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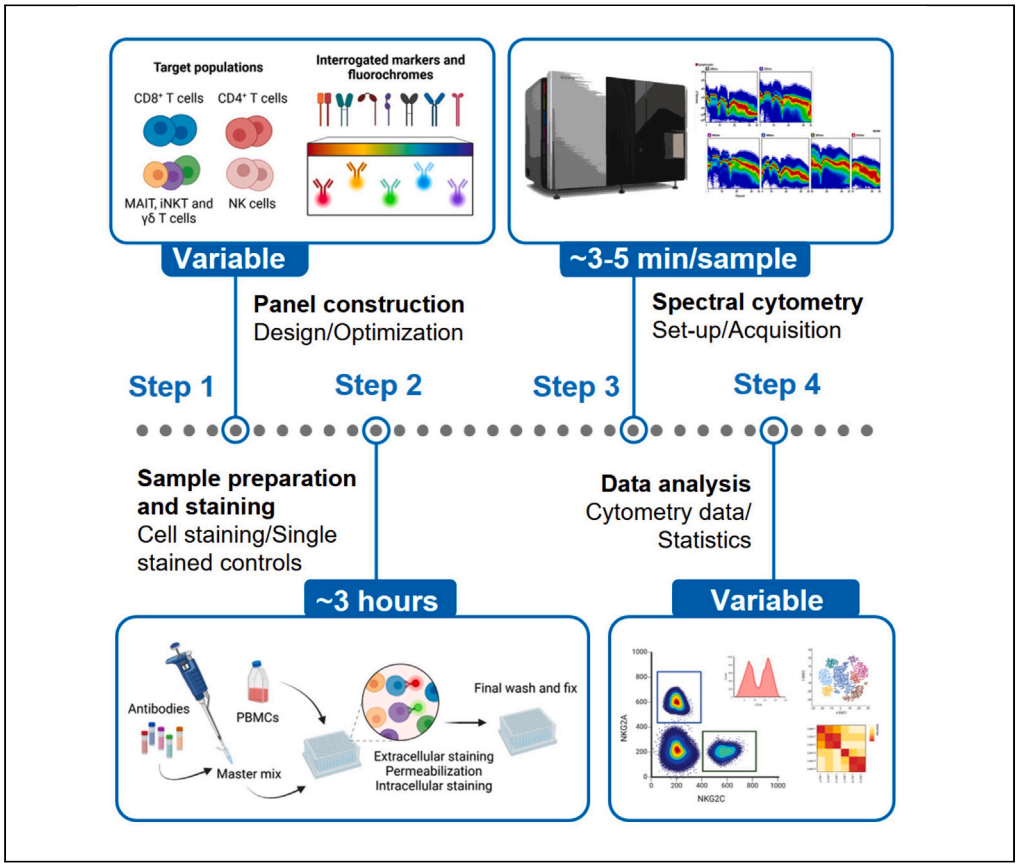
Protocol

Deep phenotyping characterization of human unconventional CD8$^+$NKG2A/C$^+$ T cells among T and NK cells by spectral flow cytometry



Here, we present a protocol for setting three spectral flow cytometry panels for the characterization of human unconventional CD8$^+$NKG2A/C$^+$ T cells as well as other T and natural killer cell subsets. We describe steps for standardizing, preparing, and staining the cells, the experimental setup, and the final data analysis. This protocol should be advantageous in various settings including immunophenotyping of limited samples, immune function evaluation/ monitoring, as well as research in oncology, autoimmune, and infectious diseases.

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

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Highlights

Protocol for deep phenotyping of human CD8$^+$ T cells expressing NKG2A/NKG2C

Design and optimization of three spectral flow cytometry panels

Preparation, staining, and analysis of human PBMCs with spectral flow cytometry

Identification and analysis of NK, classical, and unconventional T cell populations

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Deep phenotyping characterization of human unconventional CD8$^+$NKG2A/C$^+$ T cells among T and NK cells by spectral flow cytometry

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SUMMARY

Here, we present a protocol for setting three spectral flow cytometry panels for the characterization of human unconventional CD8$^+$NKG2A/C$^+$ T cells as well as other T and natural killer cell subsets. We describe steps for standardizing, preparing, and staining the cells, the experimental setup, and the final data analysis. This protocol should be advantageous in various settings including immunophenotyping of limited samples, immune function evaluation/monitoring, as well as research in oncology, autoimmune, and infectious diseases.

BEFORE YOU BEGIN

Rationalization and design of the spectral flow cytometry panels

CD8$^+$ T cells expressing inhibitory NKG2A (CD159a) and activating NKG2C (CD159c) molecules (ligands of the non-classical molecule HLA-E) have been associated with several inflammatory conditions, such as inflammatory gut diseases, tumors, aging, autoimmune and infectious diseases.[1–11] Previous data from our laboratory demonstrated that CD8$^+$NKG2A/C$^+$ T cells expressing cytotoxic and regulatory profiles expand in the gut of non-human primates (NHP) that naturally control SIV-induced disease as compared to those who develop chronic inflammation and disease.[12]

The main objective of this protocol is to phenotype human CD8$^+$ T cells expressing inhibitory NKG2A (CD159a) and activating NKG2C (CD159c) markers amongst different classical T and natural killer (NK) cell subsets, and to distinguish them from other unconventional T cells such as γδ T, invariant natural killer T (iNKT), and mucosal-associated invariant T (MAIT) cells. These populations have been studied in the context of infectious, autoimmune diseases, allergy and cancer, are found in the blood at low frequencies as compared to the other classical CD8$^+$ and CD4$^+$ T cell major populations, have unique dynamics, and can express CD94, NKG2A, and NKG2C.[13–23] We selected markers to separate all these populations and outlined a gating strategy for discriminating given cell subsets from each other.

Spectral flow cytometry allows the unambiguous simultaneous analysis of a high number of markers, and reduce the number of cells needed for a thorough phenotyping analysis. We built and standardized three panels: two focused on CD8$^+$ T cells and one on CD4$^+$ T cells. Based on literature reviews, and our own data from human donors and non-human primates (NHP), we selected markers of interest associated with the study of diverse features (homing, regulatory, cytotoxicity, exhaustion, etc.).

We incorporated classical markers of differentiation (CD27, CCR7, CD45RA, and CD45RO), which is important for the study of T cell dynamics. Furthermore, we aimed to study molecules associated with the activity of NKG2A and NKG2C (such as the non-classical HLA-E), as well as other molecules expressed in additional T cell subsets of interest such as regulatory T cells (Treg) (such as $CD4^+CD25^+FOXP3^+$ T cells), T helper 17 cells (Th17), and circulating T follicular helper cells (cTfh). Thus, the third panel (panel C), was simultaneously standardized.

We optimized the design of the panels using direct information from the reagents as well as online tools (described in the key resources table) to reduce the chances of non-compatibility between clones, host-species, and fluorochromes. For instance, we used the Cytek Full Spectrum Viewer (Cytek Biosciences 2019) (https://spectrum.cytekbio.com/) to verify that the combination of fluorochromes was maintained with the less similar dye signatures as possible. This tool provides a similarity index for every pair of fluorochromes and one complexity index for every full panel. For both type of indexes, lower values indicate low similarity between fluorochromes (Figure 1). For the final design, we took into account our gating strategies, the markers and fluorochromes available, as well as the configuration of the spectral cell analyzer. Afterwards, we standardized the panels with single stained controls (using beads), testing of regular stain buffer and brilliant stain buffer, blocking of human Fc receptors (FcR), antibody titration, different staining incubation temperature, different combinations of antibodies/fluorochromes, as well as the final fluorescence compensation (with the spectral unmixing tool), and data analysis. Table 1 shows a description of all markers included in the three panels.

Institutional permissions

Blood samples from healthy donors were obtained from the French blood bank (Etablissement Français du Sang) as part of an agreement with the Institut Pasteur (C CPSL UNT, number 15/EFS/023). The study was approved by the Ethics Review Committee (Comité de protection des personnes) of Île-de-France VII.

Technical considerations

1. Keep in mind that each panel is an individual experiment that needs to be set up and standardized independently. However, the three panels can be run in parallel.
2. Make sure to review the configurations (lasers, detectors, filters, mirrors, etc.) of the spectral flow cytometry equipment that will be used in order to adapt the fluorochromes of the panels if needed.
   a. Control the compatibility of reagents and antibodies according to your objectives and settings.
   b. Keep in mind the brightness, concentration and the use of fluorescence minus one (FMO) controls when constructing your panels.
   c. Available online tools and other resources can be accessed to determine the similarity and complexity indexes amongst the fluorochromes in order to prevent or limit potential spectrum overlaps (https://spectrum.cytekbio.com/).
3. The use of compensation beads (used for this protocol) is recommended for the fluorescence compensation (unmixing) in the spectral cell analyzer (Sony ID7000) but preparation of single stained controls for testing the antibodies in the target cells is pertinent. Specific beads exist on the market; consider the bead-antibody compatibility factor.
4. During the staining and further acquisition steps, protect the antibodies, master mixes, and stained samples from light and maintain them at 4°C of temperature.
5. Acquire the samples immediately after staining in order to avoid loss of fluorescence and sample deterioration.
6. For the preparation of your experiments, consider the amount of samples and cells to be prepared since this will decrease or increase the time and amount or reagents/consumables required.

Table 1. Target human lymphocyte cell markers

Marker (common name)	Description (type/function)
CD195 (CCR5)	Chemokine receptor, differentiation
CD196 (CCR6)	Chemokine receptor, differentiation
CD197 (CCR7)	Central memory, differentiation
CD103 (integrin αE)	Cell adhesion, homing
CD16 (FcγRIII)	NK cell differentiation, antibody dependent cellular cytotoxicity (ADCC)
CD161 (NKR-P1A)	Th17 T-cell, differentiation
CD19	B-cell marker
CD226 (DNAM-1)	Regulatory, co-stimulatory receptor
CD25 (IL2RA)	Regulatory T-cell, IL-2 receptor
CD26 (DPP-IV)	Co-stimulatory receptor, regulatory role
CD27	Co-stimulatory receptor, T and B-cell differentiation
CD28	Co-stimulatory receptor
CD3 (CD3ε)	T-cell co-receptor
CD38 (cyclic ADP ribose hydrolase)	T-cell activation, differentiation
CD39 (NTPDase1)	Differentiation, immunosuppressive marker
CD4	CD4 T-cells
CD45RA (LCA isoform CD45RA)	Differentiation marker
CD45RO (LCA isoform CD45RO)	T-cell memory marker
CD49d (integrin α4)	Integrin α4, homing
CD56 (NCAM)	NK cell marker
CD73 (ecto-5′-nucleotidase)	Differentiation, immunosuppressive marker
CD8	CD8 T-cells
CD94 (KLRD1)	NK cell receptor, NKG2/CD94 dimer
CD154 (CTLA-4)	Treg marker, immune checkpoint
CD183 (CXCR3)	Chemokine receptor, differentiation
CD185 (CXCR5)	Chemokine receptor, differentiation
CD186 (CXCR6)	Chemokine receptor
FOX-P3	Treg marker
CD357 (GITR)	Glucocorticoid-induced TNFR-related protein, TNF receptor
Granzyme B	Cytotoxicity marker
HLA-DR	MHC class II, T-cell activation marker
HLA-E	Non-classical MHC class I
CD360 (IL-21R)	IL-21 receptor
IL-23R	IL-23 receptor
Ki-67	Cell cycle marker
CD158 (KIR2DL1/S1/S3/S5)	Inhibitory/activating KIR receptors
CD158e1 (KIR3DL1)	Inhibitory KIR receptor
CD223 (LAG-3)	T cell activation, immune checkpoint
MICA	MHC class I chain-related protein A
CD159a (NKG2A)	NK cell inhibitory receptor
CD159c (NKG2C)	NK cell activating receptor
CD314 (NKG2D)	NK cell activating receptor
CD337 (NKP30)	NK cell activating receptor
CD335 (NKP46)	NK cell activating receptor
CLEC5C (NKP80)	NK cell activating receptor
CD279 (PD-1)	T-cell inhibitory receptor, immune checkpoint
TCRγδ	γδ T cells
CD336 (TIM-3)	Inhibitory receptor, regulatory marker
ULBP-2/5/6	NKG2D ligand
TCRVα2.4-Jα18	Invariant natural killer T cells (iNKT)
TCRVα7.2	Mucosal-associated invariant T cells (MAIT)
Integrin β7	Cell adhesion, homing marker

7. If you aim to include cytokines in the flow cytometry panels, consider and test if additional stimulation/activation is required for the cells in advance.

Sample preparation

Timing: 7–9 h

This section describes the preparation of the target PBMCs before the staining steps.

8. Warm Roswell Park Memorial Institute (*RPMI*) *1640* medium to 37°C before thawing the frozen *peripheral blood mononuclear cells* (PBMCs).
9. Thaw the frozen PBMCs in a water bath at 37°C.
   a. Just before full thawing of the cells, transfer the cryovials to the biosafety hood.
   b. Slowly add warm RPMI medium for a final volume of twice the initial volume originally contained in the cryovial.
10. Transfer the cell suspension to a 50-mL polypropylene tube and complete the volume to 50-mL with warm RPMI medium.
11. Centrifuge the cells at 400 g for 5 min.
12. Decant and suspend the cells into fresh new warm RPMI medium.
13. Count the cells using your local protocol (i.e., automated cell counter)
14. Suspend the cells at 1 × 10^6 cells/mL.
15. Incubate the cells between 6–8 h at 37°C and 5% CO_2 in a flask container prior to the staining steps.

Note: We recommend to assess in advance, the amount of time for resting your target cells after thawing, given that the expression of some chemokine receptors may vary. We standardized this protocol by resting the PBMCs for 8 h after the thawing process since the detection of CCR7 significantly improved as compared to recently thawed cells. In any case, we recommend staining your samples at the same conditions each time. Incubation periods longer than 8 h might have a higher negative impact in the viability of PBMCs.

Note: Although these panels were standardized with frozen PBMCs, they could potentially be used for working with fresh blood cells or other tissue-isolated cells under their particular considerations. After the preparation of tissue and cell isolation, no major modifications are likely to be needed for antibody staining; nonetheless, we suggest reviewing specific provider recommendations on equipment and reagents for particular types of tissues.

Note: This protocol was standardized for staining up to 1 × 10^6 PBMCs in 100 μL of volume. If you plan to use a few or more cells, we suggest titrating and testing the panels in advance before conducting the experiments.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Zombie UV™ Fixable Viability Kit	BioLegend	Cat#423107
APC/Fire™ 810 anti-human CD14	BioLegend	Cat#367156
APC/Fire™ 810 anti-human CD19	BioLegend	Cat#302271
Spark Blue™ 550 anti-human CD3	BioLegend	Cat#344851
BD OptiBuild™ BV480 Mouse Anti-Human γδ TCR	BD	Cat#746498
Brilliant Violet 421™ anti-human TCR Vα24-Jα18 (iNKT cell)	BioLegend	Cat#342915

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
APC/Cyanine7 anti-human TCR Vα7.2	BioLegend	Cat#351713
Brilliant Violet 510™ anti-human CD4	BioLegend	Cat#317443
Brilliant Violet 570™ anti-human CD8a	BioLegend	Cat#301037
PE/Fire™ 640 anti-human CD56 (NCAM) Recombinant	BioLegend	Cat#392431
Pacific Blue™ anti-human CD16	BioLegend	Cat#302024
FITC anti-human CD45RA	BioLegend	Cat#304105
BD Horizon™ BUV395 Mouse Anti-Human CD45RO	BD	Cat#564291
Brilliant Violet 650™ anti-human CD27	BioLegend	Cat#302828
PerCP/Cyanine5.5 anti-human CD28	BioLegend	Cat#302921
Brilliant Violet 711™ anti-human CD196 (CCR6)	BioLegend	Cat#353435
PerCP anti-human CD197 (CCR7)	BioLegend	Cat#353242
PE/Fire™ 810 anti-human CD183 (CXCR3)	BioLegend	Cat#353759
Brilliant Violet 750™ anti-human CD185 (CXCR5)	BioLegend	Cat#356941
BD OptiBuild™ BUV563 Mouse Anti-Human CXCR6 (CD186)	BD	Cat#748450
PE/Cyanine7 anti-human/mouse integrin β7	BioLegend	Cat#321241
PE/Fire™ 700 anti-human CD103 (Integrin αE)	BioLegend	Cat#350239
Alexa Fluor® 647 anti-human CD49d	BioLegend	Cat#304335
BD Pharmingen™ PE Mouse Anti-Human CD94	BD	Cat#555889
Alexa Fluor® 700 anti-human CD159a (NKG2A)	BioLegend	Cat#375120
BD OptiBuild™ BUV615 Mouse Anti-Human CD159C (NKG2C)	BD	Cat#751059
BD OptiBuild™ BUV737 Mouse Anti-Human CD314 (NKG2D)	BD	Cat#748426
PE/Dazzle™ 594 anti-human CD337 (NKp30)	BioLegend	Cat#325231
Brilliant Violet 785™ anti-human CD335 (NKp46)	BioLegend	Cat#331945
APC anti-human NKp80	BioLegend	Cat#346708
BD OptiBuild™ BV750 Mouse Anti-Human CD195 (CCR5)	BD	Cat#747224
BD OptiBuild™ BUV395 Mouse Anti-Human CD26	BD	Cat#744454
PE/Fire™ 810 anti-human CD39	BioLegend	Cat#328245
Brilliant Violet 711™ anti-human CD73 (Ecto-5'-nucleotidase)	BioLegend	Cat#344025
BD OptiBuild™ BUV737 Mouse Anti-Human CD226	BD	Cat#748428
PE/Cyanine7 anti-human CD360 (IL-21R)	BioLegend	Cat#359513
Human IL-23 R APC-conjugated Antibody	Bio-Techne	Cat#FAB14001A-100
PE anti-human CD357 (GITR)	BioLegend	Cat#311603
BD OptiBuild™ BUV805 Mouse Anti-Human KIR2DL1/S1/S3/S5 (CD158)	BD	Cat#752514
PerCP/Cyanine5.5 anti-human CD158e1 (KIR3DL1, NKB1)	BioLegend	Cat#312717
PE/Dazzle™ 594 anti-human CD152 (CTLA-4)	BioLegend	Cat#369615
Brilliant Violet 785™ anti-human CD223 (LAG-3)	BioLegend	Cat#369321
Brilliant Violet 650™ anti-human CD366 (Tim-3)	BioLegend	Cat#345027
Alexa Fluor® 647 anti-human Ki-67	BioLegend	Cat#350510
PE/Fire™ 700 anti-human CD279 (PD-1)	BioLegend	Cat#621621
PE/Cyanine5 anti-human/mouse Granzyme B Recombinant	BioLegend	Cat#372225
Alexa Fluor® 700 anti-human CD27	BioLegend	Cat#302814
Alexa Fluor® 647 anti-mouse/rat/human FOXP3	BioLegend	Cat#320013
PE/Cyanine7 anti-human CD161	BioLegend	Cat#339917
PE anti-human CD25	BioLegend	Cat#302605
Brilliant Violet 650™ anti-human CD38	BioLegend	Cat#356620
PerCP/Cyanine5.5 anti-human HLA-DR	BioLegend	Cat#307629
APC anti-human HLA-E	BioLegend	Cat#342605
Brilliant Violet 421™ anti-human CD279 (PD-1)	BioLegend	Cat#329919
BD OptiBuild™ BV605 Mouse Anti-Human ULBP-2/5/6	BD	Cat#748131
BUV805 Mouse Anti-Human MICA	BD	Cat#749768
Experimental Models: Cell Lines		
Frozen PBMCs from human donors isolated from fresh blood (information regarding sex, age, and other features of the donors is not available)	Établissement Français du Sang (France)	https://dondesang.efs.sante.fr/

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Other		
Human TruStain FcX™ (Fc Receptor Blocking Solution)	BioLegend	Cat#422301
Roswell Park Memorial Institute (RPMI) 1640 Medium	Life Technologies	Cat#21875034
Dulbecco's Phosphate Buffered Saline (1X)	Life Technologies	Cat#14190144
UltraComp eBeads™ Compensation Beads	Thermo Fisher Scientific	Cat#01-2222-42
BD Pharmingen™ Stain Buffer (BSA)	BD Pharmingen	Cat#554656
BD Horizon™ Brilliant Stain Buffer	BD Pharmingen	Cat#563794
BD Cytofix/Cytoperm™ Fixation/Permeabilization Solution Kit	BD Pharmingen	Cat#554714
Thermo Scientific™ Paraformaldehyde, 4% in PBS	Thermo Fisher Scientific	Cat#J61899
Sony ID7000™ spectral cell analyzer	Sony	https://www.sonybiotechnology.com/us/instruments/id7000-spectral-cell-analyzer/
Software and algorithms		
Cytek Full Spectrum Viewer	Cytek® Biosciences 2019	https://spectrum.cytekbio.com/
FlowJo 10.7.1	© Becton Dickinson & Company (BD)	https://www.flowjo.com/
Spectral Flow Analysis (SFA) - Life Sciences Cloud Platform	Sony	https://www.sonybiotechnology.com/us/instruments/sfa-cloud-platform/

Note: This protocol was standardized in a Sony ID7000 spectral cell analyzer set with 6 lasers (deep ultra-violet 320 nm, ultra-violet 355 nm, violet 405 nm, blue 488 nm, yellow/green 561 nm, and red 637 nm). We advise that you follow the recommendation of the supplier for the appropriate use, configuration and handling of the cell analyzer instrument. For detailed information beyond the essentials specified in this protocol regarding the instrument, you can refer to the technical data and resources available online and directly contact the provider (sonybiotechnology.com).

MATERIALS AND EQUIPMENT

Consumables and other resources		
Reagent or resource	Source	Identifier
Material and consumables		
Falcon 5 mL Round Bottom Polystyrene Test Tube, with Snap Cap	Corning	Cat#352054
Corning cell culture flasks	Corning	Cat#430639
Corning 15 mL centrifuge tubes	Corning	Cat#430791
Corning 50 mL centrifuge tubes	Corning	Cat#430829
Half-Deep Well Plate, 0.5 mL, PP, sterile, round bottom	Cole-Parmer	Cat#FV-67104-57
Other resources		
Webinar: Spectral Cytometry Software Workflows and Tools that Enable Multiparametric Flow Cytometry	2022 Sony Biotechnology Inc.	https://www.sonybiotechnology.com/us/blog/spectral-cytometry-software-workflows-and-tools-that-enable-multiparametric-flow-cytometry/
Webinar: Panel Design Considerations for Spectral Flow Cytometry	2022 Sony Biotechnology Inc.	https://www.sonybiotechnology.com/us/blog/webinar-panel-design-considerations-for-spectral-flow-cytometry/

STEP-BY-STEP METHOD DETAILS

Preparation of single stained controls

Timing: 40 min

This section describes the preparation of control beads and cells for every marker. These controls are required for the creation of spectral references (signatures) for every fluorochrome. The controls and

Table 2. Panel A (CD8$^+$NKG2A/C$^+$ T cells)

#	Specificity	Clone	Fluorochrome	ng/mL
1	Live/Dead	-	Zombie UV	-
2	CD14	63D3	APC-Fire 810	2.5
3	CD19	HIB19	APC-Fire 810	0.625
4	CD3	SK7	Spark Blue 550	2.5
5	TCRγδ	11F2	BV480	2.5
6	TCRVα2.4	6B11	BV421	0.625
7	TCRVα7.2	3C10	APC-Cy7	1.25
8	CD4	OKT4	BV510	1.25
9	CD8	RPA-T8	BV570	1.25
10	CD56	QA17A16	PE-Fire 640	1.25
11	CD16	3G8	Pacific Blue	6.25
12	CD45RA	HI100	FITC	2.5
13	CD45RO	UCHL1	BUV395	1.25
14	CD27	O323	BV650	0.625
15	CD28	CD28.2	PerCP/Cy5.5	2.5
16	CCR6	G034E3	BV711	3
17	CCR7	G043H7	PerCP	8
18	CXCR3	G025H7	PE-Fire 810	0.625
19	CXCR5	J252D4	BV750	3
20	CXCR6	13B 1E5	BUV563	6
21	Integrin β7	FIB504	PE-Cy7	2.5
22	CD103	Ber-ACT8	PE-Fire 700	0.3125
23	CD49d	9F10	AF647	1.25
24	CD94	HP-3D9	PE	0.06
25	NKG2A	S19004C	AF700	5
26	NKG2C	134591	BUV615	2.5
27	NKG2D	1D11	BUV737	6
28	NKP30	P30-15	PE-Dazzle 594	5
29	NKP46	9E2	BV785	5
30	NKP80	5D12	APC	10

unmixing matrix will allow the correct identification and separation of markers in the fully stained samples.

1. Prepare single stained controls (except for the viability dye) with compensation beads (UltraComp eBeads, Invitrogen) following instructions from the manufacturer (https://www.thermofisher.com/document-connect/document-connect.html?url=https://assets.thermofisher.com/TFS-Assets%2FLSG%2Fmanuals%2FMAN0019374_UltraComp_eBeads_and_UltraComp_eBeads_Plus_Compensation_Beads_PI.pdf).
   a. Mix the beads vigorously by pulse-vortexing and add 1 drop in a half-deep well plate (round bottom) (or single polystyrene tubes) for each antibody included in the panels.
   b. Add a single test (standardized quantity to stain 1 × 10^6 cells in 100 μL total volume, see Tables 2, 3, and 4) of conjugated antibody for each one of the single stained controls required, mix well by pulse-vortexing and incubate at 4°C for 20 min in the dark.
   c. Add 2 mL of flow cytometry stain buffer (BD) to each tube, and centrifuge at 400 g for 5 min.
   d. Decant and fix the beads with 400 μL of 4% Paraformaldehyde (PFA) for 10 min at 4°C, and maintain protected from light.
   e. Wash the beads with 1 mL of cold (4°C) 1× PBS by centrifuging at 400 g for 5 min.
   f. Decant and suspend the beads in 500 μL of cold (4°C) 1× PBS and maintain them protected from light at 4°C.
2. For the viability dye (Zombie UV, BioLegend), prepare one reaction with PBMCs and stain following the sample staining steps (see below).

Table 3. Panel B (CD8$^+$NKG2A/C$^+$ T cells)

#	Specificity	Clone	Fluorochrome	ng/mL
1	Live/Dead	-	Zombie UV	-
2	CD14	63D3	APC-Fire 810	2.5
3	CD19	HIB19	APC-Fire 810	0.625
4	CD3	SK7	Spark Blue 550	2.5
5	TCRγδ	11F2	BV480	2.5
6	TCRVα2.4	6B11	BV421	0.625
7	TCRVα7.2	3C10	APC-Cy7	1.25
8	CD4	OKT4	BV510	1.25
9	CD8	RPA-T8	BV570	1.25
10	CD56	QA17A16	PE-Fire 640	1.25
11	CD16	3G8	Pacific Blue	6.25
12	CD45RA	HI100	FITC	2.5
13	CCR5	2D7/CCR5	BV750	2.5
14	CCR7	G043H7	PerCP	8
15	CD26	M-A261	BUV395	2.5
16	CD39	A1	PE-Fire 810	1.25
17	CD73	AD2	BV711	1.25
18	CD226	DX11	BUV737	2.5
19	IL-21R	17A12	PE-Cy7	2.5
20	IL-23R	218213	APC	3 μL[b]
21	GITR	621	PE	12
22	NKG2A	S19004C	AF700	5
23	NKG2C	134591	BUV615	2.5
24	KIR2DL1/S1/S3/S5 (CD158)	HP-MA4	BUV805	2.5
25	KIR3DL1 (CD158e1)	DX9	PerCP/Cy5.5	2.5
26	CTLA4[a]	BNI3	PE-Dazzle 594	3
27	LAG-3	11C3C65	BV785	3
28	TIM-3	F38-2E2	BV650	3
29	PD-1	A17188B	PE-Fire 700	0.15
30	Granzyme B[a]	QA16A02	PE-Cy5	0.3125
31	Ki-67[a]	Ki-67	AF647	6

[a]Intracellular marker.
[b]Recommended concentration by the manufacturer: 10 μL/10^6 cells.

Note: It is recommended to run single stained controls each time that samples will be acquired, especially if there are changes of antibodies and/or reagent lot.

Optional: Add an extra sample of fully stained PBMCs (in each panel) to set the PMT configuration in the cytometer without losing events from your objective samples.

Sample staining

Timing: 2 h

This section describes the preparation of antibody master mixes, as well as incubation and washing steps for the extracellular and intracellular staining of the target PBMCs. The concentration of antibodies for each panel is listed in Tables 2, 3, and 4.

3. Count, separate and wash the cells.
   a. Centrifuge at 400 g for 5 min with 1× PBS.
   b. Suspend 1 × 10^6 cells into 100 μL of cold stain buffer (1× PBS, 20% FBS) in a 96-half-deep well plate.

Table 4. Panel C (CD4$^+$ T cell subsets)

#	Specificity	Clone	Fluorochrome	ng/mL
1	Live/Dead	-	Zombie UV	-
2	CD14	63D3	APC-Fire 810	2.5
3	CD19	HIB19	APC-Fire 810	0.625
4	CD3	SK7	Spark Blue 550	2.5
5	CD4	OKT4	BV510	1.25
6	CD8	RPA-T8	BV570	1.25
7	FOX-P3[a]	150D	AF647	2.5
8	CD161	HP-3G10	PE-Cy7	1.25
9	CD45RA	HI100	FITC	2.5
10	CCR5	2D7/CCR5	BV480	2.5
11	CCR6	G034E3	BV711	3
12	CCR7	G043H7	PerCP	8
13	CXCR5	J252D4	BV750	3
14	CXCR6	13B 1E5	BUV563	6
15	CD25	M-A251	PE	0.625
16	CD27	O323	AF700	6.25
17	CD38	HB-7	BV650	1.25
18	HLA-DR	L243	PerCP/Cy5.5	6
19	HLA-E	3D12	APC	2.5
20	PD-1	EH12.2H7	BV421	0.625
21	ULBP-2/5/6	165903	BV605	6
22	CTLA4[a]	BNI3	PE-Dazzle 594	3
23	MICA	159227	BUV805	6

[a]Intracellular marker.

4. Add 5 μL of FcR-blocking reagent (Human TruStain FcX, BioLegend) directly in the stain buffer (1× PBS, 20% FBS) containing the cells, mix and incubate for 10 min at 18°C–20°C of temperature.

   Note: This step is optional. Step 3 of the staining requires the suspension of the cells in 20% FBS-containing stain buffer, which helps to reduce non-specific FcRs binding and background fluorescence. In addition, we tested the use of an FcR-blocking solution and we did not find major differences in the markers except for the expression of CD3 in CD14$^+$CD19$^+$ cells (which are negatively selected in this protocol). Thus, we recommend performing an extra step with an Fc-blocking reagent to maximize the reduction of potential background signals especially when aiming to analyze cells containing high levels of Fc receptors such as monocytes, macrophages, and B-cells.

5. Wash the cells with cold (4°C) 1× PBS (complete to 500 μL of total volume).
   a. Centrifuge at 400 g for 5 min,
   b. Decant and suspend the cells in 100 μL of 1× PBS.
6. Add 1 μL of viability dye (Zombie UV, BioLegend) for each 1 × 10^6 cells in 100 μL cold 1× PBS (4°C) and incubate the cells for 20 min at 18°C–20°C of temperature in the dark.
7. In the meantime, prepare the different master mixes with the extracellular antibodies (panels A, B, and C) in cold (4°C) brilliant stain buffer (BD Biosciences) adjusting for a total volume of 100 μL/sample.

   Note: Antibody concentrations are indicated in Tables 2, 3, and 4.

   Note: We recommend adding each one of the polymer-based antibodies directly into the sample and gently mixing instead of adding them all together in the master mix in order to reduce the chances of additional staining artifacts due to non-specific reactivity.

a. Add 50% overhead volume to account for potential pipetting errors.
b. Mix well by gently pipetting up and down.

8. Wash the cells with cold 1× PBS (complete to 500 μL of total volume).
   a. Centrifuge at 400 g for 5 min, and decant.
9. Add the corresponding master mix of antibodies (100 μL) to the samples for extracellular staining (panels A, B, and C).
   a. Mix well by gently pipetting up and down.
   b. Incubate for 30 min at 18°C–20°C of temperature in the dark.
10. Wash the cells with cold (4°C) 1× PBS (complete to 500 μL of total volume).
    a. Centrifuge at 400 g for 5 min and decant.
11. Fix the cells without intracellular staining markers (panel A).
    a. Fix the cells with 500 μL of 4% Paraformaldehyde (PFA) for 10 min:
    b. Wash the cells with cold (4°C) 1× PBS (complete to 500 μL of total volume).
    c. Centrifuge at 400 g for 5 min.
    d. Decant by inverting the plate on absorbent clean paper towel.
    e. Suspend the cells in 500 μL of cold (4°C) 1× PBS.

Pause point: These stained and fixed cells (panel A) are ready for acquisition (maintain protected from light at 4°C if these are not immediately acquired in the cytometer).

12. Continue with the intracellular staining of the corresponding samples (panels B and C):
    a. Suspend the cells in 300 μL of cold (4°C) permeabilization buffer (Cytofix/Cytoperm solution, BD) and incubate for 20 min at 4°C in the dark.
    b. Prepare the different master mixes with intracellular antibodies (Tables 2, 3, and 4).
       i. Prepare the mixes in permeabilization/wash buffer (Perm/Wash Buffer, BD) previously diluted in distilled water (to make a 1× solution).
       ii. Adjust for a total volume of 50 μL/sample and add 50% overhead volume to account for potential pipetting errors.
       iii. Mix well by gently pipetting up and down.
    c. Wash the cells twice by adding 1× permeabilization/wash buffer (Perm/Wash Buffer, BD) (complete to 500 μL of total volume).
       i. Centrifuge at 400 g for 5 min.
       ii. Decant by inverting the plate on absorbent clean paper towel.
    d. Add the master mixes of antibodies (50 μL) to the samples.
       i. Mix well by gently pipetting up and down.
       ii. Incubate for 30 min at 4°C in the dark.
13. Wash the cells with cold (4°C) 1× permeabilization/wash buffer (Perm/Wash Buffer, BD) (complete to 0.5 mL of total volume).
14. Centrifuge at 400 g for 5 min at 4°C.
15. Decant by inverting the plate on absorbent clean paper towel.
16. Fix the cells in 500 μL of cold (4°C) 4% PFA for 10 min.
    a. Wash the cells by adding cold (4°C) 1× PBS (complete to 500 μL of total volume).
    b. Centrifuge at 400 g for 5 min.
    c. Decant by inverting the plate on absorbent clean paper towel.
    d. Suspend the cells in 500 μL of cold (4°C) 1× PBS and maintain protected from light at 4°C.

Note: Instead of a half-deep well plate, samples can be prepared in polystyrene tubes. The Sony ID7000 spectral cell analyzer supports 5-mL tube racks (24 tubes), 96-well standard height, 96-well half deep, 96-well deep plates, and 384-well standard flat bottom plates.

Note: The final volume for cell suspension should be adjusted depending on the number and type of cells, and whether tubes, deep-well or other plates will be used. Volumes in this protocol are standardized for 96-half-deep well plates.

Pause point: The stained and fixed cells are ready for acquisition (maintain protected from light at 4°C if they are not immediately acquired).

Instrument preparation and experiment set-up

Timing: 1 h

This section refers to the creation of a new experiment in the spectral cell analyzer system and preparing the instrument for sample acquisition. The steps include the calibration of the Sony ID7000 system, setting the characteristics of the experiment such as the template and groups of samples, indicating the fluorochromes in the panel, and setting the final PMT voltages to acquire the samples.

17. Set the spectral cell analyzer (Sony ID7000).
    a. Run daily purge, flow rate and QC calibrations following the instructions of the manufacturer (https://vimeo.com/847254690/e408449a02).

Note: The ID7000 spectral cell analyzer needs to be calibrated using the daily QC and performance 8-peak beads before setting-up the experiment and acquire the samples.

18. Create a new experiment (one individual experiment for each panel);
    a. In the spectral cytometer system software, indicate the type of template (tubes and/or 96-half-deep-well plates).
    b. Create a new sample group and add the samples to the group.
    c. Name the tubes or wells with the samples and single-stained controls.
    d. Add the fluorochromes to be acquired as single controls (Tables 2, 3, and 4) in the *Unmixing Settings*.
19. Adjust the PMT voltages for the experiment.
    a. Load the tube-rack or plate containing the single stained controls and/or the fully stained sample(s).
    b. Acquire in *Preview* a full stained sample and at least one single stain for each laser for setting the PMT voltage for each laser.
        i. Keep a homogeneous ratio between the lasers, and make sure dyes are higher in their corresponding excitation laser using the ribbon plots (Figure 2B).

Note: Do not change the voltage parameters after set-up; maintain the same values during acquisition of the samples and controls. PMT voltages and machine configuration for each panel must be independently set up.

20. Set the instrument settings for the experiments.
    a. Adjust the number of events to record, stopping criteria, flow rate, cleaning mode, acquisition offset, agitation and other settings as needed (i.e., taking into account sample volumes).

Note: More information from the manufacturer regarding the creation of a new experiment can be found online (https://vimeo.com/847255526/565a89c4bf).

Sample acquisition

Timing: 3–5 min/sample

This section describes the acquisition of single-stained controls and samples with auto-acquisition mode in the Sony ID7000 spectral cytometer system once the experiment settings have been fixed.

21. Acquire the samples and single-stained controls.

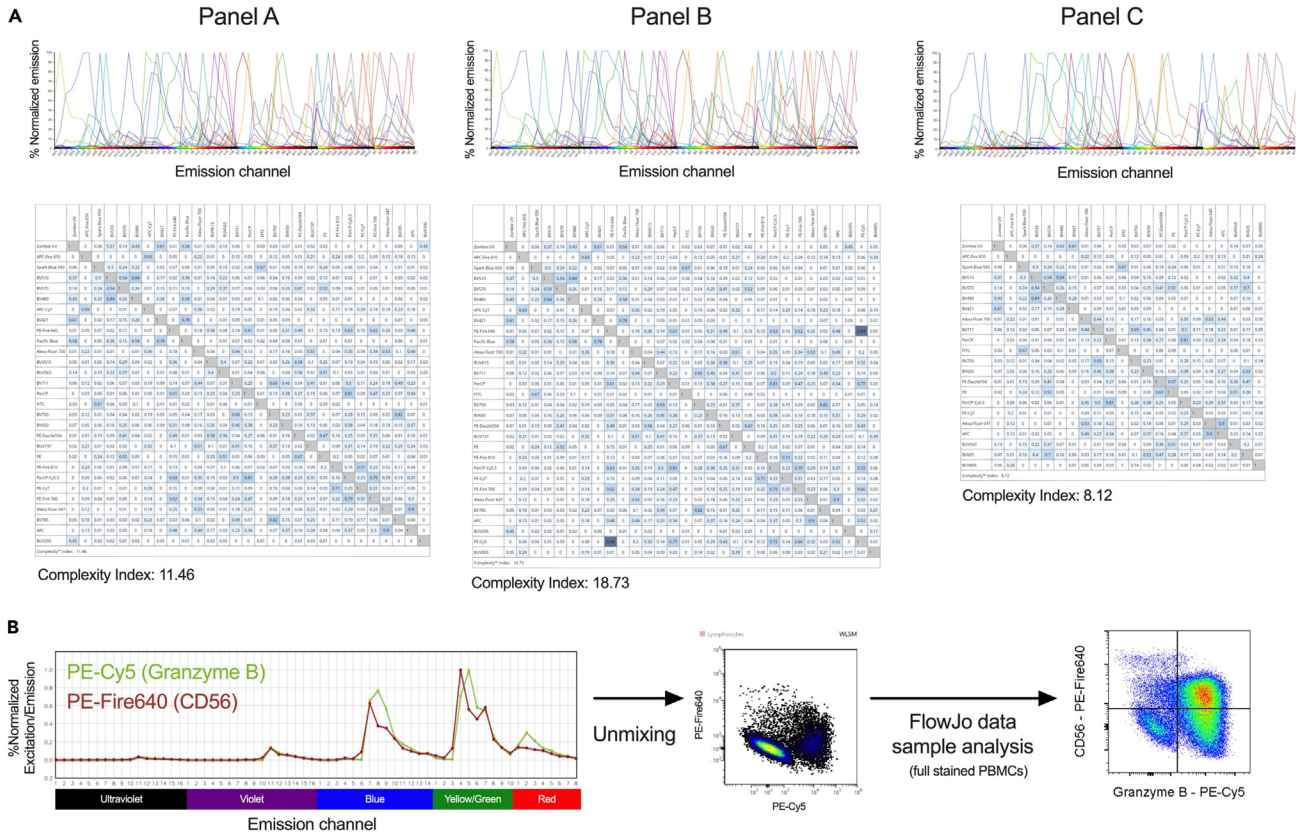


Figure 1. Overview of the panel's design according to fluorochromes combination

(A) Spectrum viewer showing the expected emission for every dye across the channels for the panels (A, B, and C) and their corresponding complexity matrix representing the full spectrum signature similarities for every dye combination. Values inside the matrix (similarity index) closer to zero indicate very different signatures between the two dyes implicated while values closer to one indicate very similar signatures (which might be more difficult to discriminate).

(B) Spectrum viewer showing the expected dye emission for PE-Cy5 and PE-Fire 640, which are the ones with the more similar signatures according to the matrix in panel B (similarity index of 0.94). However, in the experimental standardization, both fluorochromes were properly identified and unmixed, allowing the identification of the markers (CD56 and granzyme B) in the final fully stained samples as shown in the FlowJo plot gated on $CD3^{-}CD56^{+}$ NK cells.

a. Load the plate or tube-rack with the samples and set *Current Position* in the spectral cytometer system software.
b. Select the wells deemed for acquisition.
c. Right-click and add to *Auto Acquisition Target*.
d. Run *Auto Acquisition* when ready.

Note: Different groups of samples with different conditions can be recorded in the same experiment.

Unmixing analysis and file export

Timing: 1 h

This section describes how to create, set and apply the unmixing matrix for the experiments, and export the acquired sample files for further analysis. The unmixing matrix allows the correct identification of fluorescent signatures.

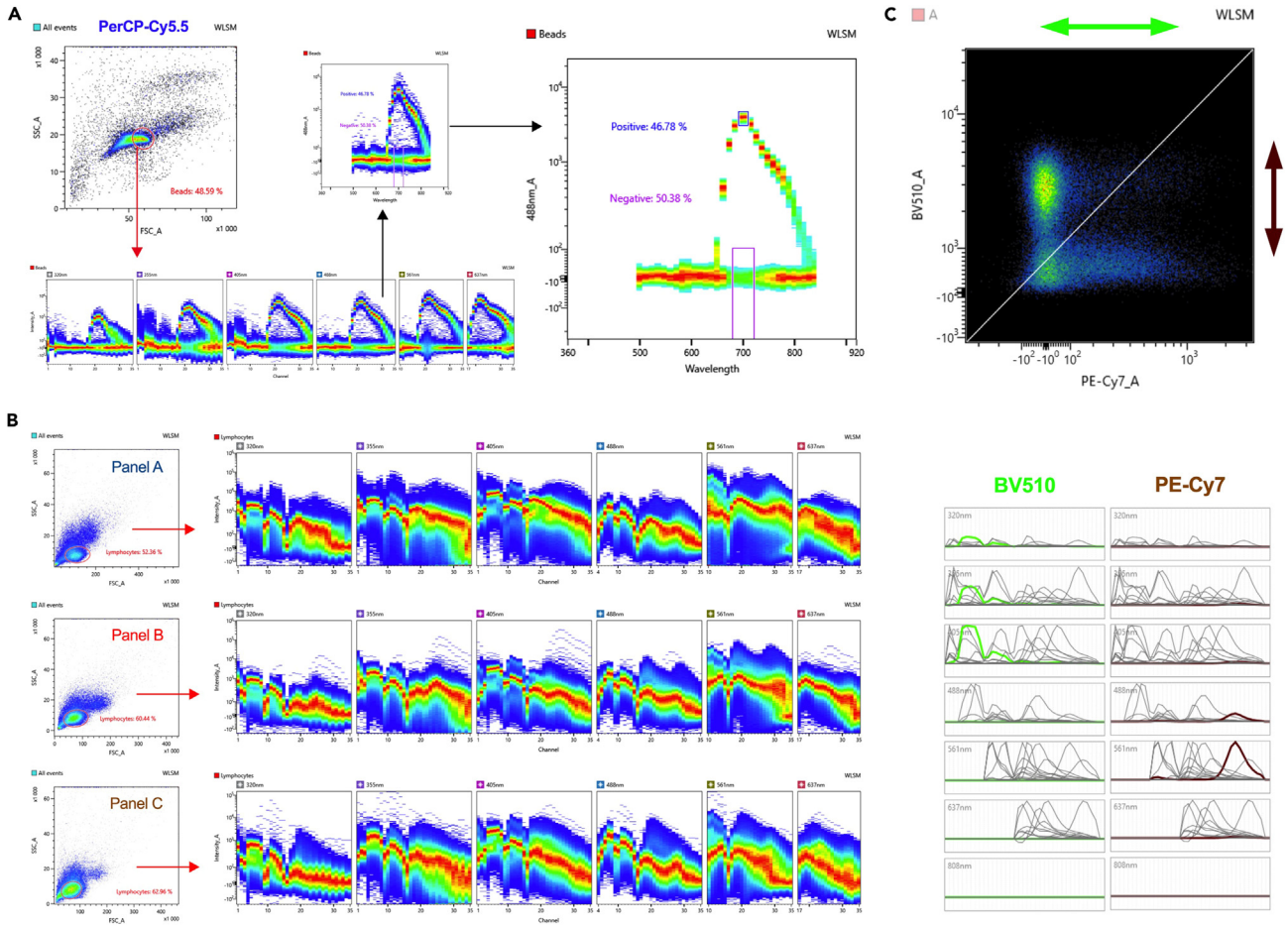


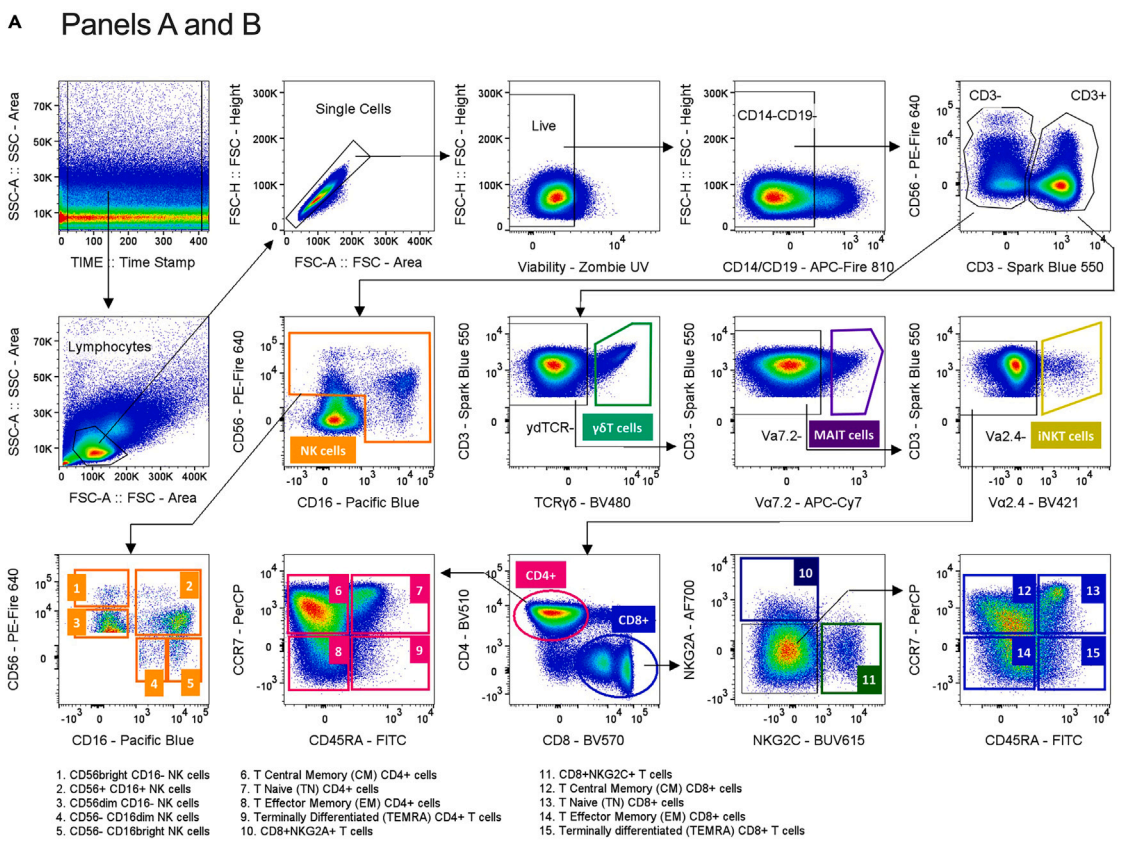
Figure 2. Acquisition of single stained controls and fully stained samples

(A) Example of single stained (PerCP-Cy5.5) control beads; the emission is detected across the lasers and then the positive and negative populations can be assigned. This should be done for every single stained control (including the viability marker) in order to run the unmixing algorithm.

(B) Target population gated by morphology and ribbon plot examples of fully stained PBMCs showing data acquisition across the channels in every laser.

(C) Spectral reference viewer/adjuster (top figure) showing BV510 versus PE-Cy7 dyes. This tool allows (if required) to manually adjusting the positive populations (following the direction of the adjacent arrows) after data acquisition and unmixing. Correspondingly, emission signatures of both fluorochromes are simultaneously shown during adjustment (bottom figure).

22. Assign the single stained controls to the corresponding beads/cells acquired.
    a. Select the single stained beads/cells acquired; right-click and select the corresponding fluorochrome.
    b. Assign the positive and negative populations using the gates (Figure 2A) (do this for every fluorochrome included in each panel).
    c. Adjust the morphology gate in the FSC/SCC graph to select the target population (beads/cells) and verify that there is one single clear signal on all lasers using the ribbon plots (Figure 2B).
23. Calculate the spectrum for each fluorochrome and apply the unmixing matrix.
    a. After assigning the single stained controls, calculate the spectrum for all fluorochromes in the color palette by clicking on the option "calculate".
    b. For applying the new calculated unmixing matrix, go to the worksheet and select "apply" (unmixing matrix).
    c. Control the results of the unmixing in the full stained samples using the flow cytometry and ribbon plots.



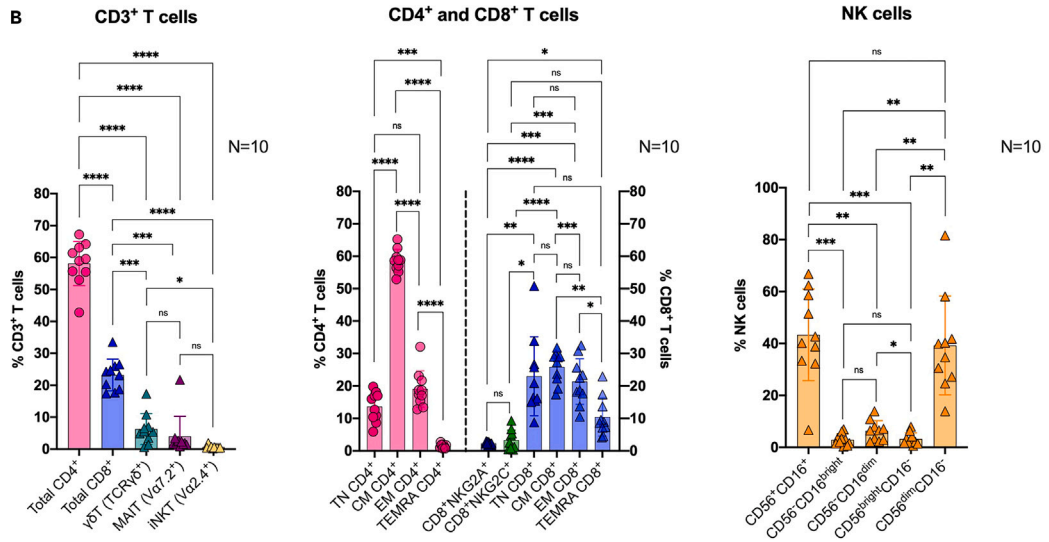


Figure 3. Main general gating strategies for panels A and B

(A) After time lapse, lymphocyte morphology, single and live events selection, samples are gated to exclude $CD14^+$ and $CD19^+$ cells (monocytes and B cells). Subsequently, $CD3^-CD56^+$ cells are gated for further natural killer (NK) cells analysis using CD56 versus CD16 markers. On the other hand, $CD3^+$ cells are gated to discriminate unconventional $TCR\gamma\delta^+$ ($\gamma\delta$T cells), $TCRV\alpha7.2^+$ (MAIT cells) and $TCRV\alpha2.4^+$ (iNKT cells) lymphocytes. From the remaining T cell population, cells are gated by $CD4^+$ versus $CD8^+$ cells. $NKG2A^+NKG2C^-$ ($CD8^+NKG2A^+$) and $NKG2A^-NKG2C^+$ ($CD8^+NKG2C^+$) cells are then identified from the $CD8^+$ T cell population. Then, the $NKG2A^-NKG2C^-$ $CD8^+$ subset as well as the total $CD4^+$ cells are gated according to differentiation

Figure 3. *Continued*
markers; CD45$^+$CCR7$^+$ T naïve (TN) cells, CD45$^-$CCR7$^+$ central memory (CM) cells, CD45RA$^-$CCR7$^-$ effector memory (EM) cells, and CD45RA$^+$CCR7$^-$ terminally differentiated (TEMRA) cells.
(B) Correspondingly, example graphs showing manually gated major populations (bottom figures) as proportions from their parent CD3$^+$ T cell population (left), CD4$^+$CD8$^-$ and CD4$^-$CD8$^+$ T cells (center), and NK cells (right).Bar graphs represent the means; error bars represent the standard deviation; and dots represent individual donors (N=10). One-way ANOVA with Tukey's multiple comparison test. Significance is indicated as ****$P \leq 0.0001$, ***$P \leq 0.001$, **$P \leq 0.01$, *$P \leq 0.05$, or $P > 0.05$ (not significant (ns)).

Note: The unmixing matrix can be further adjusted directly in the plots under the *Spectral Reference Viewer/Adjuster* and applied to all samples (Figure 2C).

24. Export the resulting flow cytometry standard (FCS) files.
    a. Select the samples to be exported and go to export files.
    b. Select the folder, type (.fcs) and export the selected files (this will take time depending on the amount of data acquired).

Data analysis

Timing: variable, objective dependent

This section describes the steps and features included in the data analysis, the gating strategy for the target populations, as well as the dimensionality reduction analysis in Figures 3, 4, and 5. These analyses were made in FlowJo (BD Biosciences), but other software and platforms compatibles with FCS files such as OMIQ (Dotmatics) and the SFA Life Sciences Cloud Platform (Sony) can be used.

25. Import and analyze the resulting files in the specialized software supporting FCS files.
    a. Open the software (FlowJo, BD Biosciences) and import the files from folder in a new experiment.
26. Analyze the data accordingly; the main general gating strategies defined for the panels are shown in Figures 3 and 4.
    a. Run quality control plug-ins (recommended) for evaluating and eventually exclude bad flow cytometry quality data from your set (i.e., deviations of parameters during time of acquisition).
    b. Transform and explore the data with other plug-ins for clustering and dimensionality reduction analysis (Figure 5).
    c. Follow the manual gating of populations of interest (Figures 3 and 4) [the expression of the markers can be analyzed by parameters such as proportion of the gated parent population and the median fluorescence intensity (MFI) of the corresponding fluorochrome].

Note: If you detect a potential unmixing over/under compensation issue during analysis, you can go back to the spectral flow cytometry system software and adjust the unmixing matrix accordingly. After, apply the new unmixing matrix to the samples and export the files for further analysis.

EXPECTED OUTCOMES

The final analysis for identifying the target CD8$^+$ T lymphocytes from other T cell populations in our data was completed in FlowJo (BD Biosciences). We concatenated the CD3$^+$NKG2A$^+$ NKG2C$^-$ and CD3$^+$ NKG2A$^-$NKG2C$^+$ T cell subsets from manually gated live single CD14$^-$CD19$^-$ lymphocyte events and performed a dimensionality reduction analysis (tSNE) using all fluorescent parameters except viability, CD14, CD19, CD3, NKG2A and NKG2C (for panels A and B in parallel) (Figure 5A). Figure 5B display the analysis of defined groups of CD8$^+$, CD4$^+$, TCRVα2.4$^+$, TCRVα7.2$^+$, and TCR$\gamma\delta^+$ cells as well as the corresponding expression of every other marker in panels A and B.

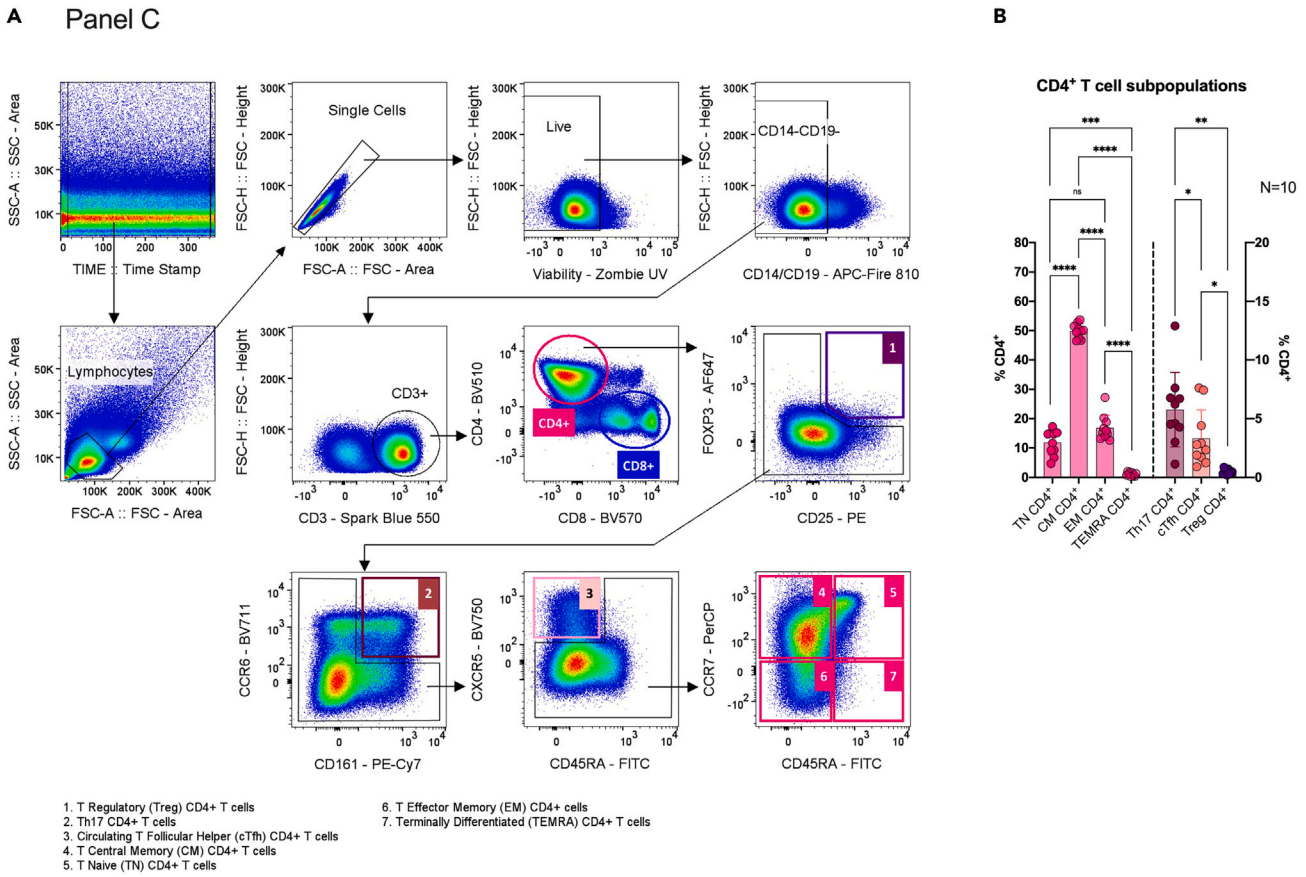


Figure 4. Main gating strategy for panel C

(A) Following the positive selection of time lapse, the lymphocyte morphology, and single and live events. Then, to exclude $CD14^+CD19^+$ cells. Then, $CD3^+$ cells are gated by $CD4^+$ versus $CD8^+$ cells. Then, $CD4^+$ lymphocytes are subsequently gated to identify $CD4^+CD25^+FOXP3^+$ regulatory T cells (Treg), $CD161^+CCR6^+$ T helper 17 cells (Th17), and $CD45RA^-CXCR5^+$ circulating T follicular helper cells (cTfh). The remaining $CD4^+$ cells are further gated on; $CD45^+CCR7^+$ T naive (TN) cells, $CD45^-CCR7^+$ central memory (CM) cells, $CD45RA^-CCR7^-$ effector memory (EM) cells, and $CD45RA^+CCR7^-$ terminally differentiated (TEMRA) cells.

(B) Correspondingly, example graph (right) showing manually gated populations as proportions from their $CD4^+CD8^-$ T cell parent population. Bar graphs represent the means; error bars represent the standard deviation; and dots represent individual donors (N=10). One-way ANOVA with Tukey's multiple comparison test. Significance is indicated as $^{****}P \leq 0.0001$, $^{***}P \leq 0.001$, $^{**}P \leq 0.01$, $^{*}P \leq 0.05$, or $P > 0.05$ (not significant (ns)).

Figures 3 and 4 show the general gating strategy for the three panels, identifying target T and NK cell populations (top figures). Corresponding graphs (column charts) show a summary of the frequency of cell subsets from their corresponding parent population (N = 10). In order to analyze every cell population (based on their differentiation and functional profiles), we followed the same exclusion gating strategy to separate and concatenate each subset. Then, we evaluated the expression of each marker through the median fluorescence intensity (MFI) (Figure 6).

All together, these deep-phenotyping analyses allow studying $CD8^+NKG2A^+$ and $CD8^+NKG2C^+$ T cells, examining their phenotype in depth, as well as screening other T and NK cell subsets in a platform providing high quality comprehensive data, with higher flexibility and sensitivity as compared to conventional flow cytometry.

LIMITATIONS

The number of markers that can be included in the panels is limited by the configuration of the spectral cell analyzer (the number of lasers and detectors), as well as the combination of available antibodies and fluorochromes. However, the ID7000, with a total of 184 detectors and 6 lasers, is the

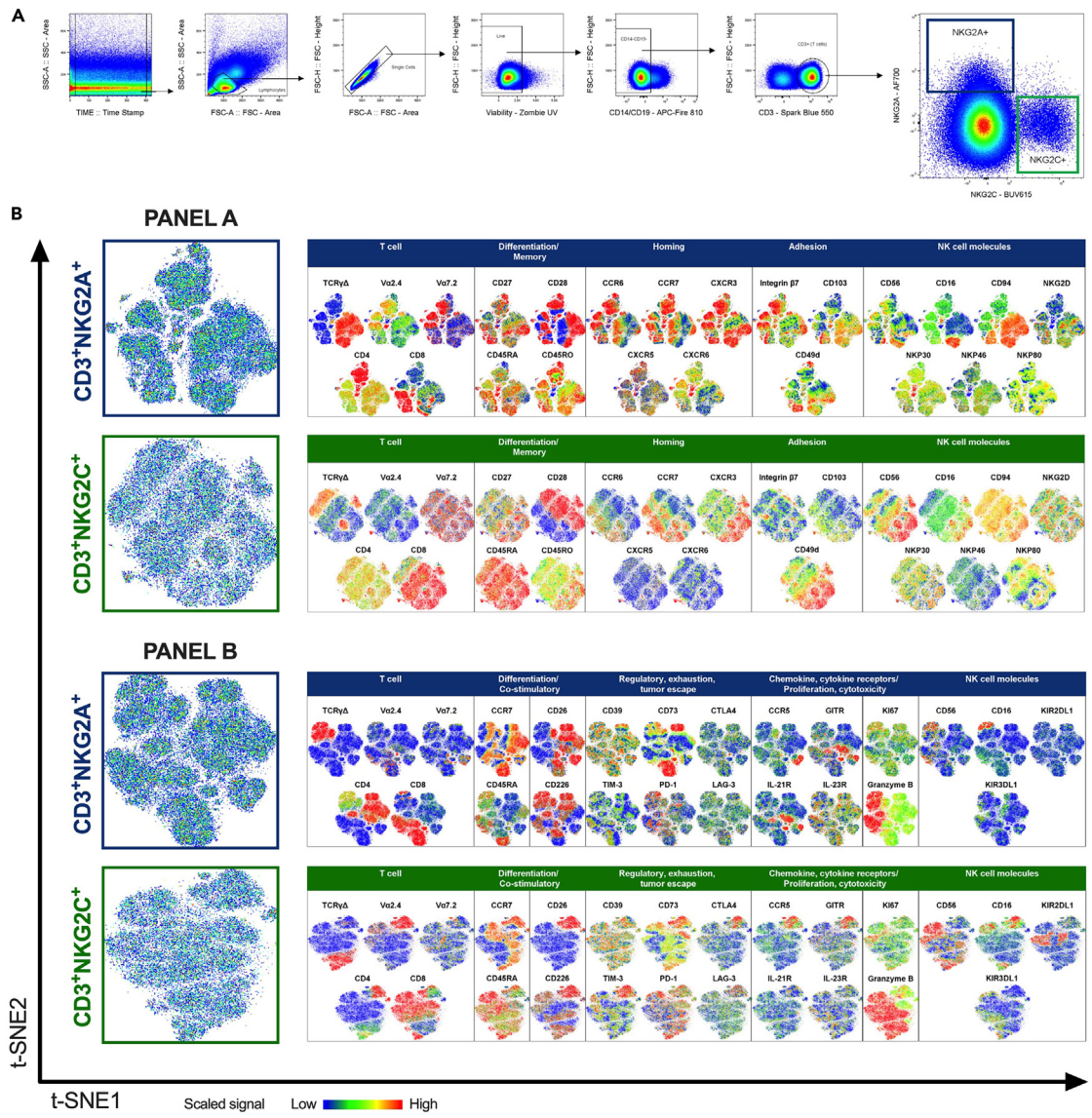


Figure 5. Clustering analysis and expression of markers between the CD3$^+$NKG2A$^+$ and CD3$^+$NKG2C$^+$ T lymphocytes

(A) We gated on CD3$^+$NKG2A$^+$NKG2C$^-$ (CD3$^+$NKG2A$^+$) and CD3$^+$NKG2CA$^-$NKG2C$^+$ (CD3$^+$NKG2C$^+$) populations (shown in blue and green gates respectively), from single events of live CD14$^-$ and CD19$^-$ lymphocytes, with the aim to corroborate our previous exclusion gating strategy by running an unsupervised clustering analysis.

(B) Using such gating strategy, we concatenated ~40,000 events for each population from fully stained PBMCs (N = 10). After, we run tSNE analyses with 3,000 iterations selecting all fluorescent parameters except for those previously gated (viability, CD3, CD14, CD19, NKG2A, and NKG2C). Relative expression of markers from panels A and B are shown across the maps for both analyzed subsets (CD3$^+$NKG2A$^+$ and CD3$^+$NKG2C$^+$ T cells). Clusters of CD8$^+$ T cells independent from CD4$^+$, TCR$\gamma\delta^+$, TCRVα7.2$^+$, and TCRVα2.4$^+$ T cells can be identified.

spectral analyzer with the highest number of detectors on the market and allows the detection of more than 40 parameters simultaneously.[24]

The number of available antibody clones also limits the analyses in other systems like non-human primate (NHP) cells. For instance, some clones do not differentiate between NKG2A and NKG2C in nonhuman primate cells. The clones included in this protocol for NKG2A (S19004C) and NKG2C (134591) have human reactivity and can differentiate between these markers on human cells.

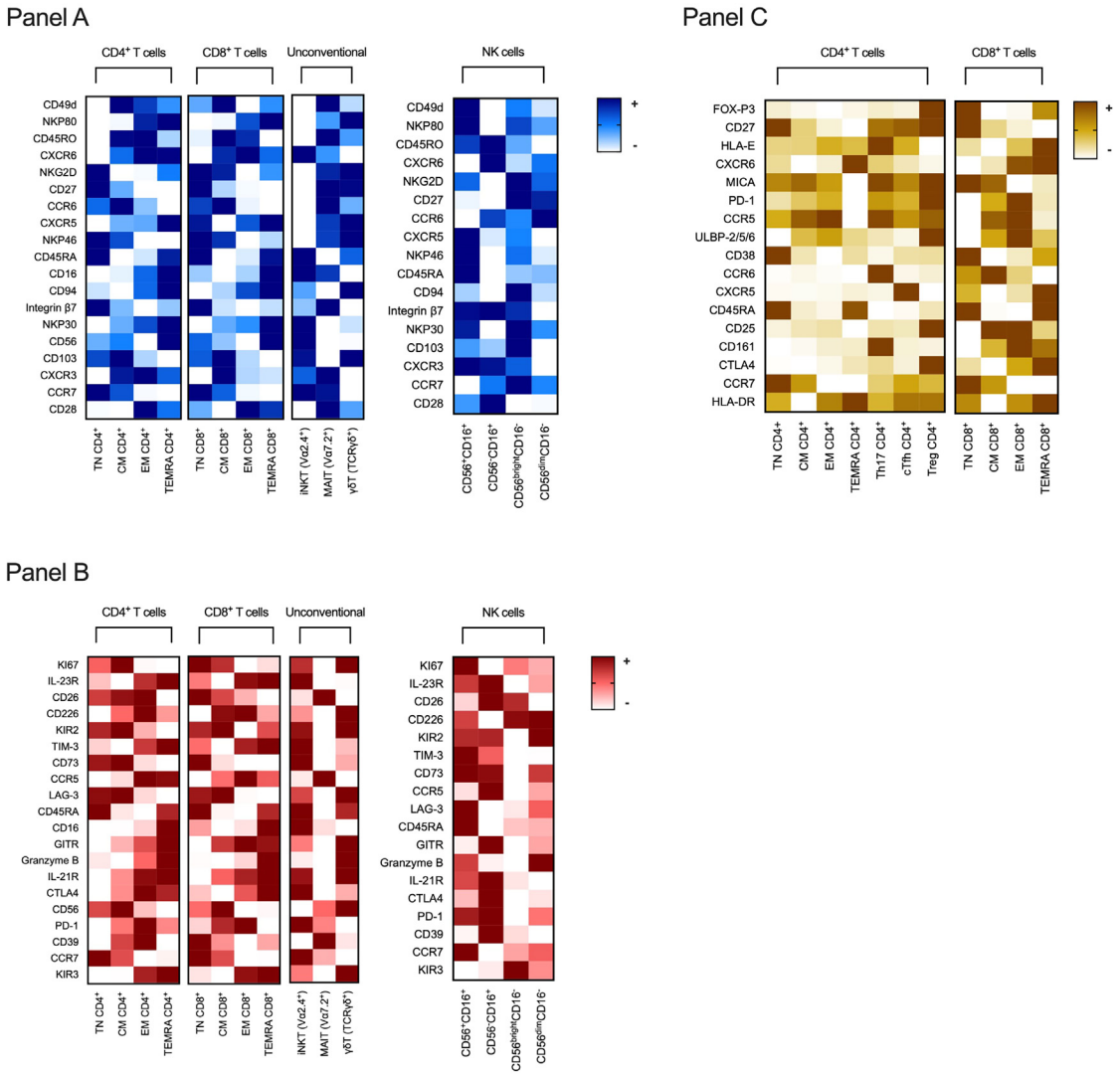


Figure 6. Expression analysis of T and NK cell populations

Heat maps showing the relative expression of markers (individually normalized MFI) across every subpopulation (~10,000-concatenated events each) of T and NK cells following the gating strategy from Figures 3 and 4. TN, T naïve cells; CM, central memory cells; EM, effector memory cells; TEMRA, terminally differentiated cells; iNKT, invariant natural killer cells; MAIT, mucosal-associated invariant T cells; γδT, TCRγδ$^+$ T cells; Th17, helper 17 cells; cTfh, circulating T follicular helper cells.

Acquiring the samples under different conditions and times may affect the values of MFI, which can be problematic for longitudinal analyses. It is important to take into account the experimental design, the expected output data to be analyzed in advance and, if possible, plan to acquire all the samples at once.

It is well known that there might be variations in the expression of different markers depending on the conditions for the preparation of the samples (including, temperature, reagents, time of incubation, etc.). For instance, we found that the expression of markers like CCR7 was very limited at 4°C as compared to higher incubation temperatures. Thus, we recommend considering this for the target markers in the panels. We standardized this protocol using recently thawed cells, however, we advise taking into account these considerations in order to prepare appropriate controls for the standardization of frozen versus fresh samples. Also, the use of the brilliant stain buffer improve the background reduction during the standardization process, so we recommend to use it when using more than one polymer dye-conjugated antibody in order to prevent non-specific polymer interactions.

TROUBLESHOOTING

Problem 1

Markers too dim or no signal (data analysis, step 26).

Potential solution

This could be due to incorrect PMT voltage set-up, incorrect or no antibodies added to the mix and inadequate permeabilization in the case of intracellular markers. If the true signal is low, meaning low expression of such marker, the use of FMO controls is recommended. Keep in mind that the expression of some markers (in particular chemokine receptors) changes in response to handling of the cells such temperature variations, thus resting cells in warm culture medium is recommended especially with recently thawed cells. However, this should be considered according to the target markers to be investigated. For instance, if cytokines are included in the panels, the use of monensin and/or brefeldin A should be considered and tested during the incubation period.

Problem 2

Incorrect double positive events, spillover, under compensation and/or background artifacts (data analysis, step 26).

Potential solution

These can be due to different causes. Bad unmixing can arise due to single stain incorrect identification and saturated events. Biological exclusive markers should be kept in fluorochromes with close emissions to minimize false double-positive events. It is recommended the use special stain buffers (as the brilliant stain buffer described in this protocol) since some fluorescent dyes, in particular polymer-based, may cause staining artifacts due to non-specific reactivity when staining the cells. In addition, the Spectral Unmixing Adjuster mode in the Sony system software allows manually adjusting and correcting unmixing matrix if necessary.

Also, bad titration and cross-reactivity might be responsible for under compensation and/or artifacts. Thus, it is recommended to titrate the antibodies in advance and potentially pre-staining problematic markers.

Problem 3

Poor quality and low rate events (sample acquisition, step 21).

Potential solution

Poor quality events might be due to inadequate sample handling or cells with low viability. In addition, it is possible that compensation beads and cells fall to the bottom of the well during long periods of sample acquisition and thus lowering the rate events. In this case, activating the continuous mixing mode in the instrument might decrease this issue. However, if many samples are being acquired it is possible to make pause intervals between certain given amount of samples and manually re-mix the volume before continuing acquisition. When gating, it is recommended to include only events with stable acquisition over time, to exclude doublets, debris and dead cells.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be answered by the lead contact, Prof. Michaela Müller-Trutwin (michaela.muller-trutwin@pasteur.fr).

Materials availability

This protocol did not generate new unique reagents.

Data and code availability

This study did not generate datasets or code.

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AUTHOR CONTRIBUTIONS

M.M.-T. and A.O.-R. conceived the study. A.O.-R. designed and developed the methodology for the panels, standardized and optimized the protocol, performed the experiments, analyzed the data, and wrote the manuscript. S.S. and S.N. standardized the local protocol for the use of the spectral analyzer instrument and provided advice and support through the standardization process. C.P. and N.H. provided scientific advice for the setup of the panels. M.M.-T. acquired the funding. B.J. coordinated and supervised the respect of the institutional guidelines. All authors reviewed and approved the final manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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24. Application Note: Forty-Two Color Flow Cytometry Panel Data Collected. Forty-two Color Flow Cytometry Panel Data Collected with the ID7000™ Spectral Cell Analyzer for Identifying Cellular Subsets in Human Peripheral Blood. Sony Biotechnology Inc. https://s3.amazonaws.com/creative.sonybiotechnology.com/ID7000/Sony+ID7000+Application+Note+42-Color+Flow+Cytometry+Panel+Data+Collected+with+the+ID7000+Spectral+Cell+Analyzer.pdf