00	5.25
149Sm	CD19	6D5	1:100	Standard BioTools	5.00	5.25
150Nd	Ly6C	HK1.4	1:100	Standard BioTools	5.00	5.25
151Eu	CD25	3C7	1:100	Standard BioTools	5.00	5.25
152Sm	CD3e	145-2C11	1:100	Standard BioTools	5.00	5.25
153Eu	PDL1	10F.9G2	1:100	Standard BioTools	5.00	5.25
155Gd	CD45	30-F11	1:100	Biolegend (c)	5.00	5.25
159Tb	CD279 (PD-1)	29F.1A12	1:100	Standard BioTools	5.00	5.25
161Dy	CD40	HM40-3	1:50	Standard BioTools	10.00	10.5
164Dy	CD62L	MEL-14	1:100	Standard BioTools	5.00	5.25
168Er	CD8a	53-6.7	1:50	Standard BioTools	10.00	10.5
170Er	NK1.1	PK136	1:50	Standard BioTools	10.00	10.5
171Yb	CD44	IM7	1:100	Standard BioTools	5.00	5.25
172Yb	CD4	RM4-5	1:200	Standard BioTools	2.50	2.625
176Yb	B220	RA3-6B2	1:100	Standard BioTools	5.00	5.25
209Bi	MHCII IA/IE	M5/114.15.2	1:100	Standard BioTools	5.00	5.25
Total Antibody Volume (μL) =						123.375
Maxpar® CSB Needed (μL) =						139.125

64. Prepare eBio™ Fixation/Permeabilization for 300 μL/tube as a 1:4 dilution of eBio™ Fixation/Permeabilization Concentrate and eBio™ Fixation/Permeabilization Diluent.
65. Prepare eBio™ Permeabilization buffer for washes by diluting eBio™ Permeabilization buffer (10×) to 1:10 using Maxpar® Water.
66. Prepare Intracellular Stain Antibody Cocktail: for example calculations of a representative intracellular stain antibody cocktail, refer to [Table 4](#) as shown above.
67. Prepare 1.6% Paraformaldehyde (PFA) enough for 400 μL/tube: dilute 16% stock Pierce™ Formaldehyde in Maxpar® PBS to 1.6% PFA.
68. Prepare Cell-ID™ Rh stain, enough for 300 μL/tube: dilute Rh in Maxpar® Fix and Perm Buffer at a 1:500 dilution.

Note: Cell-ID™ stains may need to be titrated for optimal signal.

Reagent preparation of CyTOF staining of whole blood

⌚ Timing: 1–2 h

Note: Antibody cocktails have been optimized for storage at –20°C up to one week before use; however, fresh cocktails are recommended. Other reagents should also be prepared on the day of use.

69. Prepare Surface Stain Antibody Cocktail: antibody dilutions based on the total staining volume should account for the volume of blood and EDTA solution, refer to [Table 3](#) as shown above.
70. Prepare Fix/Lyse solution.
 - a. Dilute BD Phosflow™ 5× Fix/Lyse at 1:5 in Maxpar® Water for 2 mL/tube.
 - b. Pre-warm working solutions of 1× Fix/Lyse to 37°C for 15 min prior to staining.
71. If intracellular staining is performed:

Table 4. Representative intracellular stain antibody panel design and cocktail preparation (an example of a commonly used intracellular staining panel for 10 tumors for a total volume of 300 μ L)

Mass	Marker	Clone	Dilution	Source	Volume (μ L)	Volume*1.05 (μ L)
Y89	PDPN	8.1.1	1:200	Biolegend (c)	1.2	1.58
154Sm	CTLA4	UC10-4B9	1:200	Standard BioTools	1.2	1.58
162Dy	KI67	B56	1:200	Standard BioTools	1.2	1.58
165Ho	Foxp3	FJK-16s	1:33	Standard BioTools	7.27	9.55
167Er	EOMES	Dan11mag	1:200	eBioscience (c)	1.2	1.58
169Tm	CD206	C068C2	1:100	Standard BioTools	2.4	3.15
173Yn	Granzyme B	GB11	1:66	Standard BioTools	3.64	4.77
Total Antibody Volume (μ L) =						23.79
Perm Buffer Needed (μ L) =						291.21

- a. Prepare eBio™ Fixation/Permeabilization for 300 μ L/tube for 300 μ L/tube as a 1:4 dilution of eBio™ Fixation/Permeabilization Concentrate and eBio™ Fixation/Permeabilization Diluent.
72. Prepare eBio™ Permeabilization buffer for washes for washes by diluting eBio™ Permeabilization buffer (10 \times) to 1:10 using Maxpar® Water.
 - a. Prepare Intracellular Stain Antibody Cocktail: for example calculations of a representative intracellular stain antibody cocktail, refer to Table 4 as shown above.
73. Prepare 1.6% PFA, enough for 400 μ L/tube: dilute 16% stock Pierce™ Formaldehyde in Maxpar® PBS to 1.6% PFA.
74. Thaw 20-plex Pd Barcoding Kit at 25°C just before use.
75. Prepare Cell-ID™ Ir stain, enough for 300 μ L/tube: dilute Ir in Maxpar® Fix and Perm Buffer at a 1:3000 dilution.

Note: Cell-ID™ stains may need to be titrated for optimal signal.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-Mouse B220-176Yb dilution 1:100	Standard BioTools	3176002B
Anti-Mouse CD11b-148Nd dilution 1:100	Standard BioTools	3148003B
Anti-Mouse CD11c-142Nd dilution 1:100	Standard BioTools	3142003B
Anti-Mouse CD19-149Sm dilution 1:100	Standard BioTools	3149002B
Anti-Mouse CD206-169Tm dilution 1:100	Standard BioTools	3169021B
Anti-Mouse CD25-151Eu dilution 1:100	Standard BioTools	3151007B
Anti-Mouse CD279 (PD-1)-159Tb dilution 1:100	Standard BioTools	3159024B
Anti-Mouse CD3e-152Sm dilution 1:100	Standard BioTools	3152004B
Anti-Mouse CD40-161Dy dilution 1:50	Standard BioTools	3161020B
Anti-Mouse CD4-172Yb dilution 1:200	Standard BioTools	3172003B
Anti-Mouse CD44-171Yb dilution 1:100	Standard BioTools	3171003B
Anti-Mouse CD62L-164Dy dilution 1:100	Standard BioTools	3164003B
Anti-Mouse CD69-145Nd dilution 1:100	Standard BioTools	3145005B
Anti-Mouse CD8a-168Er dilution 1:50	Standard BioTools	3168003B
Anti-Mouse CTLA4-154Sm dilution 1:200	Standard BioTools	3154008B
Anti-Mouse F4/80-146Nd dilution 1:100	Standard BioTools	3146008B
Anti-Mouse Foxp3-165Ho dilution 1:33	Standard BioTools	3165024A
Anti-Mouse Granzyme B-173Yn dilution 1:66	Standard BioTools	3173006B
Anti-Mouse KI67-162Dy dilution 1:200	Standard BioTools	3162012B
Anti-Mouse Ly6C-150Nd dilution 1:100	Standard BioTools	3150010B
Anti-Mouse Ly6G-141Pr dilution 1:50	Standard BioTools	3141008B

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Anti-Mouse MHCII IA/IE-209Bi dilution 1:100	Standard BioTools	3209006B
Anti-Mouse NK1.1-170Er dilution 1:50	Standard BioTools	3170002B
Anti-Mouse PDL1-153Eu dilution 1:100	Standard BioTools	3153016B (Discontinued)
Anti-Mouse PDL1-153Eu dilution 1:100	Standard BioTools	3153031C
Mouse BD Fc Block™	BD Pharmingen	553141
Purified Anti-Mouse CD29	Biolegend	102201
Purified Anti-Mouse CD45	Biolegend	103101
Purified Anti-Mouse CD98	Biolegend	128202
Purified Anti-Mouse Eomes	eBioscience	14-4875-82
Purified Anti-Mouse PDPN	Biolegend	127401
Chemicals, peptides, and recombinant proteins		
0.5 M EDTA	Invitrogen	15575020
10× PBS pH 7.4	Quality Biological	119-069-131
Antibody stabilizer PBS base	Boca Scientific Inc	131 050
Cell-ID™ Cisplatin	Standard BioTools	201064
Cell-ID™ Intercalator-Rh	Standard BioTools	201103A
DMEM (1×) [+] 4.5 g/L D-glucose [+] L-glutamine [-] sodium pyruvate	Gibco	11965-092
diH ₂ O	Standard Vendors	N/A
eBioscience™ Fixation/Permeabilization Diluent	Invitrogen	00-5223-56
eBioscience™ Fixation/Permeabilization Concentrate	Invitrogen	00-5123-43
eBioscience™ Permeabilization Buffer (10×)	Thermo Scientific	00-8333-56
Fetal bovine serum	Benchmark	100-106
HEPES (1 M)	Gibco	15630-080
Indium(III) chloride	Sigma-Aldrich	334065-10G
Lyse/Fix Buffer 5×	BD Biosciences	558049
Maxpar® Cell Staining Buffer	Standard BioTools	201068
Maxpar® Fix and Perm Buffer	Standard BioTools	201067
Maxpar® PBS	Standard BioTools	201058
Maxpar® Water	Standard BioTools	201069
MEM non-essential amino acids (100×)	Gibco	11140-050
Pen Strep	Gibco	15070-063
Percol™	GE Healthcare	17-0891-01
Pierce™ 16% Formaldehyde (w/v)	Thermo Scientific	28906
RPMI Medium 1640 (1×) [+] L-Glutamine	Gibco	11875-085
Sodium azide	Sigma-Aldrich	S2002-500G
Sodium pyruvate (100 mM) 100×	Gibco	11360-070
Sterile PBS 1×	Standard Vendors	N/A
Trypan Blue	Standard Vendors	N/A
Yttrium(III) chloride, anhydrous, powder, 99.99% trace metals basis	Sigma-Aldrich	451363-10G
Critical commercial assays		
Cell-ID™ 20-Plex Pd Barcoding Kit	Standard BioTools	201060
Maxpar MCP9 Antibody Labeling Kit, 112Cd—4 Rxn	Standard BioTools	201112A
Maxpar MCP9 Antibody Labeling Kit, 113Cd—4 Rxn	Standard BioTools	201113A
Maxpar MCP9 Antibody Labeling Kit, 114Cd—4 Rxn	Standard BioTools	201114A
Maxpar MCP9 Antibody Labeling Kit, 116Cd—4 Rxn	Standard BioTools	201116A
Maxpar® X8 Antibody Labeling Kit, 155Gd—4 Rxn	Standard BioTools	201155A
Maxpar® X8 Antibody Labeling Kit, 167Er—4 Rxn	Standard BioTools	201167A
Tumor Dissociation Kit, mouse	Miltenyi Biotec	130-096-730

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Experimental models: Cell lines		
C57BL/6 murine: MC-38	Kerafast	ENH204-FP
Experimental models: Organisms/strains		
Mouse: C57BL/6J, 6–10 Weeks, Female	The Jackson Laboratory	RRID:IMSR_JAX:000664
Software and algorithms		
CyTOF®	Standard BioTools	https://www.standardbio.com/products-services/software#helios-anchor
FlowJo™	BD	https://www.flowjo.com/solutions/flowjo
Premessa	Parker Institute for Cancer Immunotherapy	https://github.com/ParkerICI/premessa
Other		
1.5 mL Eppendorf tubes	Standard Vendors	N/A
10 mL, 5 mL pipette tips	Standard Vendors	N/A
15 mL High-Clarity Polypropylene Conical Tube	Falcon	352096
25 mL reagent boat	Standard Vendors	N/A
25 mL, 10 mL, 5 mL pipette tips	Standard Vendors	N/A
4°C fridge	Standard Vendors	N/A
50 mL centrifuge tube	CellTreat	229421
6-well dish plate	Standard Vendors	N/A
96-well plate flat bottom	Standard Vendors	N/A
96-well plates round bottom	Standard Vendors	N/A
96-Well DeepWell™ 1 mL Polypropylene Microplates	Fisherbrand	12-566-120
Cell strainer 40 µm	CellTreat	229482
Cell strainer 70 µm	CellTreat	229484
Centrifuge (up to 1,400 g, no brake option)	Standard Vendors	N/A
Centrifuge (up to 500 g, w/ brake option)	Standard Vendors	N/A
Centrifuge (up to 700 g, w/ brake option)	Standard Vendors	N/A
gentleMACS™ C Tubes	Miltenyi Biotec	130-093-237
gentleMACS™ Octo Dissociator with Heaters	Miltenyi Biotec	130-096-427
Goldenrod Animal Lancet	Braintree Scientific	GR-4MM
Hemocytometer	Standard Vendors	N/A
Microscope	Standard Vendors	N/A
Microvette® 100 K3EDTA Capillary Blood Collection Tube	VWR International	102971-718
Minicentrifuge	Standard Vendors	N/A
Multi-channel pipette P300	Standard Vendors	N/A
Multi-channel pipette tips P300	Standard Vendors	N/A
P1000 filtered pipette tips	Standard Vendors	N/A
P1000 pipette	Standard Vendors	N/A
P1000, P200, P20, P2 pipette	Standard Vendors	N/A
P1000, P200, P20, P2 pipette tips	Standard Vendors	N/A
Petri dish	Standard Vendors	N/A
Razor blade	Standard Vendors	N/A
Serological pipette	Standard Vendors	N/A
Vortexer	Standard Vendors	N/A
VWR® Culture Tubes	VWR	60818
Water Bath @ 37°C	Standard Vendors	N/A

MATERIALS AND EQUIPMENT

- Freezing Media: Fetal Bovine Serum with 10% of Dimethyl Sulfoxide.

Store at 4°C for up to 4 weeks.

Complete RPMI		
Reagent	Final concentration	Amount
RPMI 1640 Medium [+] L-glutamine	N/A	445 mL
Fetal Bovine Serum	10%	50 mL
Pen/Strep	1% (100 U/mL)	5 mL
Total	N/A	500 mL

Store at 4°C for up to 12 months.

Complete DMEM (MC38/MC38OVA Media)		
Reagent	Final concentration	Amount
DMEM [+] Glutamine [+] Glucose 4.5 g/L	N/A	430 mL
Fetal Bovine Serum	10%	50 mL
Non-Essential Amino Acids	1% (1×)	5 mL
Sodium Pyruvate	1% (1 mM)	5 mL
Pen/Strep	1% (100 U/mL)	5 mL
HEPES	1% (10 mM)	5 mL
Total	N/A	500 mL

Store at 4°C for up to 12 months.

△ **CRITICAL:** Reagents used for culture and freezing media should be prepared in sterile conditions in a laminar flow hood.

STEP-BY-STEP METHOD DETAILS

Section 1: CyTOF for tumors

⌚ **Timing:** 6–7 h

CyTOF for tumors—Live/dead staining

⌚ **Timing:** 1 h

The quality and consistency of samples should be ensured to secure a uniform staining environment. After tumors have been dissociated into single cells, the cells go through a series of washes. Reagents such as EDTA are added during the initial steps after plating to avoid clumping of the cells. In this example, Cell-ID™ Cisplatin is added to the sample as a live/dead stain to properly remove dead cells from downstream analysis. The Cell-ID™ Cisplatin reagent may require a titration experiment for optimal live/dead discrimination. Alternative reagents, e.g., palladium isotopes, may be used for live/dead discrimination. However, when planning, it is important to select non-overlapping channels to be used for live/dead, barcoding, and Cell-ID™ staining. Timely addition of complete RPMI media is important for quenching excess reagent. For studies that require tumor retrieval and CyTOF staining of samples on different days, see [problem 1](#).

1. Pipette at least 1.5 million cells from each individual sample onto each well of a U-bottom 96-microwell plate.
2. Centrifuge plate at 500 g for 5 min at 25°C, decant supernatant.

Note: After the first plating from the TIL, at minimum/average 2 washes are encouraged. Pellets should appear as below:



Note: For steps using microwell plates, we recommend using a multichannel pipette and reagent boats to facilitate the workflow.

3. Wash cells with 200 μ L of 2 mM EDTA per well, centrifuge at 500 g for 5 min at 25°C, decant supernatant.
4. Wash cells with 250 μ L of Maxpar® PBS per well.
5. Resuspend cells in 50 μ L/well of 20 μ M Pt (1:500) in Maxpar® PBS.
6. Incubate the plate for 2.5 min at 25°C.
7. Promptly quench excess reagent by directly adding 150 μ L/well complete RPMI, centrifuge plate at 500 g for 5 min at 25°C, decant supernatant.
8. Wash cells in 200 μ L of Maxpar® CSB, centrifuge at 500 g for 5 min at 25°C, decant supernatant.
9. Repeat the wash step two more times for a total of 3 Maxpar® CSB washes.
10. Carefully pipette off the residual supernatant off the side of each well without perturbing the cell pellets.

CyTOF for tumors—Barcode stain and batching

⌚ **Timing:** ~1 h

Upon completion of live/dead labeling, cells will be barcoded by surface epitopes, e.g., CD45, CD98, CD29, followed by batching of samples into multiplexed tubes. This improves the efficiency of the workflow while minimizing antibody consumption and batch effects.

11. Add barcode stain 75 μ L/well, gently resuspend to mix.
12. Incubate the plate for 25 min at 25°C.
13. Add 150 μ L/well of Maxpar® CSB to the samples, centrifuge at 500 g for 5 min at 25°C, decant supernatant.
14. Resuspend with 200 μ L/well of Maxpar® CSB to wash, centrifuge at 500 g for 5 min at 25°C, decant supernatant.
15. Repeat wash step.
16. Combine well contents into multiplex tubes (VWR® Culture Tubes – Polypropylene, 4.5 mL).
 - a. For each multiplex batch, use 300 μ L Maxpar® CSB to mix and pipette transfer from one well to the next, combining directly all wells that will be batched into one tube.
 - b. Pipette well mixture into multiplex tubes through a 40 μ m filter cap.
 - c. Repeat this at least two more times for a total of 900 μ L for transferring for each tube.
17. Centrifuge the multiplexed tubes at 500 g for 5 min, decant supernatant, and pipette off residual volume.

⚠ CRITICAL: Pipetting off any residual supernatant after decanting helps to ensure that antibody concentrations are not inadvertently diluted down.

CyTOF for tumors—Fc receptor block and surface stain

⌚ **Timing:** ~1 h

The Fc receptor block is first added to reduce nonspecific binding of the Fc sites on antibodies. The staining cocktail of antibodies against surface epitopes is then added directly onto the samples.

18. Resuspend cells in calculated volume of Fc block stain.
 - a. For calculations, refer to 'Reagent Preparation for CyTOF – Tumors'.
19. Incubate tubes for 10 min at 25°C.
20. Add in the calculated volume of Surface Stain Cocktail.
21. Incubate tubes for 30 min at 25°C.

22. Add 1 mL of Maxpar® CSB/tube, centrifuge tubes at 500 g for 5 min at 25°C, decant supernatant.
23. Wash samples in 1 mL of Maxpar® CSB/tube, centrifuge tubes at 500 g for 5 min at 25°C, decant supernatant off, then pipette off residual.

Optional: since tumor dissociation can be lengthy, cells can be fixed and stored in 1.6% PFA in Maxpar™ PBS for a maximum of 20 h at 4°C with stability.

CytoTOF for tumors—Fix/perm for intracellular staining

⌚ **Timing:** 1.5 h

The fix/perm step first fixes the cells and utilizes detergent-based permeabilization buffers to enable staining of the intracellular and intranuclear epitopes. All centrifugation steps are at 700 g instead of 500 g after fixation. An additional fixation step with a freshly made PFA solution is carried out at the end of all staining steps to ensure stability of the stained samples.

24. Resuspend cells in 300 µL/tube of eBio™ Fix/Perm.
25. Incubate for 30 min at 25°C.
26. Add to samples 1 mL of perm buffer/tube, spin at 500 g for 5 min at 25°C, decant supernatant.
27. Wash samples with 1 mL of perm buffer/tube, spin at 500 g for 5 min at 25°C, decant the supernatant and pipette residual volume off.
28. Resuspend cells in calculated volume of intracellular stain.
29. Incubate for 30 min at 25°C.
30. Add to samples 1 mL of perm buffer/tube, centrifuge at 500 g for 5 min at 25°C, decant supernatant.
31. Wash samples in 1 mL of Maxpar® CSB/tube, centrifuge at 500 g for 5 min at 25°C, decant supernatant, then pipette any residual volume off.
32. Resuspend cells in 400 µL/tube of 1.6% PFA in Maxpar® PBS, and fix for 10 min at 25°C if planning to proceed immediately to '[CyTOF for Tumors – Rh Staining + Cell Counting](#)'.

Optional: can be stored in 1.6% PFA in Maxpar® PBS up to 1 week at 4°C with stability.

CytoTOF for tumors—Rh staining + cell counting

⌚ **Timing:** ~1–2 h

The Rh stain, a DNA Intercalator, is used to recognize nucleated cell events during analysis. As discussed above, it is important to choose the Cell-ID™ carefully as some commonly used barcoding methods and live/dead assays can overlap in signal. For this protocol, we recommend avoiding live/dead assays with lower metal channels (such as 102–110Pd), as they would overlap in signal with the custom barcodes (metal channels 112–116) utilized in tumor CyTOF. Alternative Cell-ID™ metals for this barcoding scheme include iridium (191/193) and cisplatinum (194–198). The final cell count is critical for estimating the cell yield at the end of the data acquisition.

33. Centrifuge tubes at 700 g for 5 min at 25°C, decant the supernatant, pipette residual volume off.
34. Resuspend cells in 300 µL/tube of Rh, incubate for 30 min to 1 h at 25°C.
35. Add to samples 1 mL of Maxpar® CSB/tube.
36. Do a final cell count for each tube after resuspending in Maxpar® CSB.
37. Centrifuge tubes at 700 g for 5 min at 25°C, decant supernatant.
38. Repeat wash, after decanting, vortex tubes for 2–3 s to resuspend cells in residual volume, Store tubes at 4°C until data acquisition; Rh stain will be stable for 2–3 days in 4°C.

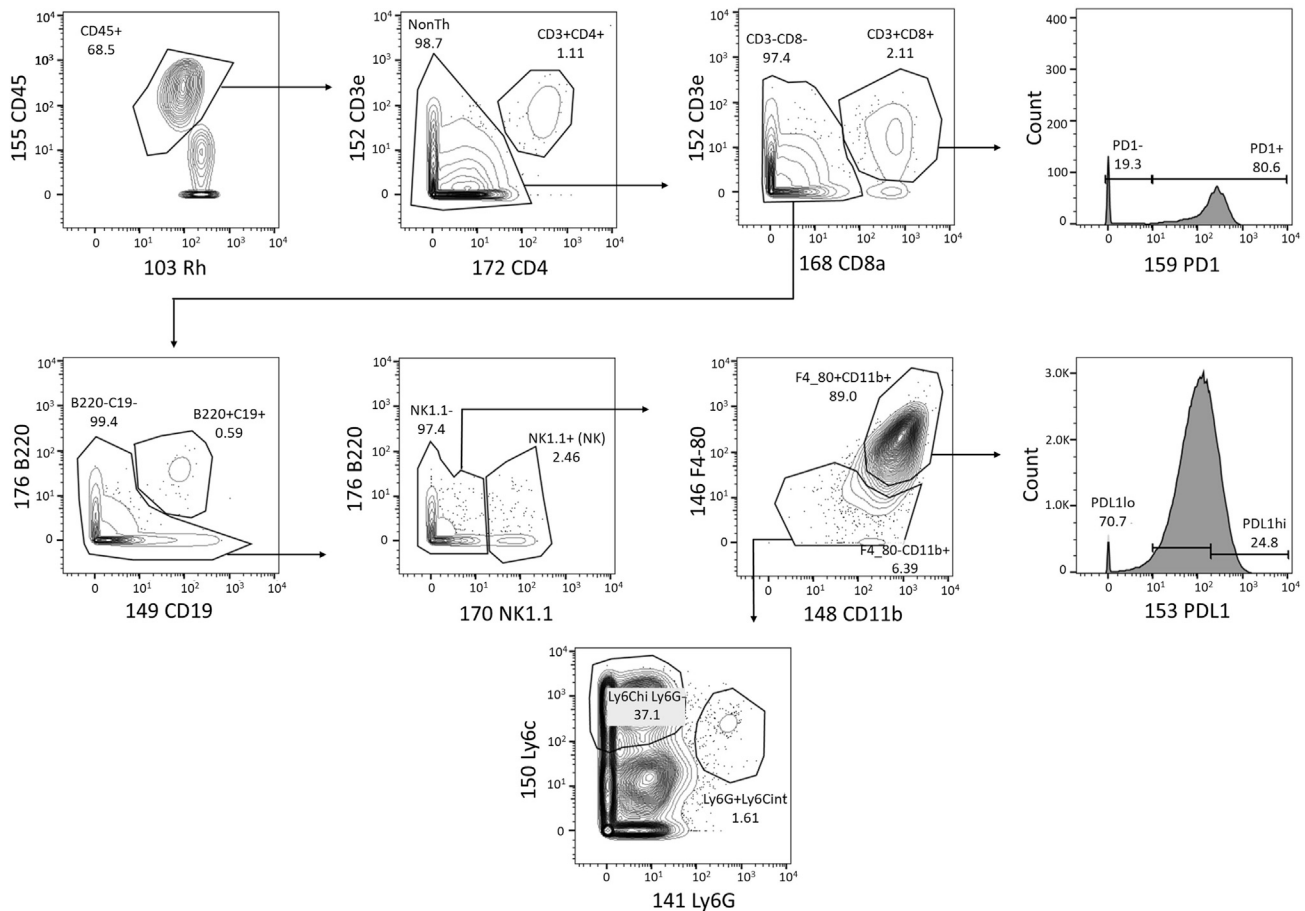


Figure 1. Gating scheme used for demonstrating expected outcomes from mouse tumor immunoprofiling

Sequential gating is performed to identify $CD4^+$ T cells, $CD8^+$ T cells ($PD1^-$ and $PD1^+$). Continued subsequent gating identifies $B220^+CD19^+$ B cells, $NK1.1^+$ NK cells, $F4/80^+CD11b^+$ macrophages, $Ly6C^+$ monocytic myeloid-derived suppressor cells, and $Ly6G^+$ granulocytic myeloid-derived suppressor cells.

Note: For visualization of a stable Rh stain, see [Figure 1](#).

Section 2: CyTOF for whole blood

⌚ Timing: 6–7 h

CyTOF for whole blood—Bleeding

⌚ Timing: 10 min

It is imperative to work quickly with fresh blood. Although the blood collection tube will come pre-coated with an anticoagulant, it is recommended to shake the tube after collection so that the blood can be mixed well with anticoagulant as much as possible. We recommend the Microvette K3EDTA collection tube (Cat: 102971-718, VWR). Additional EDTA is mixed to further prevent cell clumping.

39. Blood collection, as described in above section [Example protocol for survival blood collection.
40. Just prior to staining, pipette 90 μ L blood from the collection tube into a deep-well 96-well plate with pre-aliquoted 10 μ L of 0.5 M EDTA and mix well.

41. Proceed immediately to Fc receptor block and surface stain.

Note: We generally skip initial cell counting step or any other preprocessing methods, e.g., ACK lysis of red blood cells or live-cell barcoding for multiplexing of samples prior to staining, to avoid sample loss. We have expected cell yields from a 100–200 μL volume of blood based on prior experience. See below in Expected Outcomes.

CyTOF for whole blood—Fc receptor block, surface stain, fix/lyse

⌚ **Timing:** 1 h 15 min

The Fc receptor block is first added to reduce nonspecific binding of the Fc sites on antibodies. The staining cocktail of antibodies against surface epitopes is then added directly onto the samples. Since the staining occurs prior to multiplexing in this protocol, the use of a multichannel pipette is recommended. If the study involves assaying of time-sensitive antigens, e.g., phospho-epitopes, prompt fixation of blood samples followed by staining is recommended. However, the antibody panel must be tested to ensure that the antigens of interest are detectable post-fixation with the antibody clones used in the panel.

42. Pipette 2 μL of stock Fc receptor block directly into wells and mix.
43. Incubate for 10 min at 25°C.
44. Add 48 μL surface stain solution into the wells and mix, incubate for 30 min at 25°C.
45. Add to samples 1 mL of Maxpar® CSB/well, centrifuge plate at 500 g for 5 min at 25°C, decant supernatant.
46. Resuspend cells in 100 μL of Maxpar® CSB.
47. Pipette 2 mL of pre-warmed 1 \times BD Phosflow™ Fix/Lyse in each well.
48. Incubate plate in a 37°C water bath for 15 min, centrifuge plate at 500 g for 5 min at 25°C, decant supernatant.

Note: Make sure red blood cells have fully lysed before moving onto the next step. For additional details see [problem 5](#).

49. Wash cells in 1 mL of Maxpar® CSB/well, centrifuge plate at 500 g for 5 min at 25°C, decant supernatant.
50. If skipping intracellular stain, incubate in 400 μL /well of 1.6% PFA in Maxpar® PBS for 10 min at 4°C.

Optional: can store at 4°C for a maximum of 20 h prior to intracellular staining.

CyTOF for whole blood—Fix/perm for intracellular stain (optional)

⌚ **Timing:** ~1.5 h

The fix/perm step first fixes the cells and utilizes detergent-based permeabilization buffers to enable staining of the intracellular and intranuclear epitopes. An additional fixation step with a freshly made PFA solution is carried out at the end of all staining steps to ensure stability of the stained samples.

51. Resuspend cells in 300 μL /well of eBio™ Fix/Perm.
52. Incubate for 30 min at 25°C.
53. Add to samples 1 mL of perm buffer/well, spin at 500 g for 5 min at 25°C, decant supernatant.
54. Wash samples with 1 mL of perm buffer/well, spin at 500 g for 5 min at 25°C, decant the supernatant and pipette residual volume off.
55. Resuspend cells in calculated volume of intracellular stain.

56. Incubate for 30 min at 25°C.
57. Add to samples 1 mL of perm buffer/well, centrifuge at 500 g for 5 min at 25°C, decant supernatant.
58. Wash samples in 1 mL of Maxpar® CSB/well, centrifuge at 500 g for 5 min at 25°C, decant supernatant, then pipette any residual volume off.
59. Resuspend cells in 400 µL/well of 1.6% PFA in Maxpar® PBS, and fix for 10 min at 25°C if planning to proceed immediately to 'CyTOF for Whole Blood – Barcode Stain and Batching'.

Optional: can be stored in 1.6% PFA in Maxpar® PBS up to 1 week at 4°C with stability.

CyTOF for whole blood—Barcode stain and batching

⌚ **Timing:** ~1.5 h

Since the cell numbers from mouse survival bleeding samples are generally low, we modify the manufacturer's protocol for barcoding using the 20-plex Pd Barcoding Kit. For detailed manufacturer's information on the kit, refer to the [Cell-ID 20-Plex Pd Barcoding Kit User Guide](#).

60. Centrifuge samples at 700 g for 5 min at 25°C, decant supernatant carefully, vortex gently.
61. Resuspend each well in 250 µL of 1× Barcode Perm Buffer.
62. Thaw the stock tubes containing desired barcodes from the 20-Plex Pd Barcoding Kit, spin in minicentrifuge for 10 s.
63. Directly add 7 µL of the thawed barcode into each of the wells, incubate samples for 30 min at 25°C.
64. Centrifuge samples at 700 g for 5 min at 25°C, decant supernatant carefully.
65. Wash with 2 mL of Maxpar® CSB/well, centrifuge plate at 700 g for 5 min at 25°C, decant supernatant carefully.
66. Repeat wash.
67. Combine well contents into multiplex tubes (VWR® Culture Tubes – Polypropylene, 4.5 mL).
 - a. For each multiplex batch, use 300 µL Maxpar® CSB to mix and pipette transfer from one well to the next, combining directly all wells that will be batched into one tube.
 - b. Pipette well mixture into multiplex tubes through a 40 µm filter cap.
 - c. Repeat this at least two more times for a total of 900 µL for transferring for each tube.
68. Centrifuge tubes at 700 g for 5 min at 25°C, decant supernatant.

CyTOF for whole blood—Ir staining + cell counting

⌚ **Timing:** ~1–2 h

The Ir stain, a DNA Intercalator, is used to recognize nucleated cell events during analysis. As discussed above, it is important to choose the Cell-ID™ carefully as some commonly used barcoding methods and live/dead assays can overlap in signal. In this protocol for whole blood staining where Pd is used for barcoding (102, 104, 105, 106, 108–110), Ir (191/193) is selected instead of Rh (103). For low cell count recovery in survival whole blood samples, see [problem 2](#).

69. Centrifuge tubes at 700 g for 5 min at 25°C, decant the supernatant, pipette residual volume off.
70. Resuspend cells in 300 µL/tube of working Cell-ID™ Ir solution (1:3000 in Maxpar® Fix/Perm solution), incubate for 30 min.
71. Add to samples 1 mL of Maxpar® CSB/tube.
72. Do a final cell count using trypan blue.
73. Centrifuge tubes at 700 g for 5 min at 25°C, decant supernatant.
74. Wash using 1 mL of Maxpar® CSB/tube, after decanting, vortex tubes for 2–3 s to resuspend cells in residual volume, Store tubes at 4°C until data acquisition.

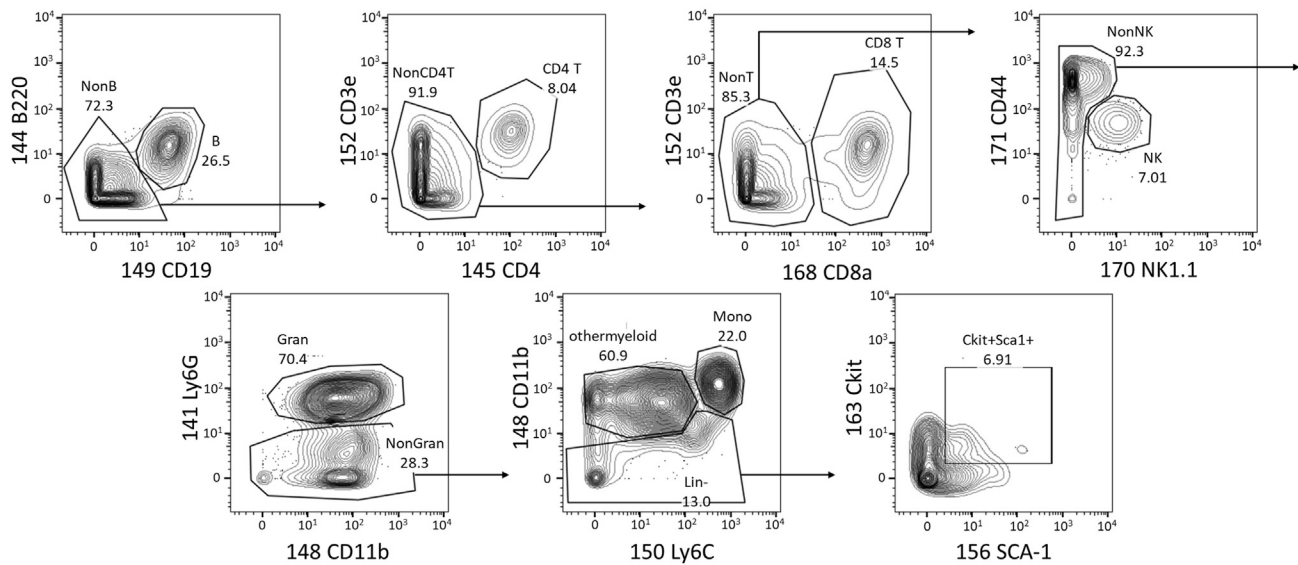


Figure 2. Gating scheme for mouse whole blood immunoprofiling

Sequential gating is performed to identify B220⁺CD19⁺ B cells, CD3⁺CD4⁺ T cells, CD3⁺CD8⁺ T cells, NK1.1⁺ NK cells, Ly6G⁺ granulocytes, Ly6Chi monocytes, CD11b⁺ Ly6Clo other myeloid cells, Lin-Ckit⁺Sca1⁺ cells.

EXPECTED OUTCOMES

Since sample acquisition with the third-generation mass cytometer Helios™ leads to approximately 50%–60% yield, a final total cell count of 5 million for a 10-plex batch that equally represents the ten samples will lead to no more than approximately 250,000 cells per tumor for analysis. Thus, in this example, a cell population that constitutes 0.1% of a given tumor would be represented by 250 cells. Using similar estimations, the protocol may be scaled up or down, most critically the antibody cocktail calculations, depending on the starting number of cells to be stained per sample to achieve the desired resolution of immune profiling. We demonstrate tumor immune profiling using a subcutaneous model based on MC38, an immunogenic colon carcinoma cell line, but we have also had success with another colon cancer model (CT26), pancreatic cancer models (Panc02 and KPC), breast cancer model (4T1), melanoma model (B16), and HCC models (Hepa1-6, Hep53.4, RIL-175, and Tib-75).^{5–7} The protocol also applies to tumors that have grown in visceral organs such as pancreas, liver, and lung.

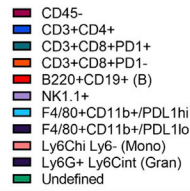
For samples obtained from survival cheek bleeding, we have observed that even volumes as low as 100 μ L lead to reproducible results with cell counts of approximately 100,000 cells. For higher blood volumes, we have noted a higher tendency to encounter clotting within the samples without proportionally higher cell recovery.

Using the presented protocol, we show a simple gating-based phenotyping strategy for tumors (Figure 1) and whole blood samples (Figure 2) to identify major immune cell types within each sample. For the analysis of mouse tumor samples, we show representative tumor profiling results from 5 untreated MC38 tumors in C57Bl/6 mice using the described protocol (Figure 3). For the analysis of mouse whole blood samples, we demonstrate the feasibility of detecting major differences in small survival bleeding volumes by comparing C57Bl/6 mice treated with a hematopoietic cell mobilization agent, CXCR4 antagonist BL-8040 vs. PBS (Figure 4).

QUANTIFICATION AND STATISTICAL ANALYSIS

Preprocessing and analysis of the acquired data are beyond the scope of this discussion and have previously been reviewed extensively.⁸ As a brief overview, major required steps include the

Freq. of Total



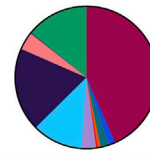
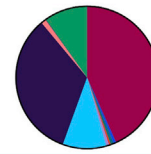
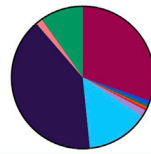
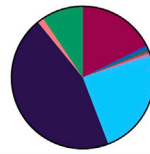
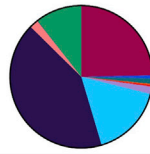
Freq of Total Tumor 1

Freq of Total Tumor 2

Freq of Total Tumor 3

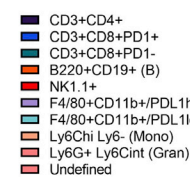
Freq of Total Tumor 4

Freq of Total Tumor 5



CELL POPULATION	MOUSE 1	MOUSE 2	MOUSE 3	MOUSE 4	MOUSE 5
CD45-	24.70	17.60	30.40	43.30	43.40
CD3+CD4+	0.68	0.44	0.76	0.59	1.47
CD3+CD8+PD1+	1.24	0.99	0.73	0.71	1.96
CD3+CD8+PD1-	0.30	0.16	0.40	0.20	0.66
B220+CD19+ (B)	0.29	0.20	0.39	0.14	0.31
NK1.1+	1.69	0.77	1.62	0.69	3.28
F4/80+CD11b+/PDL1hi	16.50	24.00	14.10	9.91	11.50
F4/80+CD11b+/PDL1lo	42.00	45.00	40.30	33.40	19.00
Ly6Chi Ly6- (Mono)	1.99	1.44	1.52	1.13	4.06
Ly6G+ Ly6Cint (Gran)	0.07	0.04	0.07	0.10	0.19
Undefined	10.55	9.37	9.71	9.83	14.17

Freq. of CD45+



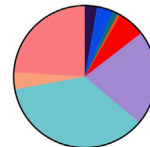
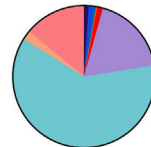
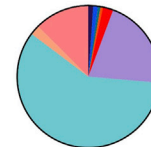
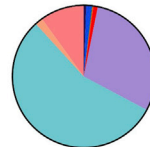
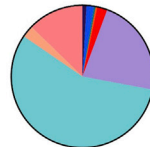
Tumor 1 (Freq. of CD45+)

Tumor 2 (Freq. of CD45+)

Tumor 3 (Freq. of CD45+)

Tumor 4 (Freq. of CD45+)

Tumor 5 (Freq. of CD45+)

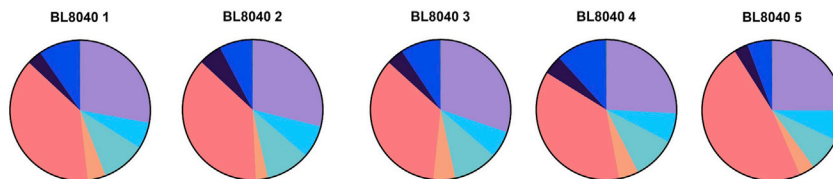
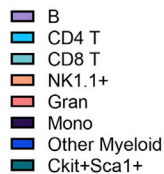


CELL POPULATION	MOUSE 1	MOUSE 2	MOUSE 3	MOUSE 4	MOUSE 5
CD3+CD4+	0.91	0.54	1.11	1.09	2.78
CD3+CD8+PD1+	1.68	1.22	1.06	1.3	3.7
CD3+CD8+PD1-	0.4	0.2	0.58	0.37	1.25
B220+CD19+ (B)	0.39	0.25	0.57	0.26	0.59
NK1.1+	2.28	0.95	2.36	1.27	6.2
F4/80+CD11b+/PDL1hi	22.3	29.6	20.7	18.2	21.7
F4/80+CD11b+/PDL1lo	56.6	55.5	58.9	61.4	35.9
Ly6Chi Ly6- (Mono)	2.69	1.77	2.22	2.07	3.77
Ly6G+ Ly6Cint (Gran)	0.087	0.043	0.096	0.19	0.14
Undefined	12.663	9.927	12.404	13.85	23.97

Figure 3. CyTOF mouse tumor immune profile—Frequency of each cell type

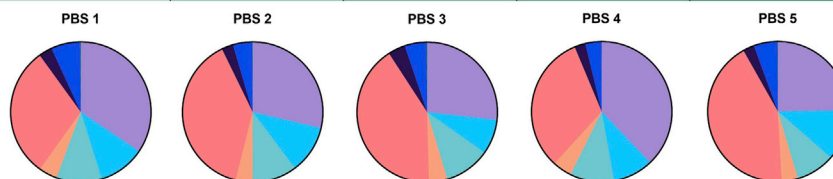
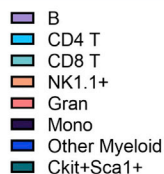
The frequency values of each immune cell population are shown as a percentage of total cells (top) and percentage of CD45⁺ cells (bottom). Pie charts are visualized at the top of each respective mouse, along with the legend to the left.

Freq. of
Total (WB)



CELL POPULATION	BL8040 1	BL8040 2	BL8040 3	BL8040 4	BL8040 5
B	26.5	27.6	28.7	24.5	23.9
CD4 T	5.8	7.2	5.9	6.5	6.7
CD8 T	9.6	9.7	9.7	9.4	7.6
NK1.1+	4.0	2.6	4.8	4.3	3.4
Gran	36.8	36.0	33.5	34.9	45.5
Mono	3.3	5.1	3.7	4.2	3.1
Other Myeloid	9.0	7.2	8.7	11.0	5.4
Ckit+Sca1+	0.13	0.16	0.16	0.20	0.15

Freq. of
Total (WB)



CELL POPULATION	PBS 1	PBS 2	PBS 3	PBS 4	PBS 5
B	32.7	27.5	25.4	36.3	23.6
CD4 T	10.2	10.6	7.7	8.9	11.2
CD8 T	9.9	9.8	10.0	9.7	8.5
NK1.1+	4.0	3.8	4.0	4.2	3.6
Gran	28.6	37.2	39.2	30.6	40.8
Mono	3.0	2.6	3.8	2.4	2.5
Other Myeloid	6.3	4.3	4.7	3.5	5.2
Ckit+Sca1+	0.23	0.08	0.24	0.06	0.13

Figure 4. CyTOF mouse whole blood immune profile with BL8040 treatment vs. negative control—Frequency of total cells

The frequency values of each immune cell population are shown as a percentage of CD45⁺ cells. Pie charts are visualized at the top of each respective mouse, along with the legend to the left. The first group represents the immune profile of mice treated with a CXCR4 antagonist BL-8040 (top), a subcutaneous injection that predominantly mobilizes less mature myeloid cells, i.e., captured here as granulocytes, monocytes, other myeloid cells, and LSK cells, from the bone marrow. The second group represents mice that have received PBS control injections (bottom).

following. Upon completion of data acquisition, the mass cytometry platform generates '.fcs' files. For analysis, the file can be further pre-processed using CyTOF software (Standard BioTools) for two processes: randomization and normalization. Randomization will smoothen the data around the integer raw data values. Normalization utilizes the signal from control beads that should be incorporated into every data acquisition to normalize the data for any change in signal over the course of data collection – over time, the detector may become saturated, resulting in a gradual diminution of signal. Subsequently, the data will require additional pre-processing to: (i) discriminate nucleated cell events using Cell-ID™; (ii) exclude live/dead cells; (iii) debarcode the samples into de-multiplexed fcs files. These steps can be done on flow cytometry analysis softwares such as FlowJo (BD). Alternatively, all of these pre-processing steps can be carried out using publicly available software packages, e.g., *premassa* using R (<https://github.com/ParkerICI/premassa/>). Downstream analysis can be performed in a variety of ways, including supervised gating strategies as shown in this protocol and unsupervised clustering algorithms.⁹

LIMITATIONS

The current tumor analysis protocol relies on enzymatic dissociation and ~40% over ~80% Percoll® enrichment of samples followed by barcoding based on CD45, CD29, and CD98 markers. We have found this to yield mixtures of both immune and non-immune cells. However, this approach still does not account for all cell types. Depending on the scientific question and the sample source, the protocol may require additional optimization. For example, to have higher yields of live hepatocytes from mouse liver specimens, we have successfully removed the debris using only 20% Percoll® with a shorter centrifugation time. Other similar caveats based on the current protocol is the inability to barcode anucleate cells, therefore unable to bind Cell-ID™ tags, or cells without CD45, CD29, or CD98 expression. Thus, each new application will require further modification of the current approach to be effective.

Another key limitation, as described above, is the need for the current protocol to be tested and optimized for evaluating more dynamic, functional markers. While protein-level evaluation is generally more robust than RNA-level measurements, the dissociation protocol may still have altered more transient protein markers from their *in vivo* states by the time that staining occurs. Examples include phospho-epitopes, secreted cytokines and chemokines, chemokine receptors that turnover in surface presentation, and transient markers of metabolic states, among others. These markers may require specialized stimulation conditions and extra incubation periods that enable further functional characterization of specific cell types which at the same time may limit the ability to globally monitor the immune profile. Furthermore, survival whole blood samples are already limited in the numbers of cells that can be assayed, so careful protocol considerations must be taken to maximize the reliability of evaluating specific markers. In one particular application, to identify CCR2⁺ monocytes in whole blood samples, we have had success in modifying the protocol to include an initial chemokine receptor staining step for CCR2 for 5 min in 37°C prior to adding in the rest of the surface antibodies without altering other steps in the protocol (Figure 5). Other applications will require additional testing.

TROUBLESHOOTING

Problem 1

Higher group-to-group batch effects in a time-course study: studies that require retrieval and staining of samples on different days (relevant to [sample collection 1: example protocol for single-cell dissociation of mouse tumors](#) and [CyTOF for tumors – live/dead staining](#)).

Potential solution

There are multiple potential solutions that may be considered.

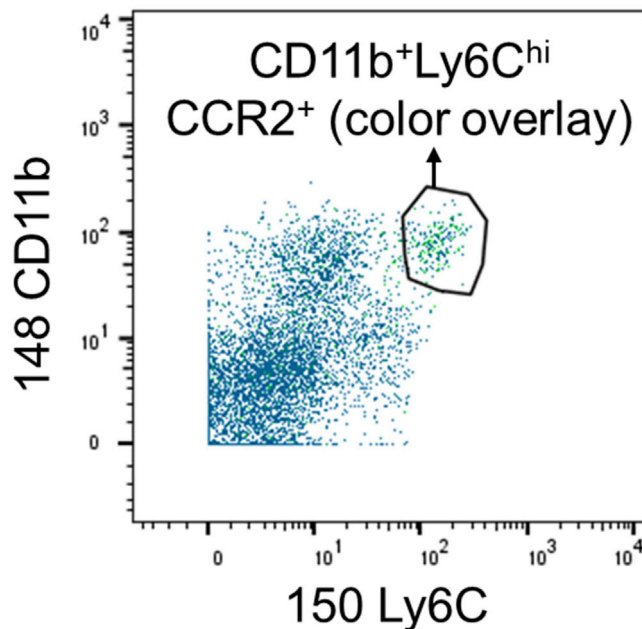


Figure 5. Representative results from a modified whole blood staining protocol for detection of CCR2

The addition of anti-CCR2 antibody at 37°C prior to adding in the rest of the surface antibodies enables the identification of CCR2⁺ monocytes (CD11b⁺Ly6C^{hi}) in whole blood samples. CCR2 expression (green) is shown as color overlay.

- Stagger the starting timepoint for the different groups so that the harvesting and staining of all groups occur at the same time. While this is the most definitive way to ensure minimal batch effects and allow for working with fresh samples, it is not always feasible. For example, tumors may be harvested only upon reaching a euthanasia point, the timing for which the model has not been well-characterized. Also, endpoints could be based on sporadic occurrences that are less predictable. In these cases, alternative solutions discussed below can be considered.
- Freeze down pre-aliquoted antibody cocktails that have been prepared in advance.¹⁰ We have had success with single freeze-thaws of barcodes and antibody cocktails. The stability of the antibodies may vary depending on the clone. Any potential changes in the staining intensity should be evaluated prior to the actual experiment. While this approach reduces the batch-to-batch variation that is specifically associated with antibody dilutions, it does not eliminate any potential batch effects related to other aspects of the protocol.
- Freeze down the samples after single-cell dissociation to be stained all at the same time. Multiple cell types, e.g., tumor cells and/or myeloid cell subtypes, depending on the tumor model, may not survive the freeze-thaw and the expression of particular antigens may be lost even on the cells that survive. This is a well-documented phenomenon and is the least preferable approach.¹¹ It is strongly recommended that the samples are compared before and after cryopreservation to ensure that the cell types of interest remain consistent.

If none of these approaches are practical, the only possibility may be to repeat the protocol several times. With robust training and experience, the protocol is overall technically consistent. Using the same lot of pre-aliquoted enzymes for dissociation, automated dissociation (e.g., Octodissociators as described in the protocol), and laboratory equipment (e.g., microplates, centrifuge, pipettes), all steps carried out by the same experimenter may help to minimize contributions to batch effects.

Problem 2

Low cell count recovery in the survival whole blood samples (relevant to [CyTOF for Whole Blood – Ir Staining + Cell Counting](#)).

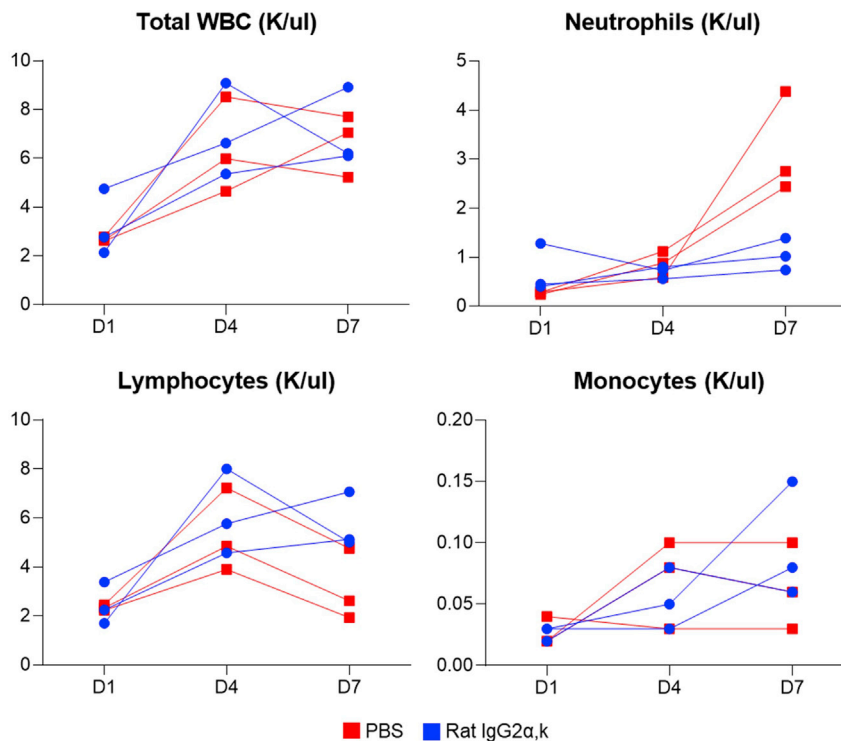


Figure 6. Effects of a control isotype antibody on specific cell types in survival whole blood samples

Using ProCyt (IDEXX), a hematology analyzer, numbers of key cell types per volume of blood (thousands of cells per μ L of blood) were quantified, demonstrating that even the use of Control Isotype Antibodies (Rat IgG2 α ,k) leads to lower numbers of neutrophils on day 7.

Potential solution

Peripheral blood samples in mice, while valuable as surrogates of systemic immune responses, are inherently limited as source of cells for profiling. If the cell counts are significantly lower than expected, i.e., \sim 100,000 cells from a 100 μ L volume of blood, other factors may be contributing. As described above, care must be taken to ensure clotting is prevented by mixing well within the EDTA-containing collection tube immediately after bleeding. Another potential cause is the use of antibody-based immunotherapies that lead to the depletion of specific cell types even in mice treated with isotype controls (Figure 6). In troubleshooting the potential effects on peripheral blood samples, a useful strategy is to first assess the overall changes in key cell types by assaying with a hematology analyzer, e.g., ProCyt (IDEXX). We have assessed our whole blood CyTOF protocol side-by-side with ProCyt to confirm that the results from the two independent assays correlate very strongly (Figure 7). Based on preliminary information, experiment designs can be revised for pooling of multiple mice, or the use of higher volume non-survival bleeding strategies, to permit cell counts sufficient for desired analyses.

Problem 3

Leukocyte layer is not distinct after Percoll[®] spin during tumor dissociation process (relevant to [sample collection 1: example protocol for single-cell dissociation of mouse tumors](#)).

Potential solution

Non-distinct leukocytic layers may result due to disrupted Percoll[®] layers or low leukocyte yields due to small tumor size. If the leukocyte layers of tumor samples are not recoverable, quench samples with 20 mL of incomplete RPMI, vortex for 2–3 s, and centrifuge samples at 500 g for 5 min at

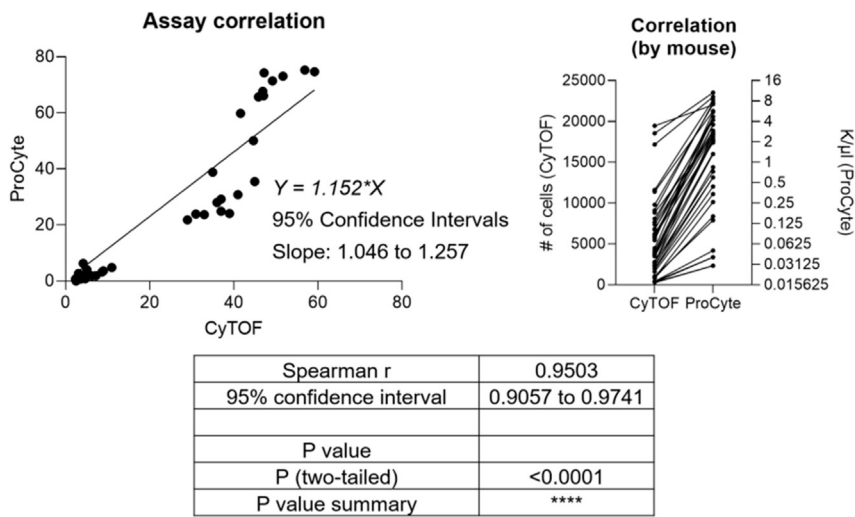


Figure 7. Correlation between CyTOF and ProCytometry hematology analyzer

Blood samples obtained for the CyTOF results shown on [Figures 3 and 4](#) were aliquoted in small amounts to be evaluated by ProCytometry in parallel. Results from the two assays for each cell type for each mouse were plotted as individual dots.

25°C. Aspirate the supernatant off, resuspend cells in 40% Percoll®, transfer cells to a 15 mL tube, pipette an 80% Percoll® underlay, and spin at 1,400 g with no brakes for 25 min at 25°C.

Problem 4

Intense channel spillover amongst Cell-ID™, barcodes, and live-dead assays (relevant to [‘key notes in barcoding schema and antibody panel designs’](#)).

Potential solution

Some Cell-ID™ options may overlap in signal with barcodes and live/dead assays as channel spillover occurs every M+1, 2, 3, 4 and +16 channels. To avoid significant spillover from critical markers in the pre-processing and de-barcoding stage, we recommend choosing the appropriate Cell-ID™ based on the surface/intracellular antibody panel. For example, in tumor CyTOF we used (metal channel 103) Rhodium as our Cell-ID™ to avoid overlap with the custom barcodes (metal channels 112–116) as well as with live-dead stain cisplatinium (metal channels 194–198).

Problem 5

Red blood cells do not properly lyse in whole blood CyTOF (relevant to [CyTOF for whole blood – Fc receptor block, surface stain, fix/lyse](#)).

Potential solution

If red blood cells do not fully lyse after the BD Phosflow™ Fix/Lyse incubation, extend the incubation for 5 to 10 more minutes in 37°C water bath. This should be indicated by a visible color transition from a clear sample solution to a dark red sample solution.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Won Jin Ho (wjho@jhmi.edu).

Materials availability

No new unique reagents were generated for this protocol.

Data and code availability

Representative data is shown directly in the published article as part of expected outcomes and shown in further detail in cited references. Raw data will be provided upon reasonable request. No code was generated for this protocol.

ACKNOWLEDGMENTS

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AUTHOR CONTRIBUTIONS

S.S. wrote the manuscript. A.H. and E.C. reviewed manuscript. S.C., N.G., and W.H. developed the tumor barcoding strategies. S.S., A.H., E.C., and W.H. all developed the final workflow. X.Y. and H.Y. acquired the mass cytometry data. S.S., A.H., E.C., K.M., S.C., and N.G. generated and analyzed the data. All authors read and approved the manuscript. W.H. conceived and oversaw the project.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

- Charmsaz, S., Gross, N., Jaffee, E., and Ho, W.J. (2021). A global live cell barcoding approach for multiplexed mass cytometry profiling of mouse tumors. *JCI Insight* 6, e143283. <https://doi.org/10.1172/jci.insight.143283>.
- Alam, A., Comer, S., Levanduski, E., and Dey, P. (2022). Isolation and adoptive transfer of innate lymphoid cells 2 to a recipient mouse model of PDAC. *STAR Protoc.* 3, 101563. <https://doi.org/10.1016/j.xpro.2022.101563>.
- Rodriguez de la Fuente, L., Law, A.M.K., Gallego-Ortega, D., and Valdes-Mora, F. (2021). Tumor dissociation of highly viable cell suspensions for single-cell omic analyses in mouse models of breast cancer. *STAR Protoc.* 2, 100841. <https://doi.org/10.1016/j.xpro.2021.100841>.
- Thrash, E.M., Kleinstueber, K., Hathaway, E.S., Nazzaro, M., Haas, E., Hodi, F.S., and Severgnini, M. (2020). High-throughput mass cytometry staining for immunophenotyping clinical samples. *STAR Protoc.* 1, 100055. <https://doi.org/10.1016/j.xpro.2020.100055>.
- Ho, W.J., Croessmann, S., Lin, J., Phyto, Z.H., Charmsaz, S., Danilova, L., Mohan, A.A., Gross, N.E., Chen, F., Dong, J., et al. (2021a). Systemic inhibition of PTPN22 augments anticancer immunity. *J. Clin. Invest.* <https://doi.org/10.1172/JCI146950>.
- Ho, W.J., Erbe, R., Danilova, L., Phyto, Z., Bigelow, E., Stein-O'Brien, G., Thomas, D.L., 2nd, Charmsaz, S., Gross, N., Woolman, S., et al. (2021b). Multi-omic profiling of lung and liver tumor microenvironments of metastatic pancreatic cancer reveals site-specific immune regulatory pathways. *Genome Biol.* 22, 154. <https://doi.org/10.1186/s13059-021-02363-6>.
- Zabransky, D.J., Danilova, L., Leatherman, J.M., Lopez-Vidal, T.Y., Sanchez, J., Charmsaz, S., Gross, N.E., Shin, S., Yuan, X., Hernandez, A., et al. (2022). Profiling of murine syngeneic HCC tumor models as a framework to understand anti-PD-1 sensitive tumor microenvironments. *Hepatology.* <https://doi.org/10.1002/hep.32707>.
- Kimball, A.K., Oko, L.M., Bullock, B.L., Nemenoff, R.A., van Dyk, L.F., and Clambey, E.T. (2018). A beginner's guide to analyzing and visualizing mass cytometry data. *J. Immunol.* 200, 3–22. <https://doi.org/10.4049/jimmunol.1701494>.
- Weber, L.M., Nowicka, M., Soneson, C., and Robinson, M.D. (2019). diffcyt: differential discovery in high-dimensional cytometry via high-resolution clustering. *Commun. Biol.* 2, 183. <https://doi.org/10.1038/s42003-019-0415-5>.
- Schulz, A.R., Baumgart, S., Schulze, J., Urbicht, M., Grützkau, A., and Mei, H.E. (2019). Stabilizing antibody cocktails for mass cytometry. *Cytometry A.* 95, 910–916. <https://doi.org/10.1002/cyto.a.23781>.
- Carlson, P.M., Mohan, M., Patel, R.B., Birstler, J., Nettenstrom, L., Sheerar, D., Fox, K., Rodriguez, M., Hoefges, A., Hernandez, R., et al. (2021). Optimizing flow cytometric analysis of immune cells in samples requiring cryopreservation from tumor-bearing mice. *J. Immunol.* 207, 720–734. <https://doi.org/10.4049/jimmunol.2000656>.