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Characterizing Phenotypes and Signaling Networks of Single Human Cells by Mass Cytometry

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Abstract

Single cell mass cytometry is revolutionizing our ability to quantitatively characterize cellular biomarkers and signaling networks. Mass cytometry experiments routinely measure 25–35 features of each cell in primary human tissue samples. The relative ease with which a novice user can generate a large amount of high quality data and the novelty of the approach have created a need for example protocols, analysis strategies, and datasets. In this chapter, we present detailed protocols for two mass cytometry experiments designed as training tools. The first protocol describes detection of 26 features on the surface of human peripheral blood mononuclear cells. In the second protocol, a mass cytometry signaling network profile measures 25 node states comprised of five key signaling effectors (AKT, ERK1/2, STAT1, STAT5, and p38) quantified under five conditions (Basal, FLT3L, SCF, IL-3, and IFN γ). This chapter compares manual and unsupervised data analysis approaches, including bivariate plots, heatmaps, histogram overlays, SPADE, and viSNE. Data files in this chapter have been shared online using Cytobank (<http://www.cytobank.org/irishlab/>).

Keywords

Single cell biology; Mass cytometry (CyTOF); Human; Immunophenotyping; Signaling network profile; Phospho-specific flow cytometry (phospho-flow)

1 Introduction

Computational tools and instrumentation advances have introduced a new era of single cell systems biology research where it is straightforward to comprehensively characterize all cell types, known and unknown, in primary tissues [1]. Traditional aggregate analysis techniques, such as Western blotting, characterize cellular features with the assumption that the total signal reflects the sum of a homogeneous underlying population of cells. However, even among clonally derived cell lines, biologically meaningful cell-to-cell differences in protein expression and phosphorylation are the rule, not the exception [2]. The presence of multiple cell subpopulations with distinct expression signatures is common in primary samples taken directly from the body, such as healthy tissue and tumor specimens [3, 4]. In

⁹Stimuli should be added as soon as the cells are taken out of the incubator to avoid cooling the cells below 37 °C.

¹⁰In general, 15-min stimulations work well for many signaling responses, including the cytokine stimulations used here. However, if this protocol should be adapted for other stimulations, it is crucial to find optimal timepoints and concentrations for each signaling response.

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the case of blood cancers, cancer cell subsets defined by abnormal signaling are associated with patient clinical outcomes, including overall survival [5, 6]. Single cell tools such as flow cytometry enable high content single cell measurements of cellular identity and functional response [7, 8]. Overlap in the emission spectra of conventional fluorescent probes can create experiment design and data analysis challenges that are particularly a drawback in quantitative single cell comparisons [9]. Moreover, autofluorescence of some tissue types can overlap with fluorophore emission spectra and complicate quantitative analysis.

Mass cytometry employs metal isotope reporters (mass tags) that are not normally found in biological specimens [10]. Typically, mass tags are coupled to an antibody or other target-specific probe so that their abundance in a cell corresponds to the abundance of a target of interest. Intercalator reagents containing iridium or rhodium are routinely used to mark single cells [11]. In addition to making cells detectable as an event to the instrument, comparison of event length and uptake of a cell marking intercalator helps distinguish single cells, cell doublets, and other particles (Fig. 1). Intercalator reagents aim to provide a consistent, strong signal that is minimally variable with experimental conditions. Regular marking of events with an invariant signal helps to identify cell events when parsing mass cytometry data into flow cytometry standard (FCS) format, gating single cells (Fig. 1), and analyzing cells with computational tools (Figs. 2 and 3).

While mass spectrometry avoids fluorescence associated problems, there are aspects of the technology that can be valuable to monitor and test. Mass cytometry issues include (1) impure isotopic mass tags, (2) spillover between closely spaced spectral channels when signal is very abundant (+1 and -1 spillover), (3) variable oxide formation (primarily +16 spillover), and (4) other less common confounding signals not originating from the cells (e.g., barium in buffers, gadolinium contrast agent from patient magnetic resonance imaging). This chapter will not specifically address these aspects of the technique except to say that they can be minimized by following best practices for instrument use, reagent quality control, and experiment design [2, 9].

A key advantage of mass cytometry is the multiplexed detection of many features of each cell. Typical experiments measure approximately 35 features of every cell [10, 12–14], with 42 being state of the art [12]. The theoretical limit on the instrument has not been approached and is likely between 100 and 200 features per cell using the current technology. Mass cytometry therefore, allows single-cell deep profiling of cell identity, phenotype, response, and functional outcome. Relative to microscopy, mass cytometry is high content and high throughput at the single cell level: a typical experiment quantifies 35 features on each of 100,000 cells from a sample in ~15–20 min. Mass cytometry has numerous applications for characterizing the cellular heterogeneity of healthy and diseased tissues and for tracking changes in populations over time in primary tissue samples [2].

Here we present protocols for two mass cytometry experiments: (1) quantifying cell surface biomarkers expressed on healthy human peripheral blood mononuclear cells (PBMCs) and (2) quantifying intracellular signaling network responses in Kasumi-1 cells using phospho-flow [15, 16]. Data from experiments provided in this chapter are available online (<http://>

www.cytobank.org/irishlab). In addition, computational tools are an integral part of analyzing multidimensional datasets. In this chapter we provide examples of multidimensional data visualization. As data analysis can be daunting in 25-dimensional datasets, this chapter compares analysis of the human PBMC cell surface immunophenotyping dataset by three methods: (1) traditional bivariate gating, heatmaps, and histogram overlays [17], (2) Spanning-Tree Progression Analysis of Density-Normalized Events (SPADE [18]), and (3) visualization of t-Stochastic Neighbor Embedding (tSNE [19]).

2 Materials

1. Ficoll-Paque solution.
2. 15 mL and 50 mL conical tubes.
3. Cell culture medium: RPMI 1640 containing 10 % fetal bovine serum (FBS), 100 U/mL penicillin, and 100 µg/mL streptomycin, stored at 4 °C. Heat by immersing in 37 °C water bath for 15–20 min.
4. Freezing medium: 12 % DMSO and 88 % FBS, keep cold on ice.
5. Cryopreservation tubes, 1.8 mL.
6. 12 × 75 mm round-bottom polystyrene cytometry tubes.
7. Water bath set at 37 °C.
8. Cell culture incubator set at 37 °C with 5 % CO₂.
9. Absolute methanol stored at –20 °C or lower.
10. 1× phosphate buffered saline (PBS).
11. Staining medium: 1 % bovine serum albumin (BSA) in phosphate buffered saline (PBS).
12. Deionized water.
13. Intercalator: 500 µM iridium. Prepare a 50× working solution (12.5 µM) by diluting with PBS.
14. Cytometry tubes with 35-µm cell strainer caps.
15. Trypan blue, prepared as recommended by manufacturer.
16. Hemocytometer.
17. 16 % paraformaldehyde (PFA) aqueous solution.
18. Antibodies: refer to Tables 1 and 2.
19. Stimuli: refer to Table 3.
20. 15–30 mL of human peripheral blood.
21. Kasumi-1 cells (ATCC; CRL-2724).

22. Mass cytometer (CyTOF) (Fluidigm).
23. Data in this manuscript were analyzed using Cytobank (<http://www.cytobank.org>).

3 Methods

3.1 Immunophenotyping of Surface Markers of Cell Identity in Human PBMCs

Human PBMCs are heterogeneous populations of cells that can be defined and categorized into distinct cell types by different surface marker expression levels. CyTOF allows simultaneous measurement of all the key surface markers of cell identity to enable deep profiling of cell subsets in human PBMCs. The protocol outlined below describes the method for live-cell surface staining of human PBMCs to characterize expression of 25 different cell surface markers.

3.1.1 Preparation and Freezing of PBMCs

1. Collect 15–30 mL of human peripheral blood from each donor in sterile tubes with heparin or EDTA anticoagulant.
2. Transfer the blood to a 50 mL conical tube and add an equal amount of 1× PBS into the same tube and pipet to mix.
3. Add Ficoll-Paque solution into two new 50 mL conical tubes at 12.5 mL per tube (*see Note 1*).
4. Slowly overlay 20 mL of diluted blood sample onto the Ficoll-Paque solution in each tube (*see Note 2*).
5. Slowly add PBS into each tube to increase the volume in each tube to 50 mL.
6. Centrifuge at $400 \times g$ for 30 min at room temperature. Set the deceleration on the centrifuge to the lowest speed to avoid mixing the PBMCs with the rest of the solution.
7. Check for a white ring of PBMCs (buffy coat) right above the red blood cell pellet. Gently aspirate and discard the majority of the plasma above the ring. Avoid disturbing the buffy coat.
8. Gently pipette the buffy coat using a wide bore pipet tip and transfer the buffy coat into a new 15 mL conical tube. Combine buffy coat from all the tubes of the same blood donor.
9. Add PBS to the 15 mL conical tube to increase the volume in each tube to 15 mL.
10. Centrifuge at $300 \times g$ for 10 min at room temperature. Set the deceleration on the centrifuge to high brake speed.
11. Aspirate to discard the supernatant.

¹Avoid spilling Ficoll-Paque solution on the side of the 50 mL conical tubes.

²Gently overlay blood and PBS mixture on top of the Ficoll-Paque solution. This can be done by adjusting the release speed of the serological pipet aid to minimum. Do not mix the Ficoll-Paque solution and the diluted blood sample since this would minimize PBMCs isolation.

12. Resuspend cell pellet in 2 mL of PBS. Count cells with trypan blue (*see* Note 3).
13. Add 10 mL of PBS to each tube and centrifuge at $300 \times g$ for 10 min at room temperature again. Repeat the wash as needed until the supernatant is relatively clear.
14. Aspirate to discard the supernatant and avoid disturbing the cell pellet.
15. Resuspend the cells in freezing medium to yield a concentration of $10\text{--}15 \times 10^6$ cells/mL.
16. Aliquot cell suspension into cryopreservation tubes at 1 mL per tube.
17. Freeze the cells slowly at the rate of $-1^\circ\text{C}/\text{min}$ in -80°C freezer (*see* Note 4). Transfer the cryopreservation tubes into liquid nitrogen the next day for long-term storage.

3.1.2 Live-Cell Surface Staining of PBMCs

1. Remove a cryopreservation tube with PBMCs from liquid nitrogen. Immediately immerse the cryopreservation tube in 37°C water bath. Remove the cryopreservation tube from the water bath once the cell suspension is completely thawed. This step should not take longer than 1–2 min.
2. Add 10 mL of warm cell culture medium into a 15 mL conical tube. Transfer thawed PBMCs from the cryopreservation tube into the same 15 mL conical tube. Pipet to mix cells with the cell culture medium.
3. Pellet the PBMCs at $300 \times g$ for 5 min at room temperature. Aspirate to discard the supernatant.
4. Add 5 mL of warm cell culture medium and pipet to mix the PBMCs with the medium. Count the cells using trypan blue (*see* Note 3) and adjust the volume to yield a concentration of $1\text{--}2 \times 10^6$ live cells/mL. Pipet the cells to get a single cell suspension.
5. Label two cytometry tubes as “Intercalator only” and “Surface panel.”
6. Aliquot the PBMC suspension into cytometry tubes at 1 mL per tube. Put the tubes with cell suspension into a 37°C incubator with 5 % CO_2 . Rest cells for 15 min before staining.
7. Take the cytometry tubes from the incubator. Pellet the cells at $300 \times g$ for 5 min at room temperature.
8. Check for cell pellets and discard supernatant by decanting (*see* Note 5).

³To count live cells, mix 10 μL of trypan blue working solution with 10 μL cell suspension and count cells under a microscope using a hemocytometer.

⁴To maximally preserve cell viability, cells should be frozen down at a slow rate close to $-1^\circ\text{C}/\text{min}$. This can generally be achieved by placing cryopreservation vials in plastic containers that are immersed in isopropanol prior to transfer to -80°C . Commercially available products can also be used. Transfer viably cryopreserved cells from -80°C to liquid nitrogen within 48 h.

⁵To avoid losing the cell pellet, decant only once and try to discard as much supernatant as possible. Avoid re-decanting after inverting the cytometry tubes to an upright position since this can loosen the cell pellet or detach it from the bottom of the tube.

9. For the tube labeled “Intercalator only,” vigorously vortex the cell pellets in void volume and add 1 mL of ice-cold methanol (stored at -20°C or lower) to permeabilize the cells. Pipet or briefly vortex to mix (*see* Note 6) and store the tube at -20°C for at least 10 min (*see* Note 7).
10. For the tube labeled “Surface panel,” resuspend the cell pellets with 75 μL of staining medium (*see* Note 8).
11. Transfer 75 μL of cell suspension from the tube labeled “Surface panel” to a new cytometry tube. Add antibodies from Surface Marker Panel (Table 1) to the tube to a final volume of 100 μL . Pipet or briefly vortex the cells with the antibodies to mix. Incubate the cells with the antibodies at room temperature for 30 min.
12. Add 1 mL of $1\times$ PBS and pipet or briefly vortex to mix. Pellet the cells at $300\times g$ for 5 min at room temperature.
13. Check for cell pellets and discard supernatant by decanting (*see* Note 5). Repeat steps 12 and 13 once.
14. Vigorously vortex the cell pellet in void volume and add 1 mL of ice-cold methanol (stored at -20°C or lower) to permeabilize the cells, pipet or briefly vortex to mix (*see* Note 6) and store the tube at -20°C with the tube labeled “Intercalator only” for at least 10 min.

3.1.3 Cell Rehydration and DNA Intercalation

1. Take the tubes from -20°C .
2. Add 1 mL of staining media to each tube, pipet or briefly vortex to mix. Pellet the cells at $800\times g$ for 5 min at room temperature.
3. Check for cell pellets and discard supernatant by decanting (*see* Note 5). Repeat steps 2 and 3 once.
4. Add 200 μL of $1\times$ PBS to each cytometry tube, pipet or briefly vortex to mix.
5. Add 4 μL of $50\times$ Intercalator to each tube. Pipet or briefly vortex to mix the cells. Incubate cells with intercalator for 30 min at room temperature.
6. Add 1 mL of $1\times$ PBS to each tube, pipet or briefly vortex to mix. Pellet the cells at $800\times g$ for 5 min at room temperature.
7. Check for cell pellets and discard supernatant by decanting (*see* Note 5). Repeat steps 6 and 7 once.
8. Add 1 mL of deionized water to each tube, pipet or briefly vortex to mix.

⁶Cell pellets may clump when methanol is added if they are not resuspended well. To prevent cell clumping, vigorously vortex the pellets in void volume prior to adding ice-cold methanol.

⁷Following permeabilization ice cold methanol for 10 min, samples can be maintained long term at -20°C or -80°C for months without additional apparent changes to target epitopes.

⁸The goal of this step is to resuspend each cell pellet to yield the *total* volume indicated in the protocol and to avoid losing cells in the following steps. Therefore, less staining media than what is indicated in the protocol might be needed depending on the void volume in the tube.

9. Strain the cells through cytometry tubes with 35- μ m cell strainer cap. Cells are now ready for CyTOF.

3.2 Phospho-flow Signaling in Kasumi-1 Human AML Cell Line

A phospho-flow signaling experiment can characterize the cellular heterogeneity of intracellular signaling responses to various stimuli among cells within a given cell population. Here, we are using Kasumi-1, a human AML cell line, to demonstrate the concept. This experiment includes addition of various stimuli (Fms-related tyrosine kinase 3 ligand (FLT3L), stem cell factor (SCF), interleukin-3 (IL-3), and interferon- γ (IFN γ)) to the cells to stimulate signaling, followed by fixation and immunostaining with antibodies specific for phosphorylated proteins (Fig. 4).

3.2.1 Cell Culture

1. Culture Kasumi-1 cells according to the manufacturer's recommendation.
2. The day before the experiment, passage cells and resuspend the cells at $0.5\text{--}1.0 \times 10^6$ cells/mL in the recommended cell culture media. Keep the cells in a 37 °C incubator with 5 % CO₂.

3.2.2 Stimulation of Cells

1. Label 6 cytometry tubes as "Intercalator only," "Unstimulated," "FLT3L," "SCF," "IL-3," and "IFN γ ."
2. Take Kasumi-1 cells from the incubator and aliquot 1 mL of cell suspension into each cytometry tube.
3. Prepare the stimuli while the cells are resting in the incubator. Make a 50 $\times$ solutions of each stimulus by diluting them in PBS (Table 3).
4. Add 20 μ L of the corresponding 50 $\times$ stimuli to each cytometry tubes. For tubes labeled "Intercalator only" and "Unstimulated," do not add any stimulus. Place all the tubes back in the incubator and incubate for 15 min (*see* Notes 9 and 10).

3.2.3 Fixation and Permeabilization of Cells

1. At the end of the stimulation timepoint, add 100 μ L of 16 % PFA to each cytometry tube and vortex the tube to mix (*see* Note 11). Allow cells to fix for at least 5 min at room temperature.
2. Wash the cells by adding 1 mL of PBS to each tube and pipet or vortex to mix. Pellet the cell suspension at $800 \times g$ for 5 min at room temperature. Check for cell pellets and discard supernatant by decanting (*see* Note 5).
3. Vigorously vortex the cell pellets in void volume to resuspend the cells.

¹¹Adding 100 μ L of 16 % PFA to each tube would yield a final concentration of approximately 1.6 % PFA. Cells must be in single-cell suspension prior to adding PFA to avoid fixing cells in clumps.

4. Add 1 mL of ice-cold methanol to each tube (stored at -20°C or lower) to permeabilize the cells. Pipet or briefly vortex to mix (*see* Note 6) and store the tube at -20°C for at least 10 min.

3.2.4 Intracellular Phospho-protein Staining and DNA Intercalation of Cells

1. Take the tubes from -20°C .
2. Add 1 mL of staining media to each tube, pipet or briefly vortex to mix. Pellet the cells at $800 \times g$ for 5 min at room temperature.
3. Check for cell pellets and discard supernatant by decanting (*see* Note 5). Repeat steps 2 and 3 once.
4. Add 200 μL of $1\times$ PBS to the tube labeled “Intercalator only.” Pipet or briefly vortex to mix and set the tube aside.
5. For the remaining tubes, resuspend the cell pellets with 95 μL of staining media per tube (*see* Note 8).
6. Transfer 95 μL of cell suspension to new cytometry tubes. Add intracellular antibodies from the signaling panel (Table 2) to each tube. Pipet or briefly vortex the cells with the antibodies to mix. Incubate the cells with the antibodies at room temperature for 30 min.
7. Add 1 mL of $1\times$ PBS to each tube and pipet or briefly vortex to mix. Pellet the cells at $800 \times g$ for 5 min at room temperature.
8. Check for cell pellets and discard supernatant by decanting (*see* Note 5). Repeat steps 7 and 8 once.
9. Add 200 μL of $1\times$ PBS to all the tubes (except the tube labeled “Intercalator only”) and pipet or briefly vortex to mix.
10. Add 4 μL of $50\times$ intercalator to all the tubes including the tube labeled “Intercalator only.” Pipet or briefly vortex to mix. Incubate the cells with intercalator for 30 min at room temperature.
11. Add 1 mL of $1\times$ PBS to each tube, pipet or briefly vortex to mix. Pellet the cells at $800 \times g$ for 5 min at room temperature.
12. Check for cell pellets and discard supernatant by decanting (*see* Note 5). Repeat steps 11 and 12 once.
13. Add 1 mL of deionized water to each tube, pipet or briefly vortex to mix.
14. Strain the cells from each tube through a cytometry tube with 35- μm cell strainer cap. Cells are now ready for CyTOF.

3.3 High-Dimensional Data Analysis

CyTOF simultaneously measures 30+ features per cell, providing a global view of biological events in individual cells. However, such high dimensional data is difficult to analyze effectively with conventional bivariate data visualization and serial manual gating alone.

Computational tools help users visualize relationships in high dimensional space and separate rare cells into populations based on distinct, multi-feature phenotypes. As data analysis can be daunting in 25-dimensional datasets, this chapter compares analysis of the human PBMC cell surface immunophenotyping dataset by three methods: (1) traditional bivariate gating (*see* Note 12), heatmaps, and histogram overlays (using Cytobank [17], Fig. 1, *see* Note 13) Spanning-Tree Progression Analysis of Density-Normalized Events (SPADE [18], Fig. 2, *see* Note 14) visualization of t-Stochastic Neighbor Embedding (viSNE [19], Fig. 3, *see* Note 15).

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References

1. Irish JM. Beyond the age of cellular discovery. *Nat Immunol.* 2014; 15:1095–1097. [PubMed: 25396342]
2. Irish JM, Dowie DB. High-dimensional single-cell cancer biology. *Curr Top Microbiol Immunol.* 2014; 377:1–21. [PubMed: 24671264]
3. Marusyk A, Almendro V, Polyak K. Intra-tumour heterogeneity: a looking glass for cancer? *Nat Rev Cancer.* 2012; 12(5):323–334. [PubMed: 22513401]
4. Kleppe M, Levine RL. Tumor heterogeneity confounds and illuminates: assessing the implications. *Nat Med.* 2014; 20(4):342–344. [PubMed: 24710377]
5. Irish JM, et al. Single cell profiling of potentiated phospho-protein networks in cancer cells. *Cell.* 2004; 118(2):217–228. [PubMed: 15260991]
6. Irish JM, et al. B-cell signaling networks reveal a negative prognostic human lymphoma cell subset that emerges during tumor progression. *Proc Natl Acad Sci U S A.* 2010; 107(29):12747–12754. [PubMed: 20543139]

¹²Bivariate analysis: employs pairwise comparisons of markers to select, or gate, cell populations. Identification of cell subsets has traditionally relied on sequential bivariate gating. Here, we use the PBMC surface immunophenotyping data to demonstrate the concept (Fig. 1) before using the high dimensional population identification tools SPADE and viSNE. In this example, established cell surface markers were used for sequential manual gating of well-characterized human blood mono-nuclear cell populations, including B cells, T cells, monocytes, dendritic cells, and natural killer (NK) cells.

¹³Heatmaps and histogram overlays: are ways to visualize a statistic, such as median expression or fold change, across a large number of populations or experimental variables. Unlike bivariate analysis and viSNE, these tools do not provide a single-cell view of the data. Instead, these tools summarize a key population feature using color. Histogram overlays illustrate the distribution of a single marker for a population and shade the graphic based on the statistic, whereas heatmaps generally just show the color. To demonstrate these data visualization tools, we have used the Kasumi-1 phospho-flow dataset (Fig. 4). Kasumi-1 phospho-flow experiment data are available online (www.cytobank.org/irishlab).

¹⁴SPADE: With high content single cell experiments measuring more than 25 features per cell, sequential bivariate gating can overlook populations of cells with low or unexpected patterns of marker expression. When applied to flow data, SPADE clusters cells based on phenotypic similarities in high-dimensional space. SPADE is a powerful complement to manual analysis approaches. After the initial clustering step, SPADE creates a minimum-spanning tree that illustrates the relationships between cell populations in two dimensions. Each node of the tree is a cluster of cells, and nodes can be colored to reflect the expression intensity of each marker [13]. Here, we subjected the human PBMC immunophenotyping dataset to SPADE analysis to create a minimum-spanning tree (Fig. 2). Known cell subsets in human PBMCs were identified as “bubbles” (Fig. 2) through manual review of biomarker expression on different populations on the tree.

¹⁵viSNE: is a computational tool that projects cells onto a two dimensional map such that the distances between cells in 2D reflect the distance between them in high-dimensional space [14]. Thus, cells that are close together on a viSNE map are phenotypically similar for the markers used to create the map. Users then identify and characterize populations of cells based on the groups formed in the viSNE map. We performed a viSNE analysis of the human PBMCs immunophenotyping dataset (Fig. 3). As with SPADE, expression intensity of each parameter can be visualized as heat intensity on the viSNE map, enabling identification of known cell subsets in human PBMCs. Importantly, viSNE allowed a global single-cell view of the data. viSNE enabled identification of rare cell subsets and cell populations without supervision or prior knowledge of the expected population distribution.

7. Krutzik PO, et al. Analysis of protein phosphorylation and cellular signaling events by flow cytometry: techniques and clinical applications. *Clin Immunol.* 2004; 110(3):206–221. [PubMed: 15047199]
8. Irish JM, Kotecha N, Nolan GP. Mapping normal and cancer cell signalling networks: towards single-cell proteomics. *Nat Rev Cancer.* 2006; 6(2):146–155. [PubMed: 16491074]
9. Bendall SC, et al. A deep profiler's guide to cytometry. *Trends Immunol.* 2012; 33(7):323–332. [PubMed: 22476049]
10. Bendall SC, et al. Single-cell mass cytometry of differential immune and drug responses across a human hematopoietic continuum. *Science.* 2011; 332(6030):687–696. [PubMed: 21551058]
11. Ornatsky O, et al. Highly multiparametric analysis by mass cytometry. *J Immunol Methods.* 2010; 361(1–2):1–20. [PubMed: 20655312]
12. Bendall SC, et al. Single-cell trajectory detection uncovers progression and regulatory coordination in human B cell development. *Cell.* 2014; 157(3):714–725. [PubMed: 24766814]
13. Newell EW, et al. Cytometry by time-of-flight shows combinatorial cytokine expression and virus-specific cell niches within a continuum of CD8+ T cell phenotypes. *Immunity.* 2012; 36(1):142–152. [PubMed: 22265676]
14. Becher B, et al. High-dimensional analysis of the murine myeloid cell system. *Nat Immunol.* 2014; 15(12):1181–1189. [PubMed: 25306126]
15. Krutzik PO, Nolan GP. Intracellular phospho-protein staining techniques for flow cytometry: monitoring single cell signaling events. *Cytometry A.* 2003; 55(2):61–70. [PubMed: 14505311]
16. Bodenmiller B, et al. Multiplexed mass cytometry profiling of cellular states perturbed by small-molecule regulators. *Nat Biotechnol.* 2012; 30(9):858–867. [PubMed: 22902532]
17. Kotecha N, Krutzik PO, Irish JM. Web-based analysis and publication of flow cytometry experiments. *Curr Protoc Cytom.* 2010 Chapter 10:Unit10.17.
18. Qiu P, et al. Extracting a cellular hierarchy from high-dimensional cytometry data with SPADE. *Nat Biotechnol.* 2011; 29(10):886–891. [PubMed: 21964415]
19. el Amir AD, et al. viSNE enables visualization of high dimensional single-cell data and reveals phenotypic heterogeneity of leukemia. *Nat Biotechnol.* 2013; 31(6):545–552. [PubMed: 23685480]

Fig. 1.

Phenotyping human PBMC subsets with traditional bivariate gating & heatmap analysis. **(a)** Bivariate plots compare features measured on healthy human PBMCs by mass cytometry. Heat corresponds to proportional cell abundance in a plot region for the population indicated above the plot. Intact cells (*grey gate*) were defined using event length and an iridium based cell marker (Ir-191 intercalator, NA). Among the intact cells, leukocytes were defined as CD45+ events. CD3 and CD19 were then used to identify T cells and B cells, respectively. Subsets of T cells and B cells were identified using additional markers (CD4, CD8, CD45RA, and IgM). The non-T non-B cells (CD45 + CD3 - CD19 -) were gated as monocytes, dendritic cells, and natural killer (NK) cell using CD14, CD11b, CD11c, HLA-DR, CD16, and CD56. **(b)** A heatmap compares expression of 27 measured features on the same cells populations shown in **(a)** using a log-like arcsinh₁₅ scale. The heat corresponds to the arcsinh₁₅ fold difference in median expression for a given marker compared to the table minimum

Fig. 2.

SPADE clusters PBMCs into populations based on similar marker expression. SPADE plots show clustered populations of healthy human PBMCs (*circles*) connected using a minimum spanning tree. *Each circle* represents a population of cells with a similar phenotype for the 21 markers shown. The size of each circle is proportional to the number of cells in that population. The *heat color for each circle* corresponds to the median expression of the indicated marker on the cells within that circle. Heat corresponds to the arcsinh₁₅ fold difference in median expression for a given marker. Note the scale min and max differ for each marker. *Black outlines* termed “bubbles” and associated population labels derive from manual interpretation of cellular identity based on marker expression

Fig. 3. viSNE arranges cells in a 2D map representing phenotypic similarity. viSNE maps show healthy human PBMCs arranged according to phenotypic similarity for the 21 displayed markers measured by mass cytometry. The axes are unitless dimensions that reflect phenotypic differences. The distance between any two cells on the map corresponds to how similar or different the cells are from each other in high-dimensional space. Heat corresponds to the arcsinh_{15} fold difference in median expression for a given marker. This allows for a global single-cell view of every parameter in every cell. Cell populations can then be identified through various techniques, including automated clustering or manual analysis of well-characterized markers

Fig. 4.

Mass cytometry phospho-flow analysis of AML cell signaling responses. **(a)** A heatmap compares phospho-protein abundance in Kasumi-1 AML cells following 15 min of stimulation by FLT3L, SCF, IL-3, or IFN γ . Each row in the heatmap or histogram overlay corresponds to a stimulation condition and each column corresponds to a phospho-protein (p-AKT, p-ERK1/2, p-STAT5, p-STAT1, or p-p38). Heat corresponds to the arcsinh₁₅ fold difference in median expression for a given marker compared to the unstimulated condition (Unstim.). **(b)** The same data as in **(a)** are shown in histogram overlay format. Histogram overlays illustrate the distribution of marker expression within a population and highlight heterogeneity (e.g., p-STAT5 response to IL-3)

Table 1

Antibody panel for cell identity

#	Target	Tag	Clone	μL^a	Catalog # ^b
1	CD19	I42Nd	HIB19	1	3142001B
2	CD117	I43Nd	104D2	1	3143001B
3	CD11b	I44Nd	ICRF44	1	3144001B
4	CD4	I45Nd	RPA-T4	1	3145001B
5	CD8a	I46Nd	RPA-T8	1	3162001B
6	CD20	I47Sm	2H7	1	3147001B
7	CD34	I48Nd	581	1	3148001B
8	CD61	I50Nd	VI-PL2	1	3150001B
9	CD123	I51Eu	6H6	1	3151001B
10	CD45RA	I53Eu	HI100	1	3153001B
11	CD45	I54Sm	HI30	1	3154001B
12	CD10	I56Gd	HI10a	1	3156001B
13	CD33	I58Gd	WM53	1	3158001B
14	CD11c	I59Tb	Bu15	1	3159001B
15	CD14	I60Gd	M5E2	1	3160001B
16	CD69	I62Dy	FN50	1	3162001B
17	CD15	I64Dy	W6D3	1	3164001B
18	CD16	I65Ho	3G8	1	3165001B
19	CD44	I66Er	BJ18	1	3166001B
20	CD38	I67Er	HIT2	1	3167001B
21	CD25	I69Tm	2A3	1	3169003B
22	CD3	I70Er	UCHT1	1	3170001B
23	IgM	I72Yb	MHM-88	1	3172004B
24	HLA-DR	I74Yb	L243	1	3174001B
25	CD56	I76Yb	CMSSB	1	3176003B

^a Microliter of antibody per 100 μL total volume

Source was Fluidigm for all antibodies_q

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Table 2

Antibody panel for cell signaling

#	Target	Tag	Clone	μL ^a	Catalog # ^b
1	p-STAT5	150Nd	47	1	3150005A
2	p-AKT	152Sm	D9E	1	3152005A
3	p-STAT1	153Eu	58D6	1	3153003A
4	p-p38	156Gd	D3F9	1	3156002A
5	p-ERK1/2	167Er	D13.14.4E	1	3167005A

^a Microliter of antibody per 100 μL total volume

^b Source was Fluidigm for all antibodies

Table 3

Stimulation conditions

Stimulus	Time (min)	50× Dose ^a
FLT3L	15	10 µg/mL
SCF	15	25 µg/mL
IL-3	15	10 µg/mL
IFNγ	15	10 µg/mL

^a Add 20 µL of 50× to 1 mL cells to yield 1×