

Application of Mass Cytometry (CyTOF) for Functional and Phenotypic Analysis of Natural Killer Cells

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Abstract

Mass cytometry is a novel platform for high-dimensional phenotypic and functional analysis of single cells. This system uses elemental metal isotopes conjugated to monoclonal antibodies to evaluate up to 42 parameters simultaneously on individual cells with minimal overlap between channels. The platform can be customized for analysis of both phenotypic and functional markers. Here, we will describe methods to stain, collect, and analyze intracellular functional markers and surface phenotypic markers on natural killer cells.

Key words CyTOF, Mass cytometry, Flow cytometry, Natural killer cell, Intracellular cytokine staining

1 Introduction

Mass cytometry is a next-generation flow cytometry platform with several technological advances that offer advantages over fluorescence-based flow cytometry when highly parametric analyses are required. Most notably, mass cytometry does not rely on detection of fluorescence, which requires compensation for spillover into adjacent channels. Instead, through the use of antibodies coupled to metal isotopes, mass cytometry can detect discrete isotope peaks without significant overlap [1, 2], thus ameliorating the need for compensation. In addition, more channels are available. The primary limiting factor is the chemistry required to conjugate the metals to the antibodies with high efficiency. Currently, mass cytometry allows for the detection of 42+ unique parameters rather than the 8–12 parameters that comprise a typical flow cytometry panel.

This increase in total parameters is critical for the study of natural killer (NK) cells, the diverse functional properties of which are influenced by combinatorial expression of multiple phenotypic markers [3, 4]. The functional properties of NK cells can be explored through multiple stimulation conditions including phorbol 12-myristate 13-acetate (PMA)+ionomycin, cytokines

(such as IL-2, IL-12, IL-15, and/or IL-18), viral stimulation (such as HIV-1-infected or influenza-infected cells), or cell lines deficient in MHC-I expression (such as K562 or 721.221 cells). To analyze intracellular cytokines and chemokines, brefeldin A and monensin are added for the final 4 h of stimulation to maintain the cytokines intracellularly for detection [5]. Because CD107a (a lysosomal protein also known as LAMP-1) is briefly revealed at the cell surface during cytotoxic granule release, anti-CD107a antibodies added during stimulation serve as an indirect measure of cytotoxicity. Most antibodies used in mass cytometry bind directly to the target protein; however, in some cases, two-stage detection is more efficient. For optimal detection of CD107a by mass cytometry, anti-CD107a-APC is added during the stimulation, followed by isotope-conjugated anti-APC antibody as a surface stain.

Antibody-metal isotope pairs are available for purchase from Fluidigm (<http://maxpar.fluidigm.com/product-catalog-metal.php>). However, optimizing a panel that both explores the desired markers and accounts for isotope spillover and varying degrees of antibody signal intensity often requires a customized panel. Conjugation of antibodies and metal isotopes is an easily performed step that results in increased options for panel design, and has been previously described in detail [6]. Here we describe a method to profile both the phenotypic and functional characteristics of natural killer cells by conjugating antibodies to selected metal isotopes, surface and intracellular labeling of cells and analysis on a mass cytometer. The process of conjugating a custom panel for phenotypic analysis has been described previously; we will mention NK-cell-specific modifications here [3]. This chapter focuses on the addition of conditions for intracellular staining and functional assessment of NK cells, but can also be adapted for other cell types.

2 Materials

All reagents and containers must be free from heavy metal contaminants. No containers exposed to soap that may contain trace metal contaminants should be used. All buffers should be prepared and stored in disposable uncontaminated containers.

2.1 Antibody Conjugation

1. Maxpar[®] Metal labeling kits (Fluidigm Corporation).
2. Purified monoclonal antibodies (Fig. 1 and Table 1 provide examples), purified IgG or polyclonal. Must not have any carrier protein; otherwise, special order is required. Sodium azide is acceptable.
3. Centrifugal Filter Unit: 50 kDa Amicon Ultra—500 μ L V bottom (Millipore) or 30 kDa Amicon Ultra 500 μ L V bottom (Millipore).



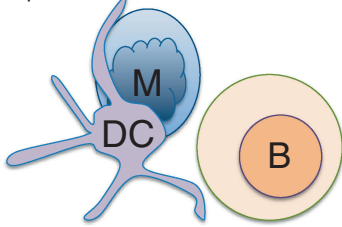
Develop a comprehensive antibody panel for NK cells Custom conjugation allows panel flexibility: simply follow the manufacturer's instructions		
<p>The Foundation: Lineage markers</p> <p>Distinguish NK cells from T cells, B cells, Monocytes, and dendritic cells Often these markers are bright and can be placed on slightly less optimal channels</p> <div style="display: flex; justify-content: space-around; align-items: flex-start;"> <div style="text-align: center;">  <p>149Sm-CD16 174Yb-CD56</p> </div> <div style="text-align: center;">  <p>112,Cd CD3-Qdot605 143Nd-CD4 144Nd-CD8</p> </div> <div style="text-align: center;">  <p>113In-CD14: "dump" channel for monocytes 113In-CD19: "dump" channel for B cells 141Pr-HLA-DR</p> </div> </div>		
<p>Phenotype: Inhibitory receptors</p> <p>148Nd-KIR3DL2 154Sm-LILRB1/ILT-2/CD85j 163Dy-KIR3DL1 165Ho-KIR3DL1/S1 166Er-KIR3DL1 169Tm-KIR2DL2/L3/S2 170Yb-KIR2DL3 171Yb-NKG2A</p>	<p>Phenotype: Activating receptors</p> <p>151Eu-CD161 153Eu-KIR2DS4 155Gd-NKp46 156Gd-NKG2D 157Gd-NKG2C 158Gd-2B4 161Dy-NKp30 164Dy-NKp44 173Yb-KIR2DL4 175Lu-KIR2DL5</p>	<p>Phenotype: Adhesion molecules</p> <p>115In-CD2 142Nd-DNAM-1 172Yb-NTB-A</p>
<p>Function: Cellular activation/differentiation, cytokine production, cytolytic activity and potential</p> <p>162Dy-APC & CD107a-APC 145Nd-CD57 146Nd-TRAIL 147Sm-GM-CSF 150Nd-MIP-1β 152Sm-TNF 159Tb-Perforin 160Gd-CD69 167Er-Granzyme B 168Er-CXCR6 176Yb-IFN-γ</p>	<p>Notes on panel design:</p> <ul style="list-style-type: none"> Optimal signal-to-noise ratios are observed with isotopes in the middle of the mass range; reserve these channels for markers that are of low frequency or intensity. When custom conjugating, be sure to mix the polymer thoroughly. Track all reagent and lot numbers. Use the same set of conjugated antibodies for a single set of experiments to limit batch effects. 127IdU can be used to assess proliferation, similar to BrdU (Behbehani et al., Cytometry, 2012) Do not forcefully eject lanthanide loaded polymer onto the filter when resuspending to avoid antibody loss 	

Fig. 1 Schematic display of CyTOF panel development

4. Centrifugal Filter Unit: 3 kDa Amicon Ultra—500 μL V bottom (Millipore).
5. Aerosol Barrier (Filter) Pipette Tips.
6. Bond-Breaker™ TCEP Solution: 0.5 M TCEP (Tris(2-carboxyethyl)phosphine) (Pierce).

Table 1
Example of table to generate antibody surface and intracellular staining cocktails

Isotope	Antibody	Clone	Supplier	Concentration (µg/mL)	Working concentration (µg/mL)	Volume to add ^a
Surface markers						
110,111,112,114Cd	CD3	UCHT1	Life Technologies (Qdot-605)	100 tests/100 µL	0.5 µL/test	10.5
113In	CD14, CD19	M5E2, HIB19	Biologend	279, 342	2.5, 2.5	9.41, 7.68
115In	CD2	RPA-2.10	BD Biosciences	302	0.625	2.17
141Pr	APC	APC003	Biologend	252	5.0	20.83
142Nd	DNAM-1	DX11	BD Biosciences	327	5.0	16.06
143Nd	CD4	SK3	Biologend	171	1.25	7.68
144Nd	CD8	SK1	Biologend	387	2.5	6.78
145Nd	CD57	HCD57	Biologend	630	0.625	1.04
146Nd	TRAIL	RIK-2	Biologend	208	2.5	12.62
148Nd	KIR3DL2		Lanier Lab	266	1.25	4.93
149Sm	CD16	3G8	BD Biosciences	385	1.25	3.41
151Eu	CD161	DX12	BD Biosciences	319	2.5	8.23
153Eu	KIR2DS4	FES172	Beckman Coulter	288	1.25	4.56
154Sm	ILT-2	GHI/75	Biologend	393	2.5	6.68
155Gd	NKp46	9E2/NKp46	BD Biosciences	385	1.25	3.41
156Gd	NKG2D	1D11	Biologend	257	5.0	20.43
157Gd	NKG2C	MAB1381	R&D Systems	138	2.5	19.02
158Gd	2B4	2-69	BD Biosciences	324	2.5	8.10
161Dy	NKp30	P30-15	Biologend	286	2.5	9.18

163Dy	KIR3DL1	DX9	BD Biosciences	415	2.5	6.33
164Dy	NKp44	P44-8	Biologend	293	1.25	4.48
165Ho	KIR3DL1/S1	Z27.3.7	Beckman Coulter	328	1.25	4.00
166Er	KIR2DL1	143211	R&D Systems	313	1.25	4.19
168Er	CXCR6	56811	R&D Systems	396	5.0	13.26
169Tm	KIR2DL2/L3/S2	GL183	Beckman Coulter	304	1.25	4.32
170Yb	KIR2DL3	180701	R&D Systems	273	2.5	9.62
171Yb	NKG2A	Z199	Beckman Coulter	428	2.5	6.13
172Yb	NTB-A	NT-7	Biologend	94	1.25	13.96
173Yb	KIR2DL4	181703	R&D Systems	840	1.25	1.56
174Yb	CD56	NCAM16.2	BD Biosciences	436	1.25	3.01
175Lu	KIR2DL5	UP-R1	Biologend	450	0.625	1.46
#Vol CyFACS 786.69						
Intracellular markers						
147Nd	GM-CSF	BVD2-21C11	Biologend	119	5.0	44.12
150Nd	MIP-1 β	D21-1352	BD Biosciences (Custom)	551	5.0	9.53
152Sm	TNF- α	MAb11	eBioscience	454	5.0	11.56
159 Tb	Perforin	B-D48	Abcam	381	5.0	13.78
160Gd	CD69	FN50	Biologend	308	2.5	8.52
162Dy	HIVp24	38/5.4A	Abcam	280	10.0	37.50
167Er	Granzyme B	2CF/F5	BD Biosciences	442	5.0	11.88
176Yb	IFN- γ	4S.B4	eBioscience	401	5.0	13.09
					#Vol CyFACS	900.018

^aVolume to add = (Working Concentration/Concentration) \times 50 \times Number of Samples (in this case 20) + 1

#Volume CyFACS = (50 \times Number of Samples + 1) – Sum Total of the Volume of Antibodies

7. Microcentrifuge, ideally 2 units.
8. Heat block incubator or water bath at 37 °C.
9. PBS-based antibody stabilization solution (Candor Biosciences).
10. Nanodrop for protein quantification.

2.2 Mass Cytometry Labeling

1. Custom-conjugated antibodies or pre-conjugated antibodies purchased from Fluidigm (<http://maxpar.fluidigm.com/product-catalog-metal.php>).
2. Thermo Scientific™ Nunc™ 96 Deep Well Plates, Polystyrene. 96-well round bottom plates can be used for stimulation and incubation, but cells should be transferred to deep-well plates prior to surface staining.
3. MilliQ dH₂O: No water should have contact with beakers or bottles washed with soap.
4. CyPBS: PBS without heavy metal contaminants, made from 10× PBS using MilliQ purified water, with no contact with glassware washed with soap.
5. CyFACS buffer: 0.1 % bovine serum albumin + 2 mM EDTA + 0.1 % sodium azide in CyPBS. Filter solution with a 0.2 µM filter.
6. Cisplatin Solution: Prepare 100 mM (Stock solution) in DMSO. Freshly prepare working solution of 10 mM cisplatin in PBS (100 µL cisplatin stock + 900 µL PBS).
7. Lysing Solution: Prepare 1× solution from 10× stock (BD FACST™ Lysing Solution, BD Biosciences) solution using MilliQ deionized water and store in a disposable plastic container.
8. 0.1 µM spin filters (Millipore).
9. Permeabilization buffer: Prepare 1× permeabilization buffer from 10× stock solution (BD FACST™ Permeabilizing Solution 2, BD Biosciences) using MilliQ deionized water and store in a disposable plastic container.
10. Interchelator-PFA solution: Dilute Iridium-Interchelator solution (Fluidigm) 1:10,000 into 2 % paraformaldehyde solution. Prepare the 2 % Paraformaldehyde solution by diluting 16 % Stock Paraformaldehyde (Electron Microscopy Sciences) in CyPBS. Freshly prepare the Interchelator-PFA solution for each use.
11. Complete RPMI medium: RPMI-1640, 10 % Fetal Bovine Serum, 1× penicillin-streptomycin and 1× L-glutamine.
12. Refrigerated centrifuge equipped with rotor for spinning 96-well plates.
13. Aspirator with vacuum trap set-up.

2.3 Cell Stimulation

1. Cell Stimulation cocktail contains PMA and ionomycin (500×) (eBioscience): Freshly prepare 1× Working solution in complete RPMI medium (1:500 dilution).
2. CD107a-APC (Biolegend, Clone H4A3).
3. EDTA 0.5 M (Stock): prepare 20 mM working solution.
4. Monensin Solution (1000×) (eBioscience): Freshly prepare 1× Working solution in complete RPMI medium (1:1000 dilution).
5. Brefeldin A Solution (1000×) (eBioscience): Freshly prepare 1× Working solution in complete RPMI medium (1:1000 dilution).

2.4 Running CyTOF Mass Cytometry

1. Ice bucket.
2. Micropipettes.
3. Normalization beads (Fluidigm).
4. Filter-top tubes (BD Biosciences).

3 Methods

All steps may be completed at room temperature (RT) unless otherwise indicated.

3.1 Antibody Conjugation Using MaxPar Metal Labeling Kit

To conjugate antibodies simply follow manufacturer's instructions provided with the MaxPar Metal Labeling Kit using the supplies listed above. An example of a customized panel to profile natural killer cell phenotype and function is shown in Fig. 1. The conjugation protocol has been described in depth previously [2–4]. Custom-conjugating antibodies gives more flexibility in panel design than would be available by purchasing pre-conjugated antibodies. The panel outlined in Fig. 1 contains receptors to identify major cell lineages (such as CD3, CD4, CD8, CD19, CD14, CD56) as well as markers for the major NK cell receptor families including the killer immunoglobulin-like receptors (e.g., KIR2DL1, KIR2DL2/L3/S2), Fc receptors (CD16), natural cytotoxicity receptors (NKp30, NKp44, NKp46), C-type lectins (NKG2A, NKG2C, NKG2D), and markers of maturity and differentiation (e.g. CD57). It also includes NK cell functional cytokines (IFN- γ , MIP-1 β , TNF- α) as well as cytotoxicity markers (CD107a, Perforin, Granzyme B). In general, greater signal-to-noise ratios can be expected on isotopes in the middle of the mass range and markers that are of low frequency or low intensity are best reserved for these channels. Please *see* **Notes 1–6** for additional suggestions regarding the conjugation of antibodies.

3.2 Surface and Intracellular Labeling of Cells for Mass Cytometry

1. Isolate human peripheral blood mononuclear cells by Ficoll gradient (*see Note 7*).
2. Wash the cells with complete RPMI medium and plate the cells at two million cells in 200 μ L in a standard 96-well plate.
3. Add stimulation condition of choice; for example 1 \times Cell Stimulation cocktail (Subheading 2.3, **item 1**), 1 \times Brefeldin A and 1 \times Monensin. To assess degranulation include CD107a-APC at a dilution of 1:25 (25-fold dilution) in this Cell Stimulation cocktail. Incubate the cells at 37 °C for 4 h.
4. During the incubation, prepare the surface and intracellular antibody staining cocktails. This is best performed using a spreadsheet to calculate the quantities based on antibody titrations (Table 1).
5. At the end of the incubation add 20 μ L of 20 mM EDTA to each well and mix by pipetting. Incubate for 10 min at room temperature.
6. Spin the plate at 750 $\times g$ for 3 min, aspirate the supernatant, and add 200 μ L CyFACS. Transfer the cells to a deep 96-well plate.
7. Add 300 μ L CyFACS to each well and spin the plate at 750 $\times g$ for 10 min. Aspirate the supernatant.
8. During centrifugation (**step 7**), dilute the 10 mM cisplatin working solution at a ratio of 1:200–1:50 (titration based on prior cisplatin batch optimization) in serum and antibiotic-free RPMI (*see Notes 8 and 9*).
9. Resuspend the cells in 400 μ L of this diluted cisplatin.
10. Incubate cells for 1 min at RT.
11. Quench (*see Note 9*) with 400 μ L of serum. Pipette to mix thoroughly.
12. Centrifuge plate at 750 $\times g$ for 10 min at RT. Aspirate the supernatant.
13. Add 500 μ L of CyFACS buffer. Centrifuge plate at 750 $\times g$ for 10 min at RT. Aspirate the supernatant. After removing plate from the centrifuge, set centrifuge to 4 °C.
14. During centrifugation (**step 13**), centrifuge the surface antibody cocktail in a 0.1 μ M Millipore spin filter for 3 min at 10,000 $\times g$.
15. Resuspend pelleted cells in 50 μ L of the antibody staining cocktail.
16. Incubate for 45 min on ice at 4 °C.
17. Add 500 μ L CyFACS buffer to each well. Centrifuge plate at 750 $\times g$ for 10 min at 4 °C. Aspirate the supernatant.
18. Resuspend each well in 100 μ L of 1 \times Lysing solution (Subheading 2.2, **item 7**) and incubate for 10 min at room temperature (*see Note 10*).

19. Add 500 μ L CyFACS buffer to each well. Centrifuge at $750\times g$ for 10 min at 4 °C. Aspirate the supernatant.
20. Add 500 μ L CyFACS buffer to each well. Centrifuge at $750\times g$ for 10 min at 4 °C. Aspirate the supernatant.
21. Resuspend cells in 200 μ L of Permeabilization solution (Subheading 2.2, **item 10**). Incubate for 10 min at room temperature.
22. Add 500 μ L CyFACS buffer to each well. Centrifuge at $750\times g$ for 10 min at 4 °C. Aspirate the supernatant.
23. During centrifugation (**step 22**), centrifuge the intracellular antibody cocktail in a 0.1 μ M Millipore spin filter for 3 min at $10,000\times g$.
24. Resuspend cells in 50 μ L of the intracellular antibody staining cocktail.
25. Incubate for 45 min on ice at 4 °C.
26. Add 500 μ L CyFACS buffer to each well, centrifuge at $750\times g$ for 10 min at 4 °C and aspirate the supernatant.
27. Repeat **step 26** two additional times.
28. Resuspend cells thoroughly in 100 μ L of freshly prepared Interchelator-PFA solution.
29. Incubate overnight at 4 °C.
30. The following day (the same day that the cells will be run on the mass cytometer), add 500 μ L of CyPBS buffer to each well. Centrifuge at $1000\times g$ for 10 min at RT. Aspirate the supernatant, leaving 100 μ L residual volume in the well.
31. Add 500 μ L MilliQ water (metal-free) to each well. Centrifuge at $1000\times g$ for 10 min at RT. Aspirate the supernatant, leaving 100 μ L residual volume in each well.
32. Repeat **step 31** twice for a total of three washes in MilliQ water.
33. Resuspend cells in the residual 100 μ L MilliQ water after the final wash.
34. Run on mass cytometer after resuspension in approximately 1 mL of MilliQ water with or without normalization beads immediately prior to the run. Pipette cells into a FACS tube through a cell-strainer cap to remove unwanted debris.

3.3 Running Samples on a CyTOF Mass Cytometer

Running samples on a CyTOF mass cytometer is normally performed by Flow Cytometry core services at most Institutions, to procure a detailed step-by-step protocol for operating CyTOF refer to the publication by Leipold M.D. et al. [7]. It is important

to note that when looking for rare cell populations, higher numbers of total cells will need to be run through the mass cytometer. Although the maximum collection rate is estimated at 1000 cells/s, to ensure the avoidance of doublets and nebulizer clots, it is prudent to use low run speeds of 300 cells/s or lower. For assay normalization within and among runs, normalization beads should be used as described in [8]. It is helpful to count the cells in each well in order to appropriately estimate the ideal resuspension volume. Cells should be stored on ice while waiting to run on the mass cytometer.

3.4 Data Analysis

Due to the increase in potential marker combinations allowed for by mass cytometry, manual gating of all possible cellular subsets is not possible. In addition, this approach does not allow for the evaluation of unexpected cellular subsets of interest [2]. However, typical manual gating schema can also be used to major known cell subsets and functions, although there are important differences to consider when analyzing in FlowJo (*see* **Notes 11** and **12** and Figs. 2 and 3). Boolean gating analysis can also be used to identify discreet cellular subsets as described by Horowitz et al. [4]. A number of analysis packages have now been developed in order to analyze CyTOF data in an unbiased manner. Spanning-tree progression analysis of density-normalized events (SPADE) is available through Cytobank and uses density-dependent down sampling, followed by agglomerative clustering, minimum spanning tree construction and upsampling to identify unique cellular subsets from CyTOF [9]. Citrus is an algorithm that identifies clusters of phenotypically similar cells in an unsupervised manner and identifies features (either functional or phenotypic) that are predictive of a selected group of samples with a specific endpoint or treatment relative to a control group [10]. Another tool to visualize these data in two dimensions is viSNE [11]. A complete step-wise protocol for analysis of CyTOF data is beyond the scope of this chapter, and we recommend that the user obtain training from their institution, Cytobank, or Fluidigm in the use of the various algorithms and analysis of these complex data sets and how to apply the programs to their specific research questions.

4 Notes

1. We have had success with IgM conjugations (e.g., CD57) although they are not technically supported by DVS/Fluidigm.
2. Qdot antibodies have a cadmium core that can be detected by mass cytometry on channels Cd111–Cd114. Bright antibodies (e.g. HLA-DR, CD3) are most effectively used on this channel or it can be designated as a dump channel.

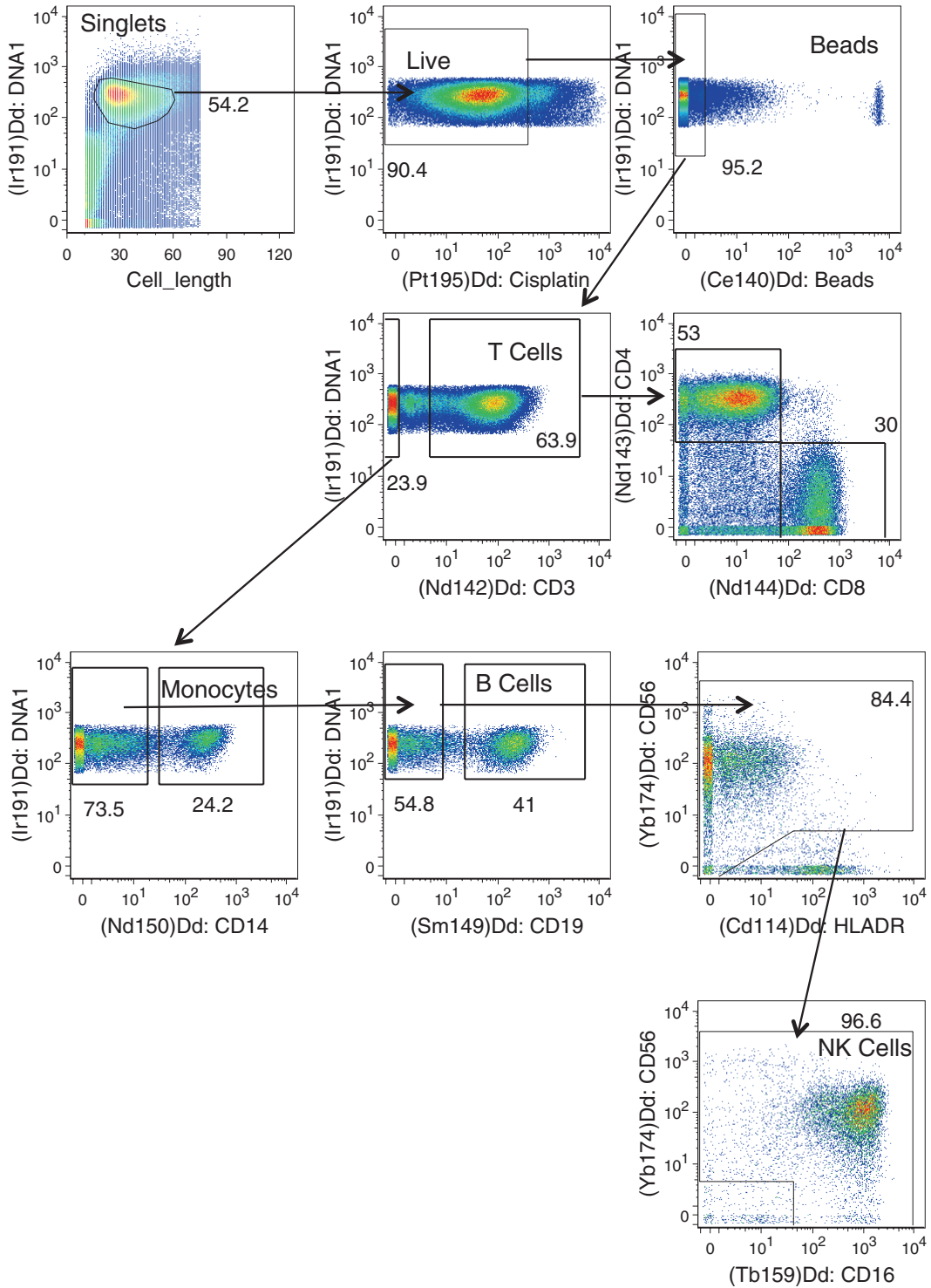


Fig. 2 Gating strategy to identify NK cells and other major cell subsets by mass cytometry. The sequential gates to identify T-cell subsets, monocytes, B cells, and NK cells are shown

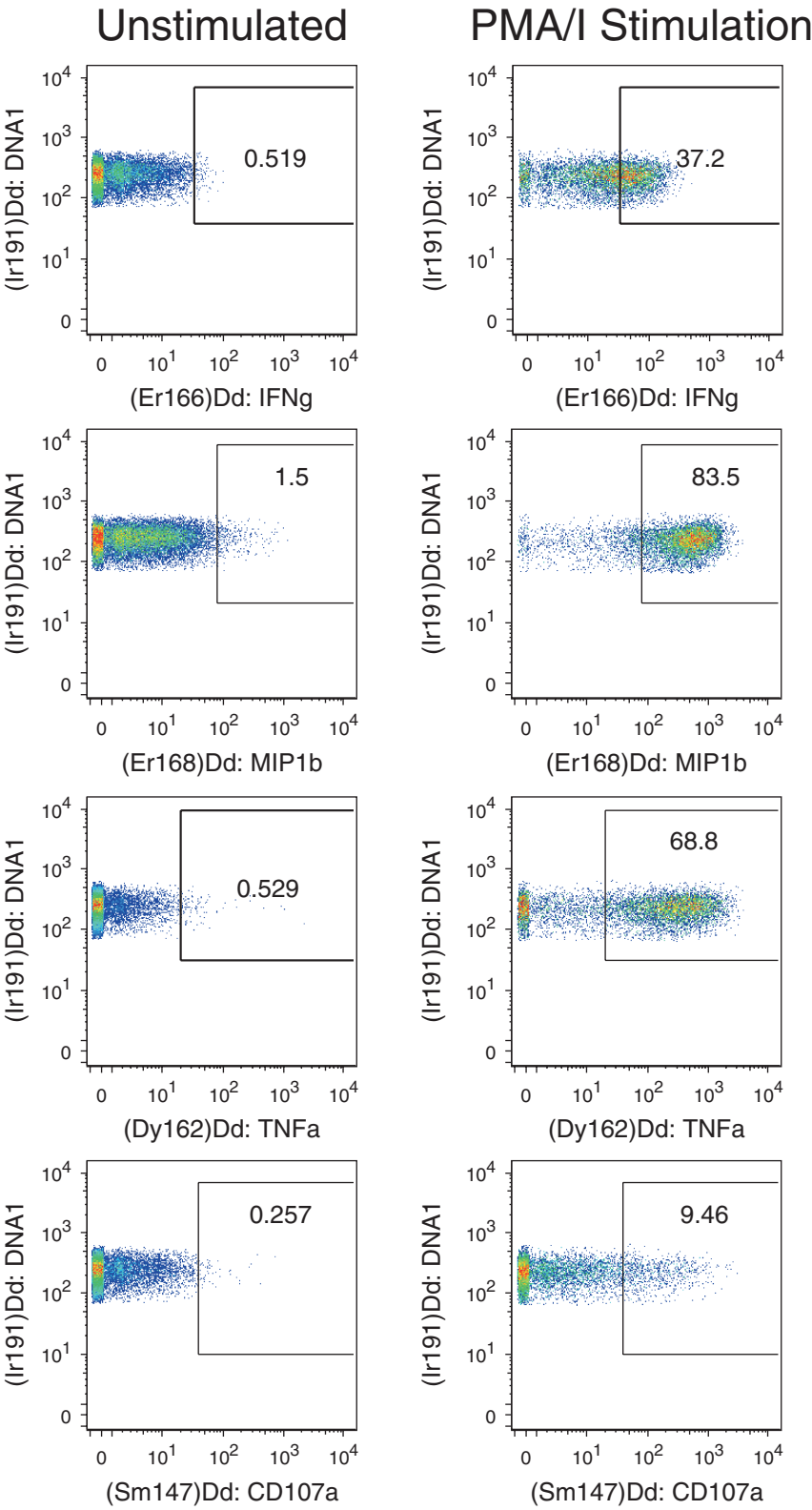


Fig. 3 Representative CyTOF plots of functional markers on stimulated and unstimulated NK cells, gated as in Fig. 2

3. Currently, Fluidigm does not sell kits to conjugate Gd157. Gd157 metals can be procured as 92%+purity from Trace Sciences International, and conjugated with polymer from MaxPar kits.
4. Following conjugation, the total concentration of antibody should be determined by the concentration of A280 protein as measured by Nanodrop. The expected yield is approximately 2/3 of the initial antibody conjugated.
5. Antibodies should be titrated to determine optimal concentration. A typical starting point is 10 $\mu\text{g}/\text{mL}$, with five serial two-fold dilutions to test the range between 0.3125 and 10 $\mu\text{g}/\text{mL}$.
6. Based on the concentration of the antibody following concentration, and the optimal titration as determined empirically, the amount of antibody added per sample can be calculated in order to make the antibody staining cocktail. It is advisable to make extra staining cocktail. Example calculations for 20 samples are shown in Table 1.
7. Peripheral blood mononuclear cells can be used fresh or cryopreserved and thawed prior to use. If the primary focus is NK cell responses, it is not necessary to rest the cells overnight prior to use.
8. Either DOTA-maleimide conjugated to a metal isotope [12] or cisplatin [13] can be used as a live-dead stain. Cisplatin use allows an additional antibody channel because its detection channel cannot be used in conjugations with other antibodies.
9. We have noted some batch-to-batch variability in the concentration of cisplatin required to see a clearly defined dead population. Validation can be performed with samples containing partially heat-killed cells. Cisplatin is a readily available platinum-based chemotherapeutic agent that passively accesses the cell interior of dead cells and rapidly reacts with protein nucleophiles such as R-SH or R-S-CH₃ [10]. Cisplatin entry into cells is stopped through the addition of 100% fetal bovine serum that reacts with residual extracellular cisplatin and prevents active transport into live cells.
10. It is acceptable to freeze the plate at -80°C for up to 1 week after adding FACS Lyse working solution.
11. In order to analyze CyTOF data, settings on FlowJo need to be customized. In order to do so go to the “Preferences” tab, and under workspace select “CyTOF” in the lower right corner and set the value to 20,000. Select the “Define” button under the “Reading Digital Data Files” heading. Select “Side Scatter” and all fluorescence parameters to display with logarithmic staining. Select “Ignore Scaling suggested by the data file”. Add Time and Cell Length as parameters that should

always be linear in the box on the top right. Set the Lowest Standard Log Conversion setting to 1 and the number of decades to display in log-converted data to 6 pulse area parameters and 6 pulse height parameters. Select the Enable Transformation box in addition to the Transform Height Parameters box. Set the number of decades to 5, additional negative display size to 0, and the width basis to -20.

12. Gating strategies to identify cell populations of interest are similar to flow cytometry, but with some important differences given the absence of forward and side scatter (Fig. 2). An example of staining and gating for NK cell functional markers is demonstrated in Fig. 3.

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Methods and Protocols

Somanchi, S.S. (Ed.)

2016, XV, 365 p. 76 illus., 49 illus. in color., Hardcover

ISBN: 978-1-4939-3682-3

A product of Humana Press