Protocol

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This protocol details the step-by-step procedure for in-depth immune phenotyping of peripheral blood natural killer (NK) cells from clinical samples by mass cytometry. The protocol consists of three main steps: PBMC incubation with a mix of metal-conjugated antibodies for extracellular phenotyping followed by fixation, permeabilization and incubation with a mix of metal-conjugated antibodies for staining of intracellular proteins, and sample acquisition on a mass cytometer. High-dimensional analysis enables the visualization of NK cell subsets and their phenotypical characteristics.

Publisher’s note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.
Protocol
High-throughput mass cytometry staining for deep phenotyping of human natural killer cells

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SUMMARY
This protocol details the step-by-step procedure for in-depth immune phenotyping of peripheral blood natural killer (NK) cells from clinical samples by mass cytometry. The protocol consists of three main steps: PBMC incubation with a mix of metal-conjugated antibodies for extracellular phenotyping followed by fixation, permeabilization and incubation with a mix of metal-conjugated antibodies for staining of intracellular proteins, and sample acquisition on a mass cytometer. High-dimensional analysis enables the visualization of NK cell subsets and their phenotypical characteristics.
For complete details on the use and execution of this protocol, please refer to Chretien et al. (2021).

BEFORE YOU BEGIN
Institutional permissions

△ CRITICAL: before collecting and working on human samples, it is mandatory to obtain permissions from the relevant institutions. In the present work, human samples from healthy volunteers were obtained from the EFS (Etablissement Français du Sang); authorization number for the conservation and preparation of products for non-therapeutic purposes, issued by the Ministry of Higher Education, Research and Innovation, is AC-2019-3428.

1. Antibodies conjugation to metal isotopes

☉ Timing: 4 h

To design a panel, we first use available commercial antibodies. However, when the relevant clones are not available or when the antibody-metal combinations are not appropriate, we perform a custom conjugation. This protocol is adapted from the Maxpar Antibody Labeling User Guide.
1. Preload the polymer with lanthanide.
   a. Thaw and quick spin X8 polymer tube then add 95 μL of L-Buffer to dissolve the polymer.
   b. Add 5 μL of 50 mM Ln metal solution and incubate at 37°C in an incubator (do not use a water bath) for 30 min.

2. Partially reduce the antibody.
   a. Add 300 μL with R-Buffer to the 50 kDa column, then add 100 μg (up to 400 μL) of purified antibody and centrifuge at 12,000 × g for 10 min at 20°C–22°C.
   b. After discarding the flow-through, add 100 μL of freshly prepared 4 mM TCEP diluted in R-Buffer on the 50 kDa column. After a quick mix by pipetting, incubate at 37°C in an incubator for 30 min.

3. Purify the lanthanide-loaded polymer.
   a. Add the polymer-metal complex to the labeled 3 kDa column containing 200 μL of L-Buffer and centrifuge at 12,000 × g for 10 min at 20°C–22°C.
   b. Discard the flow-through then add 300 μL of C-Buffer and centrifuge at 12,000 × g for 30 min at 20°C–22°C.

4. Purify the partially reduced antibody.
   a. Add 300 μL of C-Buffer to the 50 kDa column, and centrifuge at 12,000 × g for 10 min at 20°C–22°C.
   b. Discard the flow-through, then add 400 μL of C-Buffer and centrifuge at 12,000 × g for 10 min at 20°C–22°C.

Optional: A second centrifugation can be added if step 3.b is not completed.

5. Conjugate the antibody with lanthanide-loaded polymer.
   a. Discard the 3 kDa and 50 kDa column flow-through.
   b. Resuspend the lanthanide-polymer complex in 60 μL of C-Buffer by pipetting and transfer the suspension (approximately 80 μL with residual volume) to the partially reduced antibody in the 50 kDa filter. Mix well by pipetting. The final conjugation volume should be approximately 100 μL. Incubate for 60 min at 37°C.

6. Wash and recover the metal-conjugated antibody.
   a. Add 300 μL of W-Buffer to the 50 kDa column and centrifuge at 12,000 × g for 10 min. Discard flow-through and repeat this step 3 times.
   b. Elute by adding 50 μL of W-Buffer to the 50 kDa column, pipet several times and invert the filter into a new collection tube then centrifuge for 2 min at 1,000 × g. Repeat this step once.
   c. Quantify the conjugated antibody using the NanoDrop spectrophotometer with the following settings: protein A280 module, IgG sample type option, 1 μL aliquot. Use W-Buffer as the blank. Conjugated antibody must be stored at 4°C in W-Buffer supplemented with 0.05% sodium azide at a final concentration of 0.5 mg/mL.

CRITICAL: This procedure is suitable for lanthanide but not cadmium isotopes.

Pause point: Metal-conjugated antibodies can be stored for a one year. However, a monitoring of the antibodies is carried out every month with a full panel on the same PBMCs control to check the absence of signal degradation.

Figure 1A.

II. Titrations of extracellular and intracellular antibodies

Timing: 5 h for sample preparation and 1 h for the acquisition on the mass cytometer

Antibody titration is critical to determine the optimal concentration of conjugated antibody. It allows an optimal staining, strengthens population identification, and decreases unspecific binding leading to background signal. The choice of optimal titer is based on good separation between the positive and negative
population without spillover into the other channels. In-house metal-conjugated antibodies must be titrated for every new conjugation batch. A fraction of the current batch must be preserved to serve as a control. Ideally, the tests should be performed on samples of the same type as those to be analyzed.

For cell preparation please refer to step-by-step method details section. Rapidly cells were thawed, dead cells were discarded, counted and 3.10^6 cells were distributed in 96-well plate. Serial dilutions are prepared in cell staining medium (CSM) for extracellular antibodies and in perm wash for intracellular antibodies.

Note: In first intention, commercial antibodies are not titrated. Indeed, we start at the recommended concentration by the manufacturer (i.e 1 μL antibody per 100 μL cell suspension.
containing 1–3 \times 10^6 cells). However, if a strong signal or a poorly focused separation between negative and positive populations is observed during the panel validation, commercial antibodies are also to be titrated.

**Note:** The proper titration of some epitopes requires prior stimulation in order to induce their membrane expression.

**Optional:** The titration of several antibodies together is possible provided that the following rules are respected: do not titrate antibodies in the same well if metal isotopes are located at N+1, N-1 and N+16 in order to avoid a possible cross-contamination in corresponding channels.

**Figures 1B–1D.**

**Study design**

7. **Clinical Samples.**

The mean age of healthy subjects (N = 22) recruited was 50.1 ± 11.3 with a sex ratio (M/F) of 1.2. We have not retained any particular criteria for healthy volunteer selection.

8. **Reference Sample as Batch control.**

A quality control is important for longitudinal studies where multiple batches will be run and compared. PBMCs isolated from a single healthy donor serve as reference sample. These PBMCs must be included in each batch of experiment. Thus, a correction can be applied to each batch with a batch effect correction algorithm.

**KEY RESOURCES TABLE**

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| CD45-89Y (1:100)    | Fluidigm | Cat# 3089003B |
| CD19-142Nd (1:400)  | Fluidigm | Cat# 3142001B |
| CD45RA-143Nd (1:100)| Fluidigm | Cat# 3142001B |
| CD4-145Nd (1:400)   | Fluidigm | Cat# 3145001B |
| CD8α-146Nd (1:400)  | Fluidigm | Cat# 3146001B |
| CD107α 151Eu (1:400)| Fluidigm | Cat# 3151002B |
| SIGLEC7-152Sm (1:100)| Fluidigm | Cat# 3152013C |
| CD27-155Gd (1:200)  | Fluidigm | Cat# 3155001B |
| Tbet 161Dy (1:100)  | Fluidigm | Cat# 3161014B |
| NKp46-162Dy (1:100) | Fluidigm | Cat# 3162021B |
| NKG2D-166Er (1:100) | Fluidigm | Cat# 3166016B |
| DNAM-1-171Yb (1:100)| Fluidigm | Cat# 3171013B |
| CD57-172Yb (1:1600) | Fluidigm | Cat# 3172009B |
| CD56-176Yb (1:400)  | Fluidigm | Cat# 3176001B |
| CD16-209Bi (1:100)  | Fluidigm | Cat# 3209002B |
| CD3-115In (1:800)   | Immunomonitoring lab* | UCHT1 |
| TCRV62-141Pr (1:400)| Beckman Coulter | C65929 |
| NKG2C-147Sm (1:100) | Miltenyi | 130-122-278 |

(Continued on next page)
| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| TCRpan-153Eu (1:400) | Beckman Coulter | IBMULK1_TCRPGD |
| CD158b1,b2,154Eu (1:100) | Beckman Coulter | C65928 |
| CD158a,h-163Dy (1:100) | Beckman Coulter | C65928 |
| NKG2A-165Ho (1:400) | Miltenyi | 130-122-329 |
| NKp30-169Tm (1:50) | Beckman Coulter | C65929 |
| CD33-170Er (1:400) | BioLegend | BLE3034419 |
| EOMES-149Sm (1:400) | BD Biosciences | 624084-EOMES |
| BCL-2-150Nd (1:400) | BD Biosciences | 624084-BCL2 |
| BCL-XL-158Gd (1:100) | Cell Signaling Technology | 2764BF |
| Human BD Fc Block™ | BD Biosciences | Cat#564219 |
| Lymphocyte separation medium | Eurobio | Cat#CMSMSL01-01 |
| Cell-ID™ Intercalator-Ir_125 µM | Fluidigm | Cat#201192A |
| Cell-ID™ Intercalator-Rh_500 µM | Fluidigm | Cat#201103A |
| EQTM Four Element Calibration Beads | Fluidigm | Cat#201078 |
| Maxpar® Cell Staining Buffer | Fluidigm | Cat#201068 |
| Maxpar® PBS | Fluidigm | Cat#201058 |
| Fetal Bovine Serum, qualified | Gibco | Cat#10270-106 |
| RPMI 1640 Medium | Gibco | Cat#11875093 |
| Dimethyl Sulfoxide | Sigma-Aldrich | Cat#D4540 |
| eBioscience™ Fixation/Transcription Factor Staining Buffer Set | Thermo Fisher Scientific | Cat#00-5523-00 |
| Bond-Breaker™ TCEP solution, neutral pH | Thermo Fisher Scientific | Cat#77720 |
| Pierce™ Universal Nuclease for Cell Lysis | Thermo Fisher Scientific | Cat#88701 |
| 16% formic aldehyde (v/v) Pierce™, without methanol | Thermo Fisher Scientific | Cat#28906 |
| Software and algorithms | | |
| CYTOF Software V6.0 | Fluidigm | https://www.fluidigm.com/products-services/software |
| OMIQ | | https://www.omiq.ai/ |
| FlowJo V10.6 | Becton, Dickinson and Company | https://www.flowjo.com/solutions/flowjo/downloads |
| Cytosplore V2.3.1 | | https://www.cytosplore.org/ |
| Other | | |
| T15 Indium | Trace Sciences | Cat#115_in |
| Maxpar® X8 Antibody Labeling Kit, 141Pr | Fluidigm | Cat#201141A |
| Maxpar® X8 Antibody Labeling Kit, 147Sm | Fluidigm | Cat#201147A |
| Maxpar® X8 Antibody Labeling Kit, 149Sm | Fluidigm | Cat#201149A |
| Maxpar® X8 Antibody Labeling Kit, 153Eu | Fluidigm | Cat#201153A |
| Maxpar® X8 Antibody Labeling Kit, 154Eu | Fluidigm | Cat#201154A |
| Maxpar® X8 Antibody Labeling Kit, 158Gd | Fluidigm | Cat#201158A |
| Maxpar® X8 Antibody Labeling Kit, 159Tb | Fluidigm | Cat#201159A |
| Maxpar® X8 Antibody Labeling Kit, 160Gd | Fluidigm | Cat#201160A |
| Maxpar® X8 Antibody Labeling Kit, 165Ho | Fluidigm | Cat#201165A |
| Maxpar® X8 Antibody Labeling Kit, 169Tm | Fluidigm | Cat#201169A |
| Maxpar® X8 Antibody Labeling Kit, 170Er | Fluidigm | Cat#201170A |
| Maxpar® X8 Antibody Labeling Kit, 174Yb | Fluidigm | Cat#201174A |
| Sterile syringe filter 0.2 µm | Dutscher | Cat#146560 |
| Sterile syringe filter 0.45 µm | Dutscher | Cat#146561 |
| Sterile syringe filter 0.8 µm | Dutscher | Cat#146562 |
| Falcon® 5 mL Round Bottom Polystyrene Tube with Cell Strainer Snap Cap 35 µm | Dutscher | Cat#352063 |

(Continued on next page)
## MATERIALS AND EQUIPMENT

### REAGENT or RESOURCE SOURCE IDENTIFIER

| Material Description                                                                 | Source                      | Identifier          |
|--------------------------------------------------------------------------------------|-----------------------------|---------------------|
| 15 mL high clarity polypropylene conical tubes                                         | Corning Inc.                | Cat#352096          |
| 50 mL high clarity polypropylene conical tubes                                         | Corning Inc.                | Cat#352070          |
| 5 mL round bottom polypropylene tubes                                                 | Dutscher                    | Cat#351235          |
| Pre-separation filters (30 μm)                                                        | Miltenyi                    | Cat#130-041-407     |
| Amicon® Ultra-0.5 mL Centrifugal Filters UltraceI-3K                                  | Millipore                   | Cat#UFCS0396        |
| Amicon® Ultra-0.5 mL Centrifugal Filters UltraceI-50K                                 | Millipore                   | Cat#UFCS05096       |
| Micro tube 1.5 mL                                                                    | Sarstedt                    | Cat#72-690          |
| Megafuge 2.0R                                                                         | Heraeus Instruments         | NA                  |
| Biofuge fresco                                                                       | Heraeus Instruments         | NA                  |
| Eppendorf 5810R                                                                       | Eppendorf Corporate         | NA                  |
| Microscope Olympus CKx31                                                             | Olympus Life Science        | NA                  |
| Glasstic slide 10 with grids                                                         | Kova International          | NA                  |
| Vortex1 genie touch mixer                                                             | Scientific Industries       | NA                  |
| Chemical hood Astec sensair                                                          | Astec                       | NA                  |
| A set of pipettes (2.5/10/200/1,000 μL)                                              | Eppendorf Corporate         | NA                  |
| Pipet.aid                                                                             | Drummond Scientific corporation | NA                |
| MilliQ 1X pure water system                                                           | Merck                       | NA                  |
| Nanodrop 1000 spectrophotometer                                                       | Thermo Fisher Scientific    | NA                  |
| * this antibody was produced by the immunomonitoring department of the IPC.          |                             |                     |

### PROTOCOL

#### RPMI 10% FCS

| Component                        | Volume (mL) | Notes                                      |
|----------------------------------|-------------|--------------------------------------------|
| RPMI 1640 Medium                 | 450         | Can be stored at 4°C for 1 month.          |
| FCS (heat inactivated and filtered) | 50         |                                           |

#### RPMI 2% FCS

| Component                        | Volume (mL) | Notes                                      |
|----------------------------------|-------------|--------------------------------------------|
| RPMI 1640 Medium                 | 490         | Can be stored at 4°C for 1 month.          |
| FCS (heat inactivated and filtered) | 10         |                                           |

#### Pierce universal nuclease

| Component                        | Volume (μL) | Notes                                      |
|----------------------------------|-------------|--------------------------------------------|
| Pierce 1640 2% FCS               | 10,000      | This solution is prepared extemporaneously.|
| Pierce Universal Nuclease        | 1           |                                           |

#### Cisplatin

| Component                        | Volume (μL) | Notes                                      |
|----------------------------------|-------------|--------------------------------------------|
| RPMI 1640 0% FCS                 | 5,000       | This solution is prepared extemporaneously.|
| Cisplatin 5 mM                   | 1           |                                           |

#### Formaldehyde 2%

| Component                        | Volume (μL) | Notes                                      |
|----------------------------------|-------------|--------------------------------------------|
| Formaldehyde 16%                 | 125         |                                           |
| Maxpar PBS                       | 875         |                                           |

This solution is prepared extemporaneously.
CRITICAL: Pierce Universal Nuclease (final concentration=2.5 U/mL) and Cisplatin (final concentration=1 μM) must be prepared extemporaneously.

CRITICAL: In order to avoid contamination with elements likely to damage the detector of the mass cytometer, glass, material that could have been exposed to soap, and autoclaved material cannot be used. The use of polystyrene tubes or plates and unfiltered tips is prohibited as well.

### STEP-BY-STEP METHOD DETAILS

**Timing: 5 h**

This section describes a workflow for staining samples with 28-metal isotope panel in key resources table. Unless otherwise stated, cell pellets are suspended by pipetting.

### PBMC sample thawing

**Timing: 2 h**

This step details how to thaw cryopreserved PBMCs before staining.

1. Prepare 15 mL conical tubes with RPMI 10% Fetal Calf Serum (FCS).
2. Thaw each sample vial in a 37°C water bath until a small pellet of ice appears.
3. Transfer the contents of PBMC vial to a 15 mL conical tube and rinse the vial.
4. Centrifuge the cells at 400 × g for 5 min at 20°C–22°C.
5. Discard flow-through and resuspend the pellet in 10 mL of RPMI 2% FCS.
6. Add 1 μL of Pierce Universal Nuclease 25 kU and incubate at 37°C for 30 min.
7. Filter the cells with a 30 μm filter and proceed to cell count.
   a. Mix 10 μL of cell suspension with 10 μL of trypan blue.
   b. Load this mix onto KOVA slide and count cells.
8. Centrifuge all samples: 400 × g, 5 min, 20°C–22°C.
9. Resuspend the pellet in CSM, and distribute 3.10^6 cells/well up to 250 μL in 96-well polypropylene plates.

### Viability stain

**Timing: 10 min**

This step details how to label dead cells.
10. Centrifuge the plate: 1,017 \times g, 3 min, 20^\circ C–22^\circ C.

11. Prepare cisplatin working solution.

12. Add 100 \mu L / well of cisplatin diluted at final a concentration of 1 \mu M and incubate samples for 5 min at 37^\circ C.

Note: Viability stain can be performed using the same procedure replacing cisplatin by 103Rh. Use 103Rh at a dilution of 1:500 in RPMI and incubate 15 min at 20^\circ C–22^\circ C.

13. Add 100 \mu L of RPMI to stop the reaction and centrifuge the plate 1,017 \times g, 3 min, 20^\circ C–22^\circ C.

14. Aspirate the flow-through and wash the cells with 200 \mu L of CSM, centrifuge 1,017 \times g, 3 min, 20^\circ C–22^\circ C.

15. Prepare FcR blocking reagent by diluting Fc Block 1:10 with CSM.

Note: FcR blocking can be replaced by CSM supplemented with 10% FCS (Figure 4).

16. Add 100 \mu L of diluted FcR blocking reagent to each well, mix by pipetting and incubate for 10 min at 4^\circ C.

17. Add 100 \mu L of CSM and centrifuge 1,017 \times g, 3 min, 4^\circ C.

18. Add 25 \mu L of extracellular master mix per sample and incubate for 1 h at 4^\circ C.

Note: Extracellular mix is stable at –80^\circ C for one year (data not shown).

19. Add 175 \mu L of CSM and centrifuge 1,017 \times g, 3 min, 4^\circ C.

20. Aspirate the flow-through and wash the cells with 200 \mu L of CSM, centrifuge 1,017 \times g, 3 min, 4^\circ C.

Note: A second incubation for secondary antibodies can be added before 2% formaldehyde fixation step.

21. Aspirate the flow-through and add 100 \mu L of formaldehyde 2% and incubate for 15 min at 20^\circ C–22^\circ C.

22. Add 100 \mu L of CSM and centrifuge 1,017 \times g, 3 min, 4^\circ C.

Note: This procedure can be stopped at this stage and cells stored in CSM for 72 h at 4^\circ C. After 72 h, the intracellular labeling can be processed (Figure 3).

10. Centrifuge the plate: 1,017 \times g, 3 min, 20^\circ C–22^\circ C.

11. Prepare cisplatin working solution.

12. Add 100 \mu L / well of cisplatin diluted at final a concentration of 1 \mu M and incubate samples for 5 min at 37^\circ C.

Note: Viability stain can be performed using the same procedure replacing cisplatin by 103Rh. Use 103Rh at a dilution of 1:500 in RPMI and incubate 15 min at 20^\circ C–22^\circ C.

13. Add 100 \mu L of RPMI to stop the reaction and centrifuge the plate 1,017 \times g, 3 min, 20^\circ C–22^\circ C.

14. Aspirate the flow-through and wash the cells with 200 \mu L of CSM, centrifuge 1,017 \times g, 3 min, 20^\circ C–22^\circ C.

Blocking Fc receptors and extracellular staining

© Timing: 1 h 15 min

This step details how to perform FcR blocking, extracellular staining and cell fixation.

15. Prepare FcR blocking reagent by diluting Fc Block 1:10 with CSM.

Note: FcR blocking can be replaced by CSM supplemented with 10% FCS (Figure 4).

16. Add 100 \mu L of diluted FcR blocking reagent to each well, mix by pipetting and incubate for 10 min at 4^\circ C.

17. Add 100 \mu L of CSM and centrifuge 1,017 \times g, 3 min, 4^\circ C.

18. Add 25 \mu L of extracellular master mix per sample and incubate for 1 h at 4^\circ C.

Note: Extracellular mix is stable at –80^\circ C for one year (data not shown).

19. Add 175 \mu L of CSM and centrifuge 1,017 \times g, 3 min, 4^\circ C.

20. Aspirate the flow-through and wash the cells with 200 \mu L of CSM, centrifuge 1,017 \times g, 3 min, 4^\circ C.

Note: A second incubation for secondary antibodies can be added before 2% formaldehyde fixation step.

21. Aspirate the flow-through and add 100 \mu L of formaldehyde 2% and incubate for 15 min at 20^\circ C–22^\circ C.

22. Add 100 \mu L of CSM and centrifuge 1,017 \times g, 3 min, 4^\circ C.

Note: This procedure can be stopped at this stage and cells stored in CSM for 72 h at 4^\circ C. After 72 h, the intracellular labeling can be processed (Figure 3).

Intracellular staining

© Timing: 1 h

This step details how to perform the permeabilization in order to stain the intracellular and intranuclear markers.
23. Permeabilize using 200 μL per well of Foxp3 Staining Buffer Set by diluting Fixation/Permeabilization concentrate in Perm Diluent (1/4 dilution).

24. Add 25 μL of intracellular master mix per sample and incubate for 30 min at 4°C.

25. Add 175 μL of Perm/Wash working solution and centrifuge 1,017 × g, 3 min, 4°C.

**Figure 2. Gating strategy**

PBMCs from healthy volunteers were stained with metal-labeled antibodies and analyzed by mass cytometry (CyTOF Helios®). After removal of beads, cells were pre-gated as DNA−. Dead cells were excluded based on CisPt or Rh positivity. Monocytes were excluded based on CD33 expression. B cells and T cells were excluded based on CD19 and CD3 expression, respectively. Four subsets of NK cells were defined based on CD56 and CD16 expression: CD56bright NK cells, CD56dimCD16+ NK cells, CD56dimCD16− NK cells, and unconventional CD56−CD16− NK cells.
26. Aspirate the flow-through and wash the cells with 200 μL of 1× Perm/Wash, centrifuge 1,017 g, 3 min, 4°C.

Fixation and DNA intercalator staining

∡ Timing: 10 min

Finally, samples are fixed with DNA intercalator to identify cells after acquisition.

27. Prepare extemporaneously 2% formaldehyde solution by diluting fresh 16% formaldehyde with PBS.
28. Add 100 μL of 2% PFA + DNA intercalator (Cell-ID™ Intercalator-IR) diluted at 1:1,000 per sample and incubate 12–16 h.

Note: At this stage, labeled samples can be frozen or directly acquired on the Helios system.

Sample freezing

∡ Timing: 30 min
Here, we propose a freezing strategy that enables labeling stability for a maximum storage period of one week.

29. Wash the cells with 100 μL of CSM and centrifuge 1,017 × g, 3 min, 4°C.
30. Aspirate the flow-through and wash the cells with 200 μL of CSM and centrifuge 1,017 × g, 3 min, 4°C.
31. Add 100 μL of freezing media per sample and store directly in microtubes at −80°C until the acquisition.

*Note:* Labeled samples can be stored at −80°C freezer for one week without losing staining quality.

**Instrument tuning and sample acquisition**

© Timing: 45 min for 1 M cells

The startup procedure follows the instrument manufacturer’s guidelines involving checking the instrument performance, monitoring background and tuning the system.

32. Thaw samples at 20°C–22°C, transfer the cells into a 96-well plate, and add 100 μL of PBS.
33. Centrifuge samples for 1,017 × g, 3 min, 4°C.
34. Aspirate flow-through by pipette.
35. Repeat steps 32–34.
36. Add 200 μL of deionized water (DIW) and proceed to cell count.

**Note:** Wash the samples in DIW only after instrument start-up and tuning validation because samples should not remain in DIW for more than 1 h. Thus, samples must be thawed gradually.

37. Centrifuge samples for 1,017 x g, 3 min, 4°C and aspirate the flow-through.
38. Prepare the stock solution of 1:10 EQ Bead/DIW.

**Note:** Depending on the sample (cell line, tumor...) a higher dilution in EQ Beads/DIW is necessary to avoid fluidic line clogging.

39. Aspirate flow-through by pipette and resuspend one sample with EQ Bead/DIW by adjusting concentration to achieve an acquisition rate of 500 events/second i.e., 1 M/mL.
40. Normalized and concatenated the files before analysis.
41. Several algorithms can be used for data analysis.

**Note:** For analysis of immune populations that represent at least 1% of the total PBMC, a minimum of 50,000 cells should be acquired. However, when possible, we recommend the acquisition of a minimum of 500,000 cells in order to enable the analysis of rare immune subsets, or subsets that are under-represented in specific pathologic conditions such as hematologic malignancies, in which circulating tumor cells may represent up to 99% of the total PBMC.

**EXPECTED OUTCOMES**

In this section, we describe a gating strategy to identify NK cells in peripheral blood from healthy donor (Figure 2). Raw data (.FCS) were obtained after staining and acquisition then normalized using Fluidigm Software (.fcs). These data were analyzed using FlowJo Software to remove beads, clog and dead cells. On CD45+ live cells we exclude CD33+ and CD19+ cells. Based on CD3, CD56 and CD16 expression, we identified subpopulations of NK cells.

Unsupervised clustering using h-SNE also enables to visualize the main immune populations obtained from our staining protocol: myeloid cells, NK cells, B cells, T cells, \( \gamma \delta \) T cells (Figure 5). We focused on NK cells to refine their phenotype. Manually pre-gated total NK cells were exported; selected markers CD56, NKG2A, CD158b1b2j, CD158ah, CD57, NKG2C, CD16 were used to identify seven clusters of NK cells. These clusters illustrate the step of NK cell maturation from CD56bright to memory-like cells, as well as CD56- CD16+ unconventional NK cells (Figure 6).

**LIMITATIONS**

We propose a validated staining procedure with 28 markers and 5 channels used for viability and DNA identification, so we can use free channel to add more markers. Be careful, depending on cells and markers, we can lose the specific staining comparing to single labeling. It can be a steric housing problem.

This problem can be resolved using successive incubations. Antibodies targeting weakly represented antigens on the cell surface can be incubated before antibodies targeting strongly represented antigens on the cell surface. This allows overcoming steric housing problem.

**TROUBLESHOOTING**

**Problem 1**
Batch effect.
Several factors can be at the origin of batch effect such as inter-operator variability, instrument variability as well as duration of acquisition.

**Potential solution**
Several algorithms are available to correct the batch effect (Lo et al., 2022; Pedersen et al., 2022; Van Gassen et al., 2020). To do this, the use of validated and appropriate internal control is required. Important: this control must express all the markers included in the panel.

**Problem 2**
Antibodies conjugation to metal isotopes failed.

**Potential solution**
To ensure optimal metal conjugation, it is recommended to use purified antibodies that respect the following rules:

- Antibody type: monoclonal.
- Antibody class: IgG.
- Buffer: BSA- and glycerol-free.

Alternatively, it is possible to use flow cytometry antibodies labeled with a fluorochrome and to use an antibody targeting the fluorochrome labeled with a metal.

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**Figure 5. High dimensional analysis of PBMCs**
Consensus files of PBMCs from 22 healthy volunteers were generated with fixed number of cells and exported in Cytoslore for h-SNE analysis. h-SNE enables identification of myeloid cells (CD33⁺), B cells (CD33⁺ CD19⁺), NK cells (CD33⁺ CD3⁻ CD56⁺), CD8⁺ T cells (CD33⁺ CD3⁻ CD8⁺), CD4⁺ T cells (CD33⁺ CD3⁺ CD4⁺), Vβ2⁺ γδ T cells (CD33⁺ CD3⁺ Vβ2⁺), and Vβ2⁺ γδ T cells (CD33⁺ CD3⁻ Vβ2⁻). The following settings were used for hSNE analyses: number of scales: 3; #RW for Monte Carlo: 15; Random walks threshold: 1.50; Random walks length: 15; Filter percentile: 0.50.
RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Anne-Sophie Chretien (anne-sophie.chretien@inserm.fr).

Materials availability
This study did not generate new unique reagents.

Data and code availability
There is no dataset/code associated with the present article.

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Figure 6. High dimensional analysis of peripheral NK cells
Total NK cells from peripheral blood were manually pre-gated. Consensus files were generated from 22 healthy volunteers with fixed number of NK cells and exported in Cytosplore for h-SNE analysis. h-SNE enables visualization of 7 clusters of NK cells based on CD56, NKG2A, CD158a,h,CD158b1,b2,j, CD57, NKG2C and CD16 expression. The following settings were used for hSNE analyses: number of scales: 3; #RW for Monte Carlo: 15; Random walks threshold: 1.50; Random walks length: 15; Filter percentile: 0.50.
(FRM) DEQ 20180339209” (for D.O.). S.F. is supported by a doctoral fellowship from Aix-Marseille University. P.-L.B. is supported by a doctoral fellowship from the Ligue Nationale contre le Cancer, and L.G. was supported by a Fondation ARC advanced post-doctoral fellow grant “Aides individuelles” and a grant extension “Aides exceptionnelles liées à la pandémie COVID-19” (2018–2021). D.O. is Senior Scholar of the Institut Universitaire de France. The CRCM (Inserm U1068) and the Paoli-Calmettes Institute are members of OPALE Carnot Institute, The Organization for Partnerships in Leukemia, Institut de Recherche Saint-Louis, Hôpital Saint-Louis, 75010 Paris, France. Figures have been created with BioRender.com.

**AUTHOR CONTRIBUTIONS**

Conceptualization: J.A.N., N.V., H.L., E.G., D.O., and A.S.C.; methodology: H.L. and E.G.; validation: H.L., E.G., D.O., and A.S.C.; formal analysis: A.B.A., M.S.R., J.W., and A.S.C.; investigation: A.B.A., M.S.R., S.F., E.G., and N.B.; resources: H.L. and N.V.; data curation: J.W. and A.S.C.; writing – original draft: A.B.A., M.S.R., and L.G.; writing – review & editing: L.G. and A.S.C.; visualization: A.B.A., M.S.R., P.L.B., L.G., and A.S.C.; supervision: H.L., D.O., and A.S.C.; project administration: H.L., D.O., and A.S.C.; funding acquisition: J.A.N., N.V., D.O., and A.S.C.

**DECLARATION OF INTERESTS**

E.G. is currently employed by Fluidigm. D.O. is a cofounder and shareholder of Imcheck Therapeutics, Alderaan Biotechnology, Emergence Therapeutics, and Stealth IO.

**REFERENCES**

Chretien, A.-S., Devillier, R., Granjeaud, S., Cordier, C., Demerle, C., Salem, N., Wlosok, J., Orlanducci, F., Gorvel, L., Fattori, S., et al. (2021). High-dimensional mass cytometry analysis of NK cell alterations in AML identifies a subgroup with adverse clinical outcome. Proc. Natl. Acad. Sci. USA 118 e2020459118. https://doi.org/10.1073/pnas.2020459118.

Lo, Y.-C., Keyes, T.J., Jager, A., Sarno, J., Domizi, P., Majeti, R., Sakamoto, K.M., Lacayo, N., Mullighan, C.G., Waters, J., et al. (2022). Cytofin enables integrated analysis of public mass cytometry datasets using generalized anchors. Nat. Commun. 13, 934. https://doi.org/10.1038/s41467-022-28484-5.

Pedersen, C.B., Dam, S.H., Barnkob, M.B., Leipold, M.D., Purroy, N., Rassenti, L.Z., Kipps, T.J., Nguyen, J., Lederer, J.A., Gohil, S.H., et al. (2022). cyCombine allows for robust integration of single-cell cytometry datasets within and across technologies. Nat. Commun. 13, 1698. https://doi.org/10.1038/s41467-022-29383-5.

Van Gassen, S., Gaudilliere, B., Angst, M.S., Saeys, Y., and Aghaeepour, N. (2020). CytoNorm: a normalization algorithm for cytometry data. Cytometry A 97, 268–278. https://doi.org/10.1002/cyto.a.23904.