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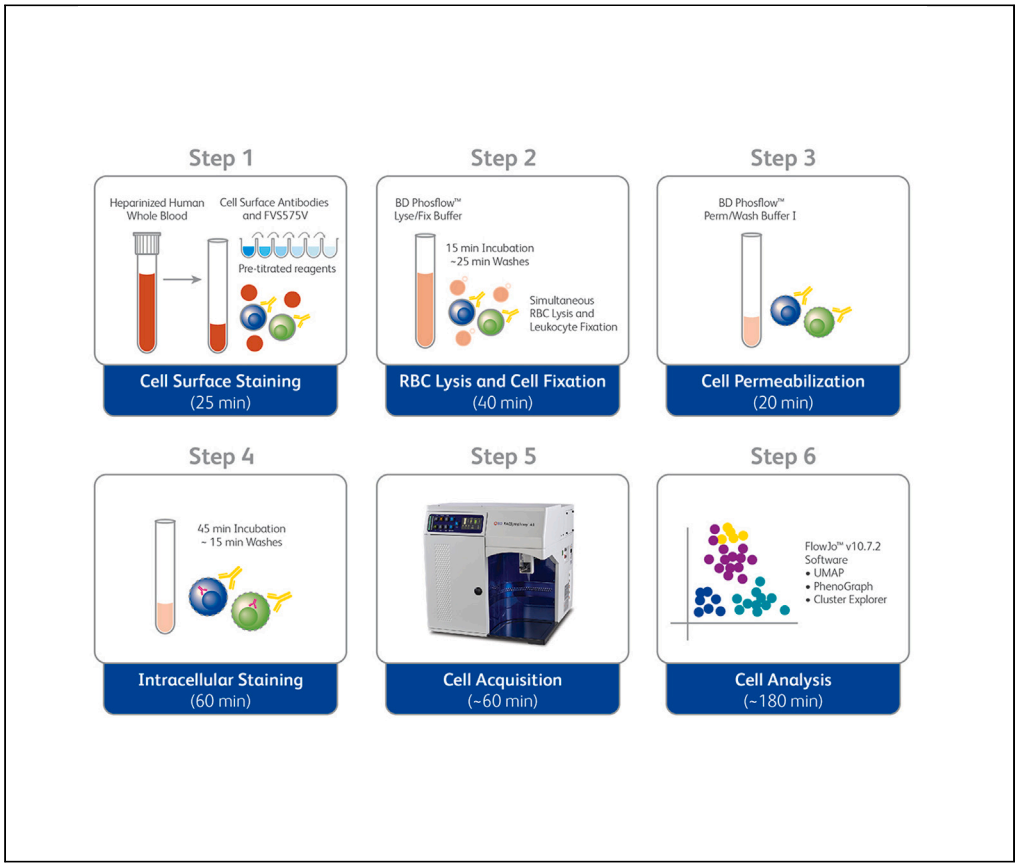
Functional phenotyping of circulating human cytotoxic T cells and NK cells using a 16-color flow cytometry panel



Cytotoxic T lymphocytes and natural killer (NK) cells are key effector cells in immune defenses against intracellular pathogens and cancer. In human blood, effector T and NK cytotoxic cells comprise a diverse and relatively rare group of cells. Herein, we describe a simplified intracellular staining workflow for classification of circulating human T and NK cells with cytolytic potential. We suggest reagents for measuring cytolytic proteins and identification of cell subsets within conventional and unconventional T cells and NK cells.

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Highlights

Intracellular staining protocol for assessment of immune cells in human blood

Detailed approaches for detection of cells comprising less than 0.5% of blood cells

Multicolor panel for distinction of NK and T cells with cytolytic potential

Insights on analytical tools for multicolor panel data analysis

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Functional phenotyping of circulating human cytotoxic T cells and NK cells using a 16-color flow cytometry panel

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SUMMARY

Cytotoxic T lymphocytes and natural killer (NK) cells are key effector cells in immune defenses against intracellular pathogens and cancer. In human blood, effector T and NK cytotoxic cells comprise a diverse and relatively rare group of cells. Herein, we describe a simplified intracellular staining workflow for classification of circulating human T and NK cells with cytolytic potential. We suggest reagents for measuring cytolytic proteins and identification of cell subsets within conventional and unconventional T cells and NK cells.

BEFORE YOU BEGIN

The protocol below provides reagents and specific steps for intracellular staining of immune cells directly in whole blood. A 16-color antibody panel was strategically designed to identify subsets of cytotoxic T cells and NK cells. However, the proposed staining method can be applied to assess other circulating immune cell types. One advantage of this protocol is the fact that red blood cell lysis and nucleated cell fixation are simultaneously performed in one step. After this step, we permeabilize the cells for intracellular analysis of granzyme K (GzmK), granzyme B (GzmB), and perforin, which aids in the analysis of the cytolytic potential of these cells. Instead of permeabilization, the fixed cells could also be promptly analyzed for the expression of surface markers or stored at 4°C for subsequent studies.

Individuals performing the experiments must have received safety training on the proper handling and disposal of human biological materials before planning experiments with human blood.

Titrations

Timing: 1–1.5 h—before the day of the experiment

1. Determine the optimal concentration of each conjugated antibody in the panel.
   a. Two-fold serial dilution ranging from 10 µg/mL to 0.31 µg/mL is sufficient for titration of mass size reagents e.g., BD OptiBuild™ Reagents in this panel.
   b. Test size reagents such as the antibodies bottled at 5 µL/test add convenience to the workflow. However, we also suggest checking beforehand whether the provided concentration is adequate for your samples and instrument settings.

Table 1. FACSymphony A1 cell analyzer configuration and antibody reagents

Laser	Filters	Fluor	Relative fluorochrome brightness	Specificity	Clone	Purpose
Violet	450/50	BV421	++++	Perforin	dG9	Cytolytic abilities
	525/50	BV480	+++	CD159a (NKG2A)	131411	Inhibitory receptor
	610/20	BV605	+++	CD19	HIB19	Exclusion/B cells
				CD14	M5E2	Exclusion/monocytes
				CD123	7G3	Exclusion/plasmacytoid dendritic cells and eosinophils
				CD141	1A4	Exclusion/myeloid cells and platelets
		FVS575V[a]	-	-	-	Viability
	670/30	BV650	++++	CD3	UCHT-1	T cells
	710/50	BV711	++++	CD314 (NKG2D)	1D11	Activating receptor
	780/60	BV786	+++	HLA-DR	G46-6	Activation marker
Blue	530/30	FITC	++	CD57	NK-1	Maturation and differentiation marker
	710/50	PerCP-Cy5.5	++	CD8	RPA-T8	Cytotoxic T cells
Yellow-Green	586/15	PE	++++	CD158 (KIRS)	HP-MA4	Maturation and differentiation marker
	610/20	PE-CF594	++++	CD56	R19-760	NK cells/activation marker
	670/30	PE-Cy5	++++	CD95 (FAS)	DX2	Differentiation marker
	710/50	PE-Cy5.5	++++	CD127 (IL-7Rα)	eBioRDR5	Differentiation marker/innate lymphoid cells
	780/60	PE-Cy-7	++++	CD38	HIT2	Differentiation marker
Red	670/30	AF647	+++	Granzyme K	G3H69	Cytolytic abilities
	710/50	R718	+++	Granzyme B	GB-11	Cytolytic abilities
	780/60	APC-H7	+	CD16 (FcγRIII)	3G8	NK cells/cytolytic abilities

+Dim ++Moderate +++Bright ++++Very Bright.
[a]BD Horizon™ Fixable Viability 575V.

⚠ CRITICAL: Check the configuration of your instrument to ensure compatibility with the reagents used in the proposed panel. Table 1 shows the configuration of a BD FACSymphony™ A1 Cell Analyzer used for acquiring this panel.

⚠ CRITICAL: Utilize optimized application settings to obtain maximum resolution of cell populations and consistent results across experiments.

⚠ CRITICAL: During titrations, note whether the positive population is within the range of detection of the instrument and that there is no significant background staining. Depending on results, test size reagents may be titrated down until signals are within the linear range of the instrument, found in the baseline report. See problem 1 for an example of titration curves of mass size versus test size reagents.

Note: For more complete details on how to set up instruments, create optimized application settings, and standardize flow cytometry experiments, please refer to Mair and Tyznik (2019).

2. We used the BD Horizon™ Fixable Viability Stain 575V (FVS575V) for discrimination between live and dead cells in the samples. The dye powder was reconstituted in 340 μL of dimethyl sulfoxide (DMSO) and stored at −20°C in 10 μL aliquots. Aliquots were thawed at room temperature (20°C–24°C), immediately prior to use, protected from light.
   a. The optimal working concentration was predetermined in a separate assay. In this assay, we spiked whole blood with heat-treated leukocytes and stained the blood with FVS575V at 1:50, 1:100, 1:200 and 1:400. In parallel, spiked blood samples were prepared for 7-aminoactinomycin D (7-AAD) staining. Additionally, we examined the actual viability profile of fresh blood cells and chose an optimal concentration based on flow cytometry results (problem 2).
   b. FVS575V is excited by the violet laser (with an excitation maximum of 396 nm) and has a fluorescence emission maximum of 572 nm. We captured the emission with the detector commonly used for BV605 (filter 610/20). As described in detail later, this detector was also used to exclude B cells and myeloid cells during analysis.

⚠ CRITICAL: Antibody conjugates and FVS575V are light-sensitive reagents and must be always protected from light. Antibody conjugates are also temperature sensitive and should not be left at room temperature unnecessarily to preserve their stability and overall shelf-life.

⚠ CRITICAL: Reconstituted FVS575V aliquots can be stored for up to one year at −20°C. Unused content in the aliquots should be discarded.

Note: Dead cells have greater autofluorescence and increased nonspecific antibody binding properties (Cossarizza et al., 2019). Thus, inclusion of a viability dye for analysis of this panel is recommended, even when using freshly drawn blood specimens.

Buffer preparation

Timing: 10 min

3. Calculate the number of samples that will be stained in the experiment, including unstained cells, single-stain controls, fluorescence minus one (FMO) controls and the full panel.
4. The day before the experiment, dilute the required amount of BD Phosflow™ Perm/Wash Buffer I at 1:10 with deionized or distilled water and store at 4°C.
5. Dilute the required amount of BD Phosflow™ Lyse/Fix Buffer 5× (1:5) with deionized or distilled water (at room temperature). In this assay, we used 20 volumes of 1× BD Phosflow™ Lyse/Fix Buffer per 1 volume of blood or 4 mL buffer/200 μL blood.

⚠ CRITICAL: The BD Phosflow™ Lyse/Fix Buffer working solution should be made fresh for each experiment, and any remaining solution should be discarded following local environmental health and safety protocols since the solution contains formaldehyde. We also recommend preparing the buffer and handling the samples in a chemical hood.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
BD Horizon™ BV421 Mouse Anti-Human Perforin (1:20)	BD Biosciences	Cat# 563393, RRID:AB_2738178
BD OptiBuild™ BV480 Mouse Anti-Human NKG2A (CD159a) (1:40)	BD Biosciences	Cat# 747923, RRID:AB_2872384
BD OptiBuild™ BV605 Mouse Anti-Human CD19 (1:155)	BD Biosciences	Cat# 740394, RRID:AB_2740124
BD Horizon™ BV605 Mouse Anti-Human CD14 (1:40)	BD Biosciences	Cat# 564054, RRID:AB_2687593
BD Horizon™ BV605 Mouse Anti-Human CD123 (1:40)	BD Biosciences	Cat# 564197, RRID:AB_2732049

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
BD OptiBuild™ BV605 Mouse Anti-Human CD141 (1:40)	BD Biosciences	Cat# 740421, RRID:AB_2740151
BD Horizon™ BV650 Mouse Anti-Human CD3 (1:40)	BD Biosciences	Cat# 563851, RRID:AB_2744391
BD Horizon™ BV711 Mouse Anti-Human CD314 (NKG2D) (1:40)	BD Biosciences	Cat# 563688, RRID:AB_2738377
BD Horizon™ BV786 Mouse Anti-Human HLA-DR (1:80)	BD Biosciences	Cat# 564041, RRID:AB_2738559
BD Pharmingen™ FITC Mouse Anti-Human CD57 (1:20)	BD Biosciences	Cat# 555619, RRID:AB_395986
BD Pharmingen™ PerCP-Cy™5.5 Mouse Anti-Human CD8 (1:40)	BD Biosciences	Cat# 560662, RRID:AB_1727513
BD Pharmingen™ PE Mouse Anti-Human KIR2DL1/S1/S3/S5 (CD158) (1:40)	BD Biosciences	Cat# 567158, RRID:AB N/A
BD Horizon™ PE-CF594 Mouse Anti-Human NCAM-1 (CD56) (1:40)	BD Biosciences	Cat# 564963, RRID:AB_2869631
BD Pharmingen™ PE-Cy™5 Mouse Anti-Human CD95 (1:10)	BD Biosciences	Cat# 559773, RRID:AB_397317
Mouse monoclonal anti-human CD25, PE-Cy5.5 (1:155)	Thermo Fisher Scientific	Cat# 35-1278-42, RRID:AB_2744722
BD Pharmingen™ PE-Cy™7 Mouse Anti-Human CD38 (1:80)	BD Biosciences	Cat# 560677, RRID:AB_1727473
BD Pharmingen™ Alexa Fluor® 647 Mouse Anti-Human Granzyme K (1:20)	BD Biosciences	Cat# 566655, RRID:AB_2869812
BD Horizon™ R718 Mouse Anti-Human Granzyme B (1:80)	BD Biosciences	Cat# 566964, RRID:AB_2869975
BD Pharmingen™ APC-H7 Mouse Anti-Human CD16 (1:40)	BD Biosciences	Cat# 560195, RRID:AB_1645466
Biological samples		
Human healthy peripheral whole blood	Any Supplier	N/A
Chemicals, peptides, and recombinant proteins		
Fixable Viability Stain 575V	BD Biosciences	Cat# 565694, RRID:AB_2869702
Phosflow Lyse/Fix Buffer 5×	BD Biosciences	Cat# 558049, RRID:AB_2869117
Phosflow Perm/Wash Buffer I	BD Biosciences	Cat# 557885, RRID:AB_2869104
Brilliant Stain Buffer Plus	BD Biosciences	Cat# 566385, RRID:AB_2869761
Stain Buffer (FBS)	BD Biosciences	Cat# 554656, RRID:AB_2869006
Software and algorithms		
Diva 9.3	BD Biosciences	https://www.bdbiosciences.com/en-us
FlowJo v10.7.2	BD Biosciences	https://www.flowjo.com/exchange/#/
Uniform Manifold Approximation and Projection	BD Biosciences	https://www.flowjo.com/exchange/#/
PhenoGraph	BD Biosciences	https://www.flowjo.com/exchange/#/
ClusterExplorer	BD Biosciences	https://www.flowjo.com/exchange/#/
Other		
FACSymphony A1 Cell Analyzer	BD Biosciences	https://www.bdbiosciences.com/en-us
FACSymphony A5 Cell Analyzer	BD Biosciences	https://www.bdbiosciences.com/en-us

Note: Approximate antibody dilution is provided in the reaction – 200 μL blood + 185 μL cocktail + 2 μL FVS575V.

STEP-BY-STEP METHOD DETAILS

Sample preparation and cell staining

This section describes a workflow for staining two donor samples with the 16-color panel shown in Table 1. In addition, we included FMO controls to aid in identifying lowly expressed markers and single-stained control cells for all 16 parameters (problem 3). As described in Table 2, we stained 200 μL of freshly drawn heparinized whole blood with the full panel. Conversely, the control samples, including FMOs and single-stained control cells, were prepared with 100 μL of blood. Staining 200 μL of blood allowed us to acquire 500,000 lymphocytes for good visualization and characterization of rare subsets. A total of 30,000–50,000 lymphocytes were acquired from the control samples.

⚠ CRITICAL: Red blood cell lysis and cell fixation require a volume of 1× BD Phosflow™ Lyse/Fix Buffer that is 20 times the volume of blood or 4 mL for 200 μL of blood. Thus, 200 μL of blood is the maximum volume that can be stained in 5 mL polystyrene tubes.

Table 2. Antibody cocktails for two or more samples

Laser	Fluor	Specificity	Antibody volume (μL)/100 μL blood or perm buffer[a]	200 μL blood	2 samples of 200 μL blood + 20% overage	4 samples of 200 μL blood + 40% overage	8 samples of 200 μL blood + 40% overage
Violet	BV421	Perforin	5	0	0	0	0
	BV480	CD159a (NKG2A)	5	10	22	44	84
	BV605	CD19	1.25	2.5	5.5	11	21
		CD14	5	10	22	44	84
		CD123	5	10	22	44	84
		CD141	5	10	22	44	84
	FVS575V*	-	1	2	-	0	0
	BV650	CD3	5	10	22	44	84
	BV711	CD314 (NKG2D)	5	10	22	44	84
	BV786	HLA-DR	2.5	5	11	22	42
Blue	FITC	CD57	10	20	44	88	168
	PerCP-Cy5.5	CD8	5	10	22	44	84
Yellow-Green	PE	CD158 (KIRS)	5	10	22	44	84
	PE-CF594	CD56	5	10	22	44	84
	PE-Cy5	CD95 (FAS)	20	40	88	176	336
	PE-Cy5.5	CD127 (IL-7Rα)	1.25	2.5	5.5	11	21
	PE-Cy-7	CD38	2.5	5	11	22	42
Red	AF647	Granzyme K	5	0	0	0	0
	R718	Granzyme B	1.25	0	0	0	0
	APC-H7	CD16 (FcγRIII)	5	10	22	44	84

Perm, Permeabilization.

[a]See Certificate of Analysis for antibody concentration.

Note: We recommend creating antibody cocktails with at least 20% overage to ensure adequate volumes for labeling. We suggest increasing the percentage overage to 30%–40% when staining more than two samples with the full panel.

Note: We used BD Vacutainer® Heparin Tubes for blood collection but ethylenediaminetetraacetic acid (EDTA) or other anticoagulants can also be used in this analysis. However, heparin instead of EDTA may provide better results in protocols that require cell stimulation and analysis of signaling pathways, since EDTA could disrupt processes that depend on divalent cations.

Note: Although we have not tested in this protocol, compensation beads can potentially be used as single-color controls for compensation. However, we would recommend treating the beads similarly to the cells, including the incubation steps in fixation and permeabilization buffers.

Surface staining

Timing: ~30–40 min making antibody cocktails; 25 min cell surface staining

1. Place BD Pharmingen™ Stain Buffer (FBS) and BD Horizon™ Brilliant Stain Buffer (BSB) Plus on ice.
2. Bring antibodies and FVS575V to room temperature (RT), protected from light.
3. Quickly spin the antibody vials and pipette volumes from the top of the tube to avoid possible antibody aggregates.
4. Calculate the number of polystyrene tubes required, and label the tubes as suggested in Figure 1.
5. Pooling antibodies for the full panel.

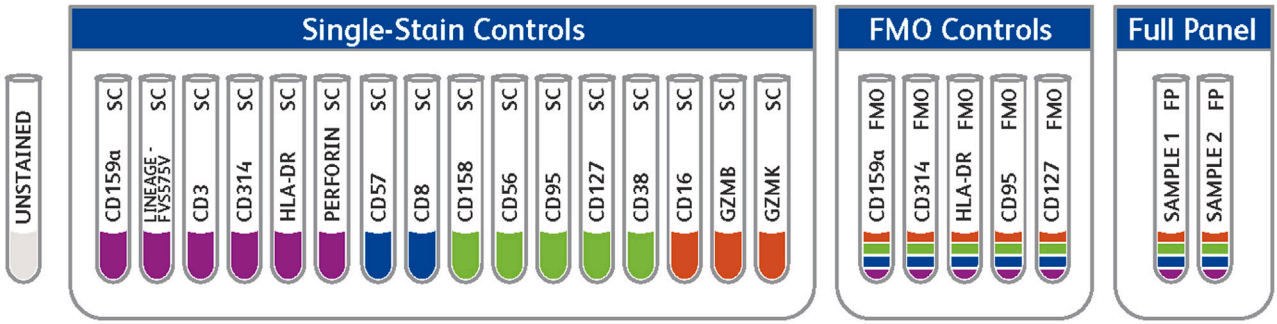

Figure 1. Experiment setup

The tubes are labeled with the names of the specificities and abbreviations for single-stain controls (SC), fluorescence minus one (FMO) and full panel (FP). The colors indicate fluorochrome-conjugated antibodies primarily excited by the violet, yellow-green, blue and red lasers of the instrument.

a. Determine the volume of each antibody based on predetermined optimal concentrations as shown in Tables 1 and 2.
b. First pipet BSB Plus into a polystyrene tube and subsequently add the antibodies to cell surface markers (Table 3).

6. Pooling antibodies for FMO controls
   a. Similarly, as described in step 5, add BSB Plus and all surface antibodies, except one. Herein we created 5 FMO controls, each missing one of the following antibodies: CD159a-BV480, CD314-BV711, HLA-DR-BV786, CD95-PE-Cy5 and CD127 PE-Cy5.5.
7. Preparing single-stain controls
   a. Add BSB Plus (10 μL per sample) to a total of 14 labeled polystyrene tubes, one for each surface marker in the panel and one for unstained cells.
   b. Add each antibody to its respective labeled tube.
8. Gently vortex and place tubes on ice, protected from light. Here we set the vortex to run continuously at 300 rpm and press the tubes against the vortex for 5 s, carefully to avoid spills.
9. Distribute equal volumes of the full panel cocktail into new polystyrene tubes, one tube per sample.

Example in Table 2:

1 donor sample: 1 cocktail = 185 μL

2 donor samples: 2 cocktails + 20% overage = 407 μL total > > 185 μL/donor

10. Dispense 100 μL of blood into the single control tubes for cell surface markers and FMO tubes and 200 μL of blood into the full panel tubes.
11. Add 1 μL of FVS575V into the FMO tubes and the FVS575V/BV605 single control tube and 2 μL into the full panel tubes.
12. Briefly vortex the tubes and incubate at RT for 25 min, protected from light.
13. While the cells are staining for cell surface markers, prepare the single-stain control tubes for intracellular markers and unstained cells by adding 100 μL of blood to the tubes, labeled as unstained, GzmB, GzmK and perforin.

⚠ CRITICAL: BD Horizon™ Brilliant Stain Buffer (BSB) has been developed to reduce possible staining artifacts when using multiple BD Horizon Brilliant™ Reagents in the same tube. BSB Plus is formulated to allow for reduced test volume (10 μL/test) to reduce the final size of the cocktails and avoid excessive dilution of the antibodies during staining.

Table 3. Continuing antibody cocktails for two or more samples

	200 µL blood	2 samples of 200 µL blood + 20% overage	4 samples of 200 µL blood + 40% overage	8 samples of 200 µL blood + 40% overage
BSB Plus (µL)	10	22	44	84
Antibody cocktail volume (µL)	175	385	770	1470
BSB+ antibody cocktail (µL)	185	407	814	1554
Cocktail volume per sample (µL)	185	185	185	185

BSB, Brilliant Stain Buffer; Note that antibody cocktails DO NOT include FVS575V.

⚠ CRITICAL: Because FVS575V is used in its concentrated form, we recommend agitating the samples by vortexing immediately after adding the dye, to guarantee uniform dye incorporation in the cells.

Alternatives: This panel was designed for a FACSymphony A1 and tested in a FACSymphony A5. It can potentially be used in any instrument equipped with Blue, Violet, Yellow-Green and Red lasers and filter sets indicated in Table 1. Table 1 also shows relative brightness of fluorochromes for easier selection of alternative reagents that are compatible with these filter sets.

Red blood cells lysis and cell fixation

Timing: ~15 min incubation for simultaneous red blood cell (RBC) lysis and leukocyte fixation; ~25 min washes

14. After incubation with the antibodies against surface markers and FVS575V, add 2 mL of BD Phosflow™ Lyse/Fix Buffer to the tubes with 100 µL of blood (unstained cells, single-stain controls and FMO controls) and 4 mL to the tubes with 200 µL of blood.
15. Vortex the tubes or pipet the blood/buffer solution up and down 5 times very gently to avoid spills.
16. Incubate at room temperature for 15 min to permit complete RBC lysis and leukocyte fixation.
17. Spin down the tubes at 500 *g* and RT for 7 min, and aspirate the supernatants.
18. Homogenize cell pellets by flicking the bottom of the tubes or gently vortexing. Then, wash the cells with 2 mL of BD Pharmingen™ Stain Buffer (FBS) by centrifugation at 500 *g* and RT for 7 min.
19. Repeat step 18 once.

⚠ CRITICAL: Adding a volume of BD Phosflow™ Lyse/Fix Buffer working solution that is 20 times greater than the blood volume is important for proper RBC lysis.

Pause point: After RBC lysis/cell fixation and washes, the cells can be stored for 16–24 h at 4°C in 200 µL of BD Pharmingen™ Stain Buffer (FBS). However, loss of fluorescence signal might be observed with prolonged storage (Diks et al., 2019).

Cell permeabilization and intracellular staining

Timing: 20 min cell permeabilization; 45 min intracellular staining; ~15 min washes

20. Use a pipette to resuspend the cell pellets in 100 µL of BD Phosflow™ Perm/Wash Buffer I and incubate at 4°C for 20 min for cell permeabilization.
21. Add the antibodies for intracellular staining to the respective single-stain control, FMO and full panel tubes.
22. Mix with a pipette and incubate at 4°C for 45 min protected from light.

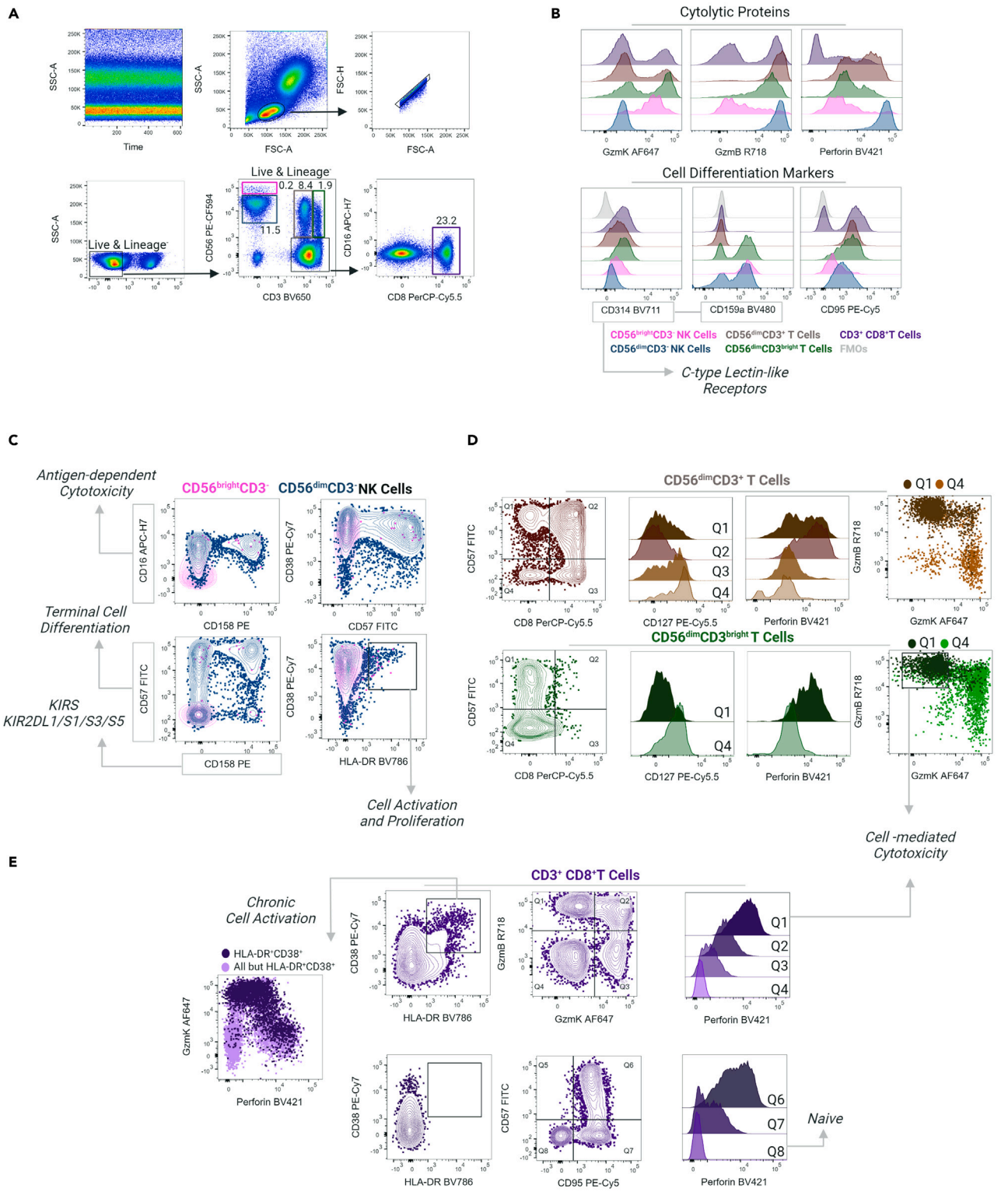

Figure 2. Manual gating strategy for identification of main cytotoxic cell populations in peripheral blood

(A) Cytotoxic cell populations are represented in different colors and the numbers next to the gates correspond to the respective percentages in the Live & Lineage⁻ gate.

(B) Histogram overlays showing the expression of cytolytic proteins and differentiation markers in each population.

Figure 2. *Continued*
(C) Overlay of NK cell subsets highlighting the expression of key functional and differentiation markers (CD16, CD57, CD158 and HLA-DR).
(D) Comparison between $CD3^+$ and $CD3^{bright}$ unconventional T cells, showing a strong correlation between CD57, perforin and GzmB expression, indicating these are terminally differentiated cells with cytotoxic potential (Q1). Conversely, CD57 negative cells expressed higher levels of GzmK than GzmB and perforin (Q3/Q4).
(E) Clear detection of $CD38^+HLA\text{-}DR^+CD8^+$ T cells, a rare subset of chronically activated T cells in blood. The HLA-DR FMO staining helped to determine the gating boundaries for proper detection of the double positive cells. The figure also depicts different CD8 T cell subsets based on the expression of GzmB versus GzmK or CD95 versus CD57.

23. Wash with 2 mL of BD Phosflow™ Perm/Wash Buffer I by centrifugation at 500 *g* and 4°C for 7 min.
24. Repeat step 23 once.
25. Resuspend the cell pellets in 300 μL of BD Pharmingen™ Stain Buffer (FBS) and acquire the cells in the flow cytometer.

⚠ CRITICAL: BD Phosflow™ Perm/Wash Buffer I is an aqueous buffer solution containing saponin. Thus, the permeabilization step is reversible and intracellular staining must be carried out in the presence of the buffer (Lacaille-Dubois and Wagner, 1996).

Pause point: We have not extensively tested saving the samples in the fridge overnight after intracellular staining. However, in the case that samples cannot be immediately acquired by the flow cytometer, the cells can be saved for 16–24 h at 4°C, protected from light.

Note: The BD Phosflow™ Lyse/Fix Buffer 5× is also compatible with other permeabilization buffers such as BD Phosflow™ Perm/Wash Buffer II, BD Phosflow™ Perm/Wash Buffer III and BD Cytoperm™ Permeabilization Buffer Plus.

Note: BSB Plus or BSB are routinely added to the cell sample during the surface staining step and there is no need to add it again during intracellular staining.

EXPECTED OUTCOMES

In this section, we show representative results from one healthy donor, in which we could clearly detect five main populations of circulating cytotoxic cells: (1) $CD3^-CD56^{bright}$ NK cells; (2) $CD3^-CD56^{dim}$ NK cells, (3) $CD3^+CD56^{dim}$ T cells; (4) $CD3^{bright}CD56^{dim}$ T cells; and (5) $CD3^{+-}CD56^-CD8^+$ T cells. However, the frequencies of these cell subsets may vary from one donor to another, especially among $CD56^{dim}$ T cell subsets (limitations). We evaluated a total of five donors. The following gating strategy and cell analysis was performed using cells from donor 5.

The gating strategy began by electronically plotting time versus side scatter area (SSC-A) to inspect stability of cell acquisition in the cytometer. After detecting stable events, we sequentially removed cell doublets and excluded dead cells, B cells and most myeloid cells from the analysis. To exclude B cells, myeloid cells and dead cells altogether, we used BV605-conjugated antibodies against CD19, CD14, CD123, CD141 and FVS575V, then selected the negative events in the BV605 detector (Figure 2A). The negative events contained mostly live T cells and NK cells (Live & $Lineage^-$ gate). Next, we analyzed the expression of CD3 and CD56 within the Live & $Lineage^-$ gate to separate T cells and NK cells subsets. Despite their overall high expression on the cell surface, CD3 and CD56 analyses were performed by staining the cells with antibodies conjugated with very bright dyes, BD Horizon™ BV650 and PE-CF594, respectively (problem 4). This allowed a clean separation between $CD3^{+-}CD56^{dim}$ and $CD3^{bright}CD56^{dim}$ T cells and $CD3^-CD56^{bright}$ and $CD3^-CD56^{dim}$ NK cells. Other markers in the panel that were expressed at moderate or low levels could also be interrogated with bright or very bright dyes on a BD FACSymphony™ A1 Cell Analyzer (Table 1).

In addition to manually gating and further inspecting the cell populations (Figures 2B–2E), we also applied a clustering algorithm for an unbiased identification of cell populations. We used

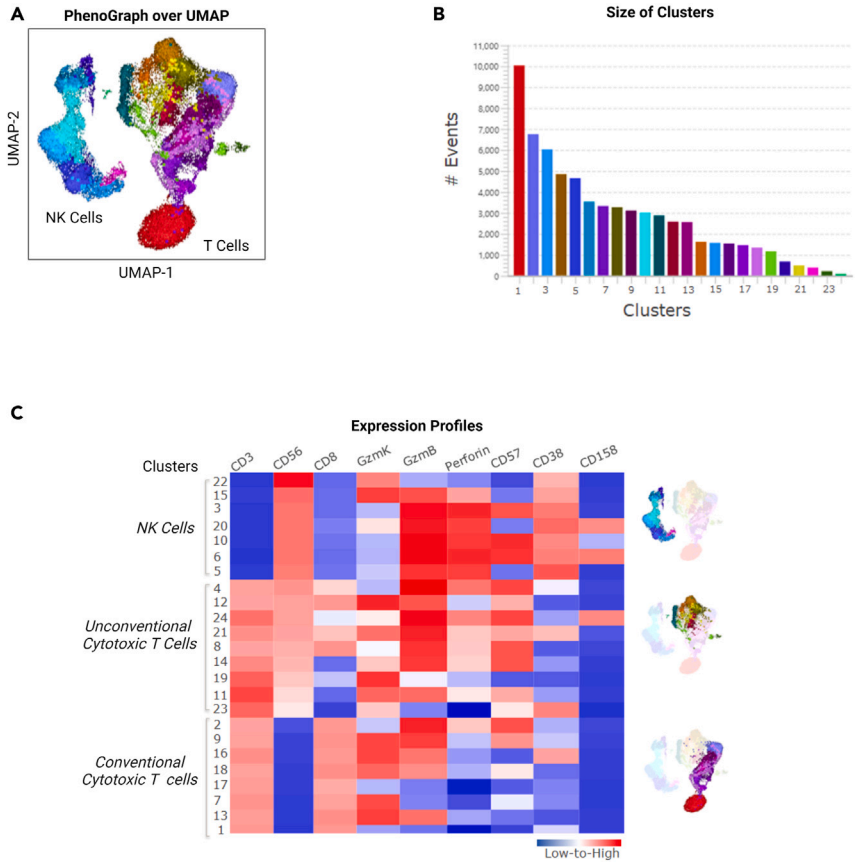

Figure 3. Profiles of circulating cytotoxic cells revealed with a 16-color panel

(A) UMAP displaying 67K circulating cytotoxic T cells and NK cells. The PhenoGraph algorithm identified 24 cell clusters among these cells that were overlaid on the UMAP and represented in different colors.

(B and C) The ClusterExplorer algorithm was used to determine the number of cells in the PhenoGraph clusters and to generate the heatmap for analysis of the protein expression patterns in each cluster.

PhenoGraph for clustering due to its robustness compared to other clustering algorithms (Liu et al., 2019) and because it can be uploaded in the plugins folder of FlowJo™ v10.7.2 Software. We also utilized the ClusterExplorer algorithm to investigate the expression profiles of individual clusters and Uniform Manifold Approximation and Projection (UMAP) for simple visualization of the data set (Figure 3). The use of a clustering algorithm was critical for evaluation of all cells in the experiment, including cells that express intermediate levels of certain markers and are often excluded from analysis during manual gating. In doing so, PhenoGraph detected a total of 24 clusters. One of the smallest clusters (cluster 22) contained all $CD56^{bright}CD3^{-}$ NK cells confirming the robustness of PhenoGraph in identifying cell populations (Problem 5).

> ***Note:*** This 16-color panel was designed for a BD FACSymphony™ A1 Cell Analyzer, which is equipped with four lasers and 16 fluorescence detectors. If using an instrument with five lasers, the markers can be distributed across the laser lines to further minimize impact from spillover-spreading errors in population resolution. This panel has also been successfully used in a BD FACSymphony™ A5 Cell Analyzer.

LIMITATIONS

Donor-to-donor variations are expected and may preclude the analysis of all cell populations shown in this data set. Also, since we used stained cells as reference controls for compensation, it was important to use cells from a control donor with a known expression profile. In this example, we

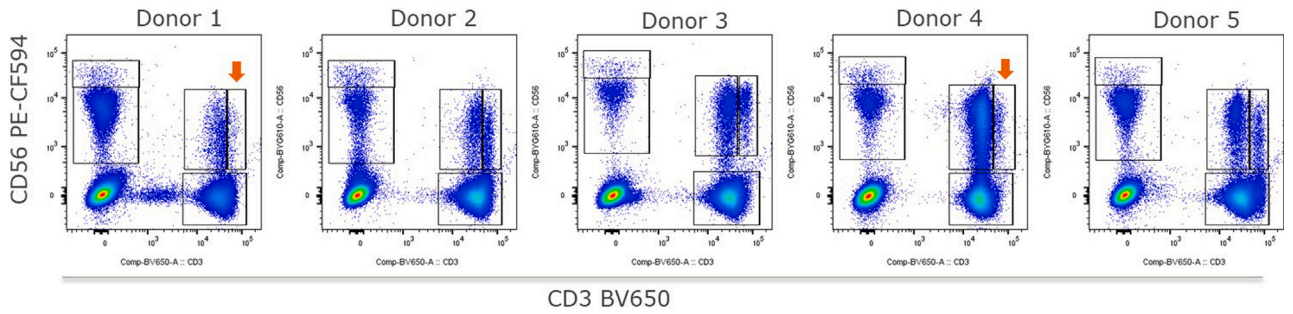

Figure 4. Assessment of different healthy donors

show two donors with lower frequencies of $CD3^{bright}CD56^{dim}$ populations compared to the other donors, as indicated by the orange arrows (Figure 4).

TROUBLESHOOTING

Problem 1

The performance of the panel greatly depends on instrument sensitivity and settings as well as reagents. Using pre-titrated reagents or failing to properly titrate reagents may result in off-scale events or lack of maximum separation between negative and positive populations. Adjusting PMT voltages, while acquiring the data may perturb the efficiencies of the detectors, which then may not operate at their peak of performance (step 1 titrations).

Potential solution

To avoid possible technical variances that may affect data interpretation, it is critical to properly set up the instrument and titrate reagents before the experiment. Titration of reagents is recommended to ensure the data can be acquired using the optimal settings of the instrument. Herein we show an example of how to titrate BD OptiBuild™ Reagents, which are provided at a 0.2 mg/mL concentration, and how to define the optimal concentration for test size reagents (Figure 5).

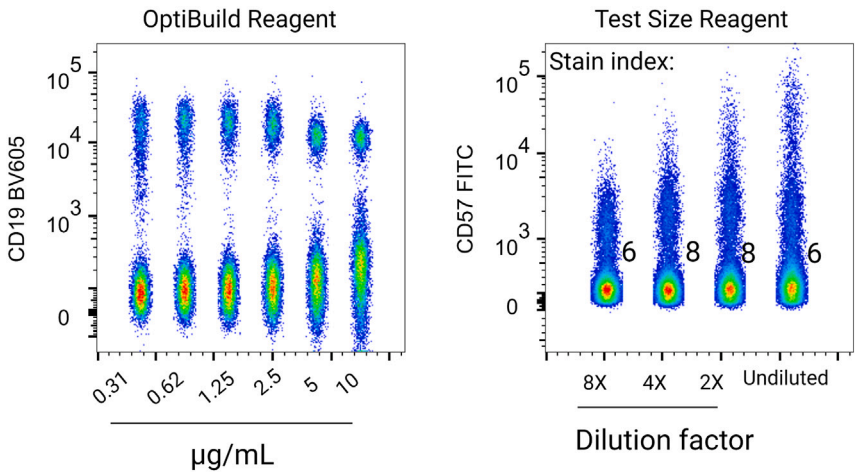

Figure 5. Antibody titrations

(Left) Serial dilutions of CD19-BV605 antibody ranging from 10 µg/mL to 0.3 µg/mL. Note that the background on the negative cell population increases proportionally to the antibody concentration. The optimal concentration ranges from 0.62 to 1.25 µg/mL, in which the positive population is maximally separated from the negative population.
(Right) CD57-FITC titration showing all bright cell events on scale after 2-fold dilution of the original test size reagent (undiluted). At this concentration, the stain index is also optimum (SI = 8 compared to SI = 6).

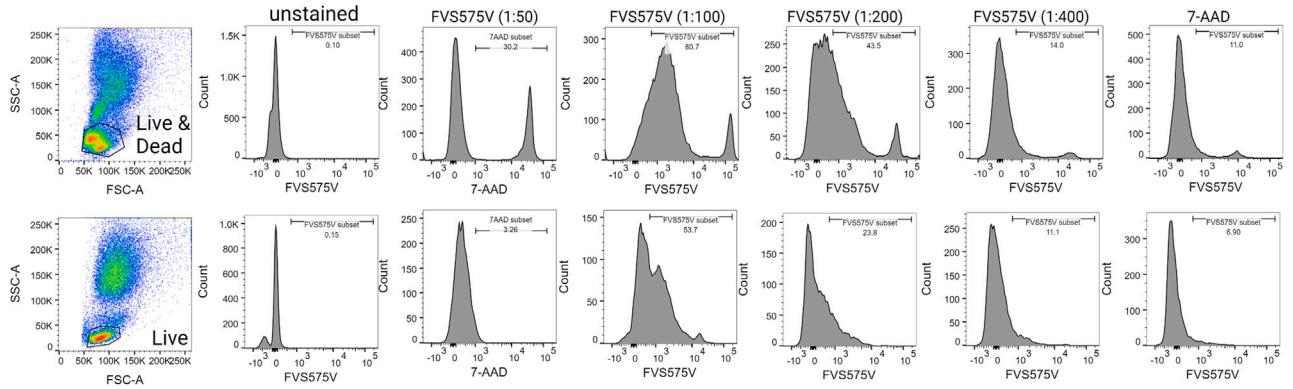

Figure 6. Titration of BD Horizon™ Fixable Viability Stain 575V (FVS575V)

Four milliliters of fresh blood were treated with BD Pharm Lyse™ Lysing Buffer for red blood cell lysis for 15 min at RT. After two washes with PBS, the cell pellet was resuspended in 2 mL of PBS and incubated for 5 min at 65°C. (Top row) One hundred microliters of fresh blood were mixed with 100 μL of heat-treated cells. (Bottom row) One hundred microliters of fresh blood were mixed with 100 μL of PBS. The samples were incubated with different concentrations of FVS575V for 20 min at room temperature (RT). Then, 2 mL of BD Pharm Lyse™ Lysing Buffer were added to the samples and after 10 min incubation at RT the cells were washed twice with BD Pharmingen™ Stain Buffer (FBS). In parallel, unstained samples were also lysed for subsequent staining with 7-AAD or for generation of negative control samples (unstained cells). The histograms show the percentages of dead cells. Based on these results, at the 1:100 dilution, there is some background staining that is not observed in the next dilutions (1:200 and 1:400). However, when compared to 7-AAD, the 1:200 dilution stained approximately half of the dead cells (30.2% versus 14%, respectively). This led us to use the 1:100 dilution to ensure maximum exclusion of dead cells from the analysis.

Problem 2

Since this panel was designed to capture cells that are found at low frequencies in the blood, it was critical to exclude dead cells and debris to ensure accurate measurements. To generate this data set, we used FVS575V for live/dead cell discrimination. This fluorescent dye belongs to a class of amine reactive dyes that are stable after fixation with paraformaldehyde and cell permeabilization. These dyes cannot permeate intact plasma cell membranes and thus can bind only to cell-surface amines on living cells. However, the dye can penetrate damaged cell membranes of necrotic cells and stain intracellular amines in addition to cell-surface amines. As a result, necrotic cells show brighter FVS fluorescence than live cells, which helps with the discrimination between live and dead cells. Because the reagent reacts minimally with live cells, it may introduce a high background if not used at the right concentration. Blood samples show a bi-modal FVS-staining profile such as that live cells are dimly stained and dead cells are brightly stained. In this regard, even in the presence of a high background, live cells can be distinguished from dead cells. However, the amount of FVS fluorescence in live cells (background) can be detrimental and introduce spread to the data (step 2 titrations). See more details on how to identify spreading errors in problem 4.

Potential solution

As described in the protocol below (Figure 6), we recommend titrations to determine the optimal concentration of FVS dyes. During titrations of the reagents, it is also interesting to include 7-AAD as a control for detection of dead cells. In contrast to FVS reagents, 7-AAD is efficiently excluded by intact cells. Thus, all 7-AAD-labeled cells correspond to dead cells. However, 7-AAD can permeate live permeabilized cells, which makes it less suitable for analyses of cell death in intracellular protocols.

Problem 3

Donor-to-donor variations, blood storage conditions, the amount of time elapsed between sample collection and analysis and other factors may result in differences in the expression levels of proteins (sample preparation and cell staining).

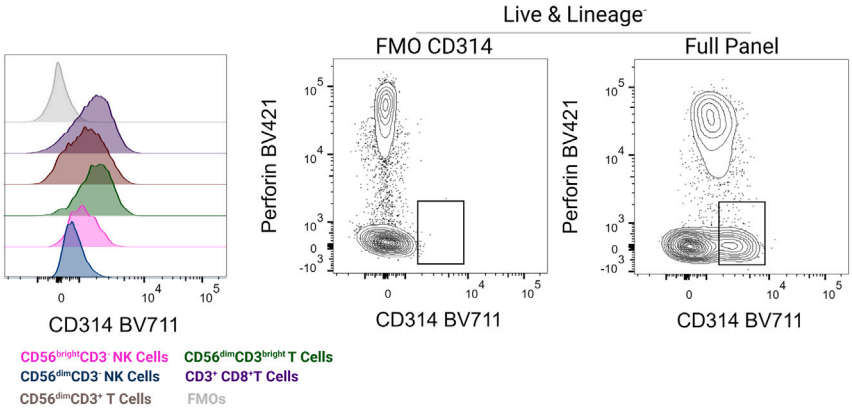

Figure 7. Using fluorescence minus one control to determine the boundaries between negative and positive populations

The histograms and contour plots show the expression of CD314 in different cell populations. Because CD314 expression levels are relatively low or might be impacted by spreading of the negative population, we used an FMO control to define the positive population.

Potential solution

To circumvent these issues, biological control samples and experimental samples should be processed and analyzed concurrently. Technical control samples, such as FMO controls are essential to help to define the boundaries between negative and positive populations. Using gates drawn based on FMO controls enables the analysis of markers that are expressed even at very low levels (Figure 7).

Problem 4

One caveat of using bright dyes for detection of highly expressed antigens is the fact that some bright dyes may introduce spread into the data. BV650-CD3, for example, introduced spread into the Alexa Fluor™ 647 (AF647) detector, as observed when GzmK-AF647 was analyzed within $CD3^{bright}$ versus $CD3^{+}$ and $CD3^{-}$ cell populations. However, this result did not prevent proper analysis of $GzmK^{+}$ cells in the panel (Figure 2 expected outcomes).

Potential solution

In the example below, we determined the impact of spillover-spreading errors in the resolution of GzmK-AF647. Due to cross-laser excitation, CD3-BV650 spills into the AF647 channel, as indicated by the arrow (Figure 8A). As a result, there is more spread of the GzmK-AF647 negative population within the CD3-BV650 bright population (Figure 8B) compared to the $CD3^{+}$ (Figure 8C) and $CD3^{-}$ (Figure 8D) populations. Despite the spread observed in the negative population, the GzmK positive cells were satisfactorily resolved in the panel. Alternatively, AF647 could be swapped with another dye that is not affected by BV650. BV421, for example, is a good choice because it receives very little residual signals from BV650 (Figure 8E) or other dyes, as illustrated in the Figures 8F–8H.

Problem 5

The PhenoGraph algorithm is initialized by calculating the *k*-nearest neighbors (*KNN*) of each cell, to detect connectivity and density peaks among cells in high-dimensional spaces. The parameter *k* denotes the number of neighboring cells used to make these estimates. Because initial *k* values can impact the clustering results and can be entered by the user, there is a potential for heuristic application of the algorithm (Figure 3 expected outcomes).

Potential solution

We used the PhenoGraph v3.0 algorithm in the FlowJo™ v10.7.2 Software, which automatically calculates an appropriate *k* value, based on the size of the data set, to create the optimal *KNN* size. Furthermore, the *k* value can affect the performance of the algorithm but does not determine the

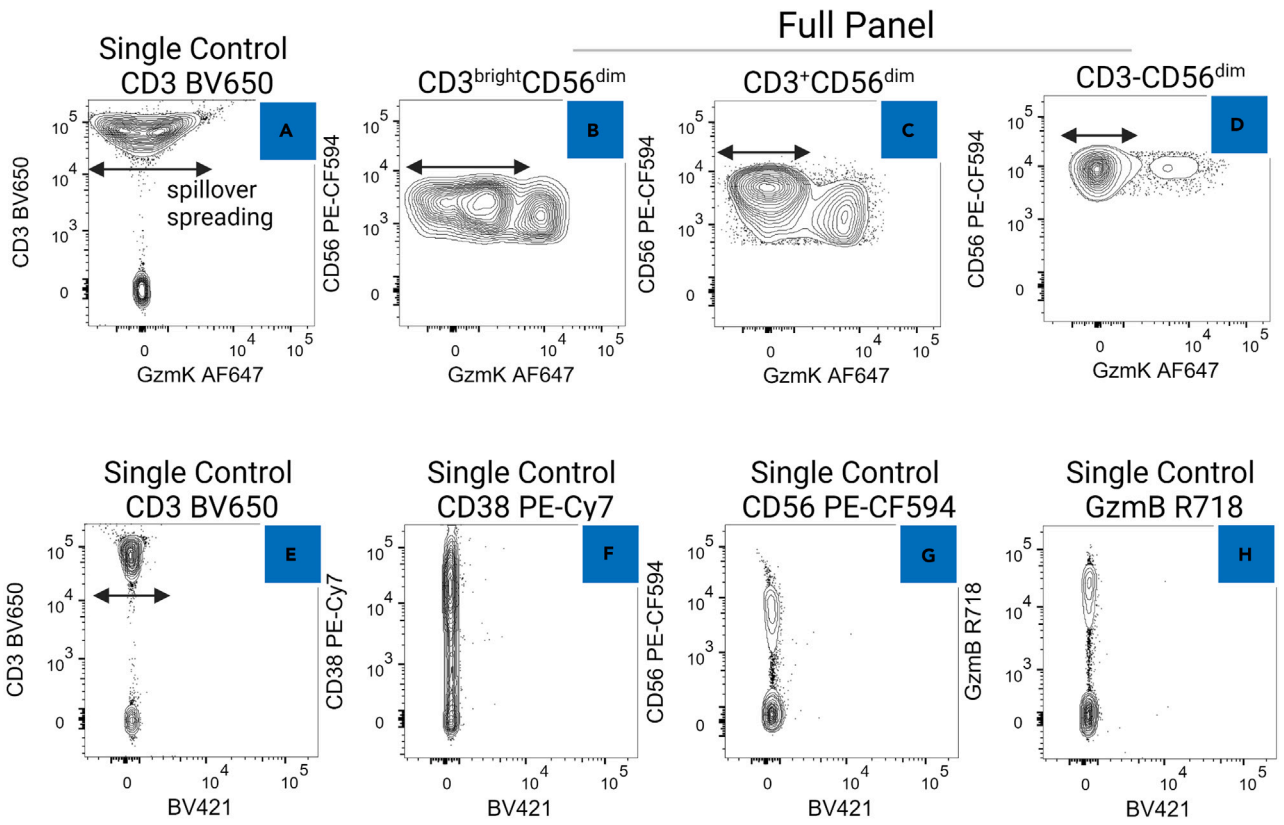

Figure 8. Investigating the impact of spill-over spreading in cell population resolution

(A–D) Analysis of the impact of BV650-CD3 in the resolution of GzmB-AF647.

(E–H) Analysis of the spread of different very bright dyes into the BV421 channel. As exemplified, BV650 as well other very bright dyes introduce little or no signal into the BV421 channel.

number of clusters. Once the KNN network is created, a partitioning algorithm identifies the distinct phenotypes based on density, and thus divides the data into clusters algorithmically. In the initial PhenoGraph publication (Levine et al., 2015) the authors demonstrated consistent results for a wide range of initial *k* values, demonstrating the elasticity of the algorithm. Finally, to confirm the composition of the automatically created clusters, we selected a few PhenoGraph clusters and compared them to manually assigned cell populations. As shown in the following example, PhenoGraph precisely identified and distinguished $CD56^{dim}CD3^{+}CD8^{+}$ cells (cluster 8) from $CD56^{dim}CD3^{+}CD8^{-}$ cells (cluster 14). PhenoGraph also accurately identified one of the smallest cell populations (cluster 22) containing all $CD56^{bright}CD3^{-}$ NK cells (Figure 9).

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Aaron J. Tyznik, aaron.j.tyznik@bd.com

Materials availability

This study did not generate new unique reagents.

Data and code availability

The dataset supporting the current study did not generate code and is available from the corresponding author on request.

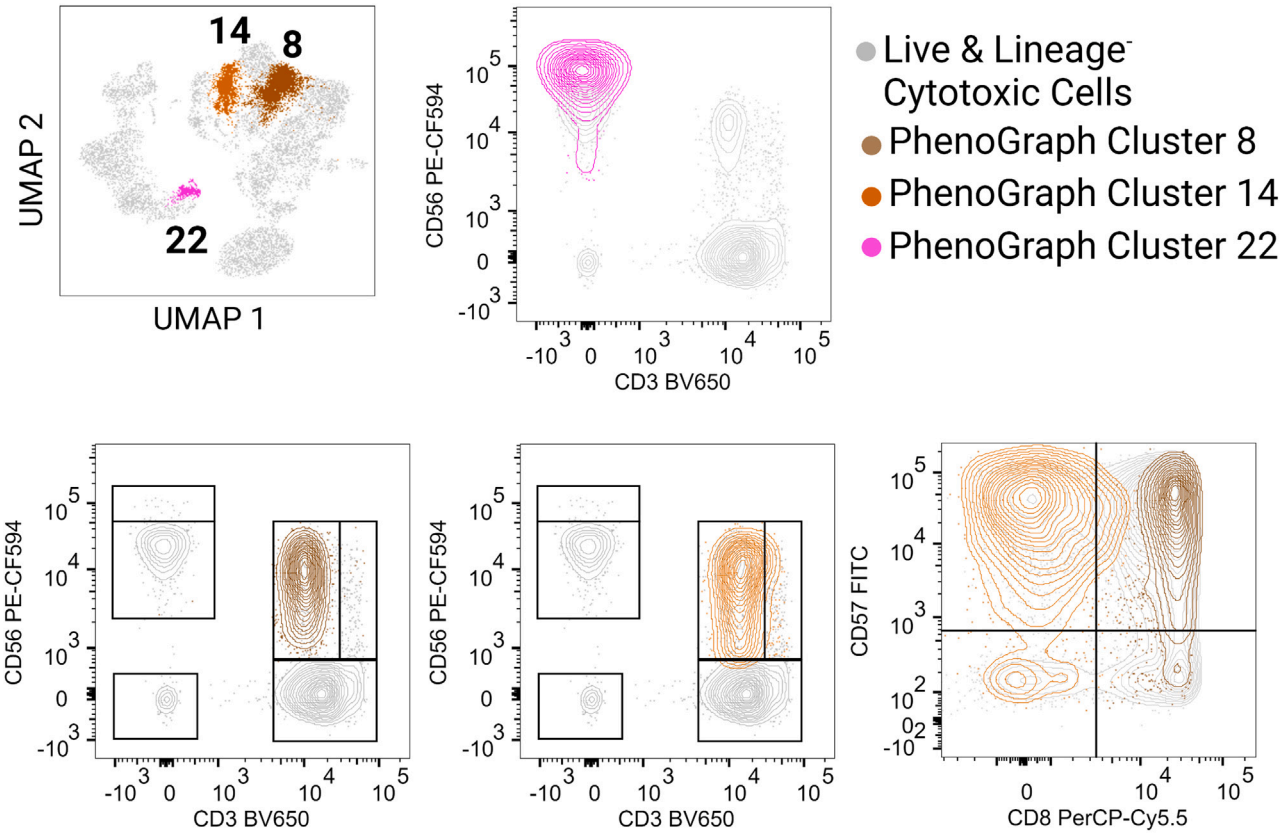

Figure 9. Comparison between PhenoGraph clusters and manually assigned cell populations

Overlay of PhenoGraph clusters 8, 14 and 22 on UMAP or contour plots containing Live & Lineage⁻ cytotoxic cells.

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AUTHOR CONTRIBUTIONS

G.V.B. conceived the idea, designed the protocol, analyzed the data, and wrote the manuscript. N.K. executed all the experiments (unless otherwise indicated) and provided insights for troubleshooting and manuscript. E.L. performed experiments for triage of healthy donors. S.R. helped to elaborate the idea and performed initial tests showing the feasibility of the BD Phosflow™ Buffer system for detection of granzyme K. S.J.W. provided insights on panel design and inputs for the manuscript. A.J.T. read and provided critical inputs for the manuscript. All the authors read and approved the final version of the manuscript.

DECLARATION OF INTERESTS

G.V.B., N.K., S.R., S.J.W., and A.J.T. are employees of BD Biosciences. E.L. is a former employee of BD Biosciences.

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