HHS Public Access

Author manuscript

Curr Protoc Cytom. Author manuscript; available in PMC 2018 October 02.

Published in final edited form as:

Curr Protoc Cytom. ; 82: 9.54.1–9.54.12. doi:10.1002/cpcy.27.

CyTOF Measurement of Immunocompetence across Major Immune Cell Types

Priyanka B. Subrahmanyam, Ph.D. [Post-doctoral Research Fellow] and
Immunity, Transplantation & Infection, Stanford University School of Medicine, 299 Campus Drive, Stanford, CA 94305-5124

Holden T. Maecker, Ph.D. [Professor]
Dept. of Microbiology & Immunology, Stanford University School of Medicine, 299 Campus Drive, Stanford, CA 94305-5124

Abstract

The central role of the immune system is becoming appreciated in a wide variety of diseases. Cancer immunotherapy is one area that has yielded much recent success, although not all patients benefit equally. At the same time, recent studies have highlighted the heterogeneity of the human immune system. Despite this heterogeneity, we do not routinely measure immune competence in clinical practice, and there are no consensus assays of healthy immune function. Using mass cytometry (CyTOF), we can simultaneously detect ~40 markers to identify various cell subsets and determine their function by the expression of cytokines, cytotoxicity, and activation markers. This can help assess 'immunocompetence' and facilitate better implementation of immunotherapies, both in specific disease settings and perhaps eventually as a prognostic tool in healthy subjects. Here we introduce the concepts behind this assay and provide a protocol that we have successfully implemented to identify possible predictive biomarkers of immunotherapy outcome.

Keywords

CyTOF; immunotherapy; immune profiling; biomarkers

INTRODUCTION

The immune system is composed of a very large number of cell types that perform many different functions. Perhaps because of this complexity, there is little consensus on what aspects of immune cell phenotypes and functions are truly important for the healthy functioning of the immune system (which we can generically call "immunocompetence"). The exceptions are the genetic and acquired immune deficiencies, which generally present with a gross defect in one or more major immune cell types, such as the lack of $CD4^+$ T cells in HIV disease. However, a wide heterogeneity exists among healthy individuals with regard to many cell subset frequencies and functions (Brodin et al., 2015; Brodin and Davis, 2017). Normal ranges have been established only for the broadest of immune cell subsets, and not at all for their functional capacities (Apoil et al., 2017; Valiathan et al., 2014).

Our lack of ability to assess relevant aspects of immune competence might be forgiven as long as there were no therapeutic interventions that could alter immune function. But in the

field of organ transplantation, immune suppression is routinely carried out, with only very crude monitoring of immune cell counts and clinical parameters. Furthermore, cancer immunotherapy has recently become much more successful with the advent of immune checkpoint inhibitors. But these can lead to autoimmune syndromes on one hand, and lack efficacy in certain patients on the other hand. Better understanding of the immune landscape prior to (and during) treatment is needed to allow tailoring of these therapies (Gnjatic et al., 2017). In fact, one could argue that accurate assessment of immune competence could benefit many areas of clinical medicine, since the immune system is central not only to cancer and transplant rejection, but also plays a major role in cardiovascular disease, infection, and autoimmunity, for example.

Because blood is a suspension of many heterogeneous cells, single-cell technologies such as flow cytometry are ideally suited to its characterization (Maecker and Harari, 2015). And because blood is the most easily accessible immunological tissue, it would seem the most obvious for the development of an immunocompetence assay. But assessing only cell frequencies in blood is unlikely to be fully informative. An *in vitro* stimulation to determine functional response capacity is much more likely to yield informative biomarkers (Bjornson et al., 2013). One stimulus condition that can evoke a full complement of functions from virtually all immune cell types is the combination of phorbol myristate acetate (PMA) and the calcium ionophore ionomycin. In this protocol, we use PMA+ionomycin to read out the broadest array of functions in all major cell types, despite the fact that it is not a physiologically relevant stimulus. We combine this *in vitro* stimulation with secretion inhibitors (brefeldin A and/or monensin), followed by cell-surface and intracellular staining for cytokines and other markers (Lovelace and Maecker, 2011).

The protocol described here combines the staining of cell-surface markers and intracellular cytokines and other proteins, using CyTOF (Lin et al., 2015). Using patient peripheral blood mononuclear cells (PBMC), we analyze all major subsets of B cells, T cells, NK cells, and monocytes, as well as activation markers, cytokines, and cytotoxic proteins.

We have effectively used this assay to discover potential biomarkers of cancer immunotherapy response (Hiniker et al., 2016; Chang et al., 2014). Of course, much more work is needed to determine how generalizable these results will be across different immunotherapies and tumor types. However, we could envision eventually determining not only whether a patient is suitable for a given immunotherapy, but perhaps even which of several immunotherapy options are most appropriate. These options could one day include therapies that are designed to reinvigorate specific aspects of the immune system that are required for downstream cancer immunotherapy. We propose that simplified versions of this immunocompetence assay could become useful in many different clinical settings.

BASIC PROTOCOL 1

CyTOF Intracellular Cytokine Staining Protocol for human PBMC—In this protocol, we describe an immunocompetence assay using CyTOF for a comprehensive phenotypic and functional analysis of immune cells. The staining panel (Table 1) includes a large number of lineage markers to identify immune cell subsets, as well as a host of

functional markers like cytokines, cytotoxic markers and activation markers. Here we use cryopreserved PBMC, although this protocol is also compatible with fresh human PBMC.

First, we begin with thawing and resting of PBMC, which is followed by stimulation (Fig. 1). Using PMA/Ionomycin, we seek to understand the ability of the various immune cell subsets to produce cytokine and express activation markers. Following stimulation, the cells are stained for surface markers, which are typically important to identify different immune cell types. Next, we use a CyTOF-compatible live/dead stain to be able to detect viable cell events after data acquisition. After this, cells are fixed and permeabilized gently using a saponin-based solution, and stained for intracellular markers. Finally, since CyTOF lacks light scatter parameters that are used in conventional flow cytometry to detect cells, we use a metal-conjugated DNA intercalator to detect intact cell events. Samples can then be run on a CyTOF version 1, CyTOF version 2, or Helios mass cytometer from Fluidigm (San Francisco, CA).

Materials: All solutions should be stored in metal-free containers. Typically sterile plasticware, tubes, filters and new, never-washed glassware are metal-free. Glassware used should not have ever been washed, since soap can cause barium contamination.

1. Dry ice and regular ice
2. Complete medium (see recipe)
3. Pierce Universal Nuclease, 25kU (ThermoFisher Scientific, Catalog #88701)
4. CyPBS (see recipe)
5. CyFACS (see recipe)
6. Brefeldin A (Sigma-Aldrich, Catalog #B7651)
7. Monensin 1000× (BioLegend, Catalog #420701)
8. Phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich, Catalog #P8139)
9. Ionomycin (Sigma-Aldrich, Catalog #I0634)
10. EDTA, 0.5M (Gibco, Catalog #15575)
11. Maleimide-DOTA loaded with ^{115}In, 5mg/ml (Macrocyclics, Catalog #B-272)
12. Paraformaldehyde, 16% w/v (Alfa Aesar, Catalog #43368)
13. 10× permeabilization buffer (eBioscience, Catalog #00-8333-56)
14. Cell ID Ir-Intercalator (^{193}Ir/^{195}Ir) 1000× (Fluidigm, Catalog #201192A)
15. EQ Four Element calibration beads (Fluidigm, Catalog #201078)
16. 96-well U-bottom plates (Falcon, Catalog #353077)
17. Centrifugal filter units, 0.1 μm (Millipore, Catalog #UFC30VV00)
18. 5 ml polystyrene tubes with cell-strainer cap (Falcon, Catalog #352235)
19. 1.5 ml, 15 ml and 50 ml conical tubes, 10 ml sterile disposable pipettes

Equipment

1. Filters for sterilization of media and other solutions (Millipore, Catalog #SCGPU11RE)
2. Water bath (maintained at 37°C)
3. Biosafety cabinet
4. Centrifuge (adaptors to fit 15 ml conical tubes, and 1.5 ml conical tubes)
5. Cell counter or haemacytometer
6. Incubator, pipettes, tips and other standard laboratory supplies

Protocol Steps (Fig.1)

I. Sample preparation

1. Warm complete medium in 37°C water bath and use this warm medium for all steps.
2. Add 1:10,000 Pierce Universal Nuclease to complete medium (20 ml of complete medium with nuclease is needed for each sample).
3. If frozen, remove samples from liquid nitrogen storage and keep them on dry ice.
4. Label and prepare 15 ml conical tubes (1 per sample) with 10 ml of complete medium with nuclease. Work in a biosafety cabinet to prevent contamination.
5. Thaw one or two samples at a time in the 37°C water bath (2–3 min) until just thawed.
6. When thawed, add 1 ml of medium from the 15 ml tube prepared for that sample in a drop wise manner. Gently pipette up and down.
7. Add these cells to the correct 10 ml conical tube labeled for that sample.
8. Centrifuge the cells at room temperature @ 300 × G for 5 min.
9. Aspirate the medium, being careful to leave the pellet intact.
10. Add 10 ml of complete medium with nuclease and gentle pipette up and down to mix.
11. Centrifuge again at room temperature @ 300 × G for 5 min.
12. Aspirate the medium and resuspend the cells in 1 ml of complete medium (no nuclease).
13. Count cells using an automated cell counter or a haemacytometer and adjust the concentration to 10×10^6 cells/ml or maximum available.
14. Add 200 µl of cells (2×10^6 cells/well) or maximum available of each sample to a separate well in a 96-well U-bottom plate.

15. Rest the cells for a minimum of 6 hrs or up to overnight (recommended) in an incubator at 37°C with 5% CO_2. (Before finalizing study design, make sure to test the effect of different rest times for your particular cell type of interest).

II. Stimulation to measure immunocompetence

1. After cells have been rested, add stimulation reagents and secretion inhibitors as shown below (Table 3). Mix by pipetting gently.
2. Other study-specific stimulation reagents such as anti-CD3/CD28, peptide mixes, etc. may also be included.
3. If using CD107a in the staining panel, add metal-conjugated antibody along with stimulation reagents. Use the titer recommended by the supplier if using commercially available conjugate, or use the optimum pre-determined titer for in-house conjugates.
4. Place in incubator for 4 hours at 37°C, 5% CO_2. (Prior to finalizing study design test all stimulation reagents, secretion inhibitors, etc.)
5. After stimulation add 2mM EDTA to each well and incubate at room temperature for 15 min. Mix by pipetting gently.
6. Wash cells 2× in CyFACS using 200 μl/well. Centrifuge at 300 × G at room temperature for 5 min. To remove supernatant, aspirate, or ‘flick’ the plate (quickly but gently invert the plate over a waste container).
7. Prepare surface staining antibody cocktail in CyFACS (70 μl/well) and add it to a 0.1 μm centrifugal filter unit. Centrifuge at highest speed in a microfuge for 5 min to remove antibody aggregates by filtration.
8. Add this antibody cocktail (70 μl/well) to the samples and mix by pipetting gently. Incubate for 45 min on ice.
9. Wash cells 2× in CyFACS as in Step 6.
10. Prepare live-dead stain by diluting Maleimide-DOTA ^{115}In (1:3000 or optimal concentration from titration) in CyPBS.
11. Add 100 μl per well and incubate for 30 min on ice.
12. Wash 3× in CyFACS as described in Step 6.
13. Dilute 16% PFA to 2% in CyPBS and add 100 μl per well. Fix cells overnight at 4°C. If needed, this incubation can be cut down to 30 min at room temperature. After 30 min, proceed to the permeabilization and intracellular staining steps described below.
14. After fixation, wash cells 2× in permabilization buffer (10× diluted to 1× in MilliQ water). Add 200 μl per well and centrifuge at 500 × G for 5 min. Aspirate or flick the plate to remove supernatants.
15. Prepare intracellular staining cocktail in 1× permeabilization buffer. Centrifuge for 5 min at highest speed in a microfuge, using a 0.1 μm centrifugal filter unit.

16. Add 70 µl intracellular staining cocktail and incubate for 45 min on ice.
17. Wash 3× in CyFACS adding 200 µl/well, and centrifuging at 500 × G for 5 min.
18. If desired cells can be left in CyFACS overnight at this stage. Otherwise, proceed to Iridium staining as described below.
19. Dilute Iridium intercalator ($^{193}Ir/^{195}Ir$) (1000× to 1×) in 2% PFA in CyPBS. Add 100 µl per well and incubate for 20 min at room temperature.
20. Wash 2× in CyFACS as in Step 17. If samples are being run on the same day, proceed to MilliQ water washes in Step 21. Otherwise leave cells as pelleted (after centrifugation) in CyFACS at 4°C overnight and run within 1–2 days.
21. Wash in MilliQ water 3×, adding 200 µl/well and centrifuging at 500 × G for 5 min.
22. Dilute calibration beads 1:10 in MilliQ water. These calibration beads will allow normalization of data based on standard bead intensities. Count the cells and adjust the concentration to 7–8×10^5 cells/ml using the 1:10 diluted beads(or optimum concentration to run at desired rate (usually 200–300 events/sec)). Transfer to the 5 ml tubes passing the cell suspension through the strainer cap to remove any cell aggregates that might cause clogging during data acquisition.
23. Acquire data on a CyTOF version 1, CyTOF version 2 or Helios mass cytometer. Collect 200,000–300,000 events to be able to identify rare cell subsets and low frequencies of cytokine producing cells.

REAGENTS AND SOLUTIONS

1. Complete medium
   a. RPMI 1640 (Hyclone, Catalog #SH30027.01)
   b. Fetal bovine serum (FBS) (Atlanta Biologicals, Catalog #S11150)
   c. 100× Penicillin/Streptomycin/Glutamine (Hyclone, Catalog #SV30082.01)

   Filter-sterilize RPMI-1640 with 10% FBS and 1× Penicillin/Streptomycin/Glutamine to make complete medium, and store at 4°C, protected from light.
2. CyPBS (metal-free PBS)
   a. 10× PBS (Ca/Mg free) (Rockland Immunochemicals, MB-008)
   b. MilliQ grade water (metal free)

   Dilute 10× Rockland PBS to 1× in MilliQ water and filter-sterilize to make CyPBS.
3. CyFACS
   a. CyPBS (see recipe above)
   b. Bovine serum albumin (BSA), 30% (Sigma-Aldrich, Catalog #A7284)

c. 2mM EDTA, 0.5M (Gibco, Catalog #15575)

d. Sodium azide, 10% (Teknova, Catalog #S0209)

Take CyPBS (recipe above) and add 0.1% BSA, 2mM EDTA, and 0.05% Sodium azide. Filter-sterilize and store at 4°C.

COMMENTARY

Background Information

Conventional flow cytometry can now measure more than 20 parameters in parallel. However, we sought to use the most high-dimensional single cell proteomics approach possible. Mass cytometry (CyTOF) combines the principles of flow cytometry and mass spectrometry to allow the simultaneous detection of >40 different markers on each cell (Bandura et al., 2009; Bendall et al., 2011). Unlike conventional flow cytometry, which uses fluorophores as tags for antibodies and other probes, CyTOF uses heavy metal-tagged antibodies. This allows for parallel readout of many more antibody specificities, without the significant spillover between detector channels inherent in fluorescence flow cytometry. As a result this CyTOF intracellular staining protocol allows high-dimensional phenotypic and functional analysis of a larger number of immune cell subsets.

Critical Parameters and Troubleshooting

Sample Selection—Before using this protocol, it is important to consider the end goal for the measurement of immunuocompetence. For this, appropriate sample selection is critical. Be sure that there are enough samples in each comparison group to yield statistically significant results. Also, ensure that the samples have enough cells for the assay (we recommend 2×10^6 PBMC per stimulation condition), and that their viability is good (ideally >90%). We recommend the use of cryopreserved PBMC for convenience and for the ability to batch samples (Maecker et al., 2005; Costantini et al., 2003). However, poor cryopreservation, storage, and/or thawing procedures can severely compromise cell viability and recovery, which in turn renders functional assays useless (our unpublished—and unpublishable—data).

Fresh vs. Cryopreserved PBMC—Despite the advantages of cryopreserved PBMC, the expression levels of some markers are compromised upon freeze-thaw. The decision to use fresh versus cryopreserved PBMC samples should be considered carefully in the context of the staining panel, number of samples and ability to batch them, etc. Whether fresh or frozen, all samples in one study should be of the same type.

Panel Design—The antibodies used in this protocol are metal-tagged, many of which can be purchased in conjugated form from Fluidigm (South San Francisco, CA). However, some antibodies may not be available with the desired metal label and need to be custom conjugated. As shown in our sample staining panel, we use a combination of commercially available and in-house conjugated antibodies (Table 1). Conjugation of purified carrier-free antibody to a metal tag can be easily performed in the laboratory using the MaxPar labeling kit from Fluidigm (Table 2). Standard antibody panels, as well as online panel design tools

are available on the Fluidigm website (Table 2). For in-house conjugated antibodies, titration and testing for each batch of conjugated antibody is recommended to determine the optimum staining titer. Once decided, the panel should be kept consistent in terms of antibodies, titers used, as well as channels selected. Finally, it is important to consider the markers that are necessary to answer the basic research question being addressed. For example, while studying samples from cancer patients undergoing checkpoint blockade therapy, important costimulatory molecules like CTLA-4, PD-1 and PD-L1/2 can be included. Similarly, studies focused on a particular cell type such as B cells, T cells or NK cells, should include markers specific for that cell type. Staining of intracellular markers like cytokines and cytotoxic markers can be easily accomplished using this protocol, however, staining of transcription factors will require different cell permeabilization conditions that would need to be optimized in the context of the cell-surface markers and cytokines being targeted. Since CyTOF doesn't have forward and side light scatter parameters to aid in cell subset identification, markers for each cell population to be discriminated must be included. For example, we include CD14 and/or CD33 in the panel to separate lymphocytes and monocytes during analysis (Fig. 2), even if monocytes are not a primary target of interest.

Data Analysis and Understanding the Results

Data pre-processing

1. After data have been acquired, make sure that there is a single file for each sample. If not, concatenate the files using Fluidigm software or FlowJo (Treestar Inc.).
2. Normalize the data for instrument performance using either the Fluidigm normalizer or the Matlab-based Nolan lab normalizer, which is freely available on Github (Table 2). Thus signal intensities from the channels being analyzed will be normalized based on bead channel signal intensities.

High-Dimensional Data Analysis—CyTOF data are similar to flow cytometry data, but have higher dimensionality. Manual gating is an important part of detailed analysis of CyTOF data for drawing conclusions on immunocompetence (Fig. 2). However, there are some advanced analysis tools that can also be employed to effectively deal with high-dimensional CyTOF data.

SPADE: Clustering algorithms like SPADE can be a valuable tool to represent high-dimensional data in the form of cluster dendograms in 2-dimensional space (Table 2) (Qiu et al., 2011). This algorithm clusters cells according to a subset of specified markers (usually major cell lineage markers), and the resulting dendogram of clusters can be colored by expression of specific markers of interest (Fig. 3).

viSNE: This dimension reduction algorithm condenses the channels to give a 2-dimensional representation of cell subsets (Table 2) (Amir et al., 2013). Expression levels of different markers can be visualized by a color scale (Fig. 4). viSNE results can be similar to SPADE, but without overt clustering, and with a less stochastic nature to the structure of the visualization. Both SPADE and viSNE are great tools for visual representation. In addition, they also provide an opportunity to discover unexpected changes in expression. Using an

unbiased approach to the input data, these algorithms can often help discover completely new findings that even experts might not find due to their focused approach.

Citrus: Citrus is a good tool to detect group-level differences in samples (Table 2) (Bruggner et al., 2014). For example, in a study comparing responders to non-responders or healthy to diseased individuals, Citrus can identify clusters or marker intensities within clusters that are differentially expressed. It can also help generate predictive models and provide means for visual representation of the findings. Citrus uses hierarchical clustering to generate a dendogram, similar to SPADE, but where peripheral clusters are subsets of the central parents.

Besides SPADE, viSNE and Citrus, many new methods for high-dimensional data analysis are constantly being developed. These tools each have strengths and weaknesses, but can be useful adjuncts to manual analysis, especially in studies where measurement of immunocompetence is directed toward biomarker discovery.

Time Considerations

This protocol is typically implemented over a period of 3–4 days, starting from thawing of PBMC to data acquisition. However the precise length of time required will vary as some steps like resting of cells and fixation can be adjusted according to study design. Furthermore, the time required for data acquisition must be carefully considered. Typically samples are run at 200–300 events/sec. So the total number of samples to be run, total number of events collected per samples, as well as start-up and shut-down procedures for the machine will all dictate the amount of time that must be invested in completing this protocol.

Acknowledgments

This work was supported by grants 5R21CA205430, S10RR027582, and 5U24AI118648 from the National Institutes of Health.

LITERATURE CITED

Amir ED, Davis KL, Tadmor MD, Simonds EF, Levine JH, Bendall SC, Shenfeld DK, Krishnaswamy S, Nolan GP, Pe'er D. viSNE enables visualization of high dimensional single-cell data and reveals phenotypic heterogeneity of leukemia. Nature Biotechnology. 2013; 31:545–552.

Apoil PA, Puissant-Lubrano B, Congy-Jolivet N, Peres M, Tkaczuk J, Roubinet F, Blancher A. Reference values for T, B and NK human lymphocyte subpopulations in adults. Data in brief. 2017; 12:400–404. [PubMed: 28491945]

Bandura DR, Baranov VI, Ornatsky OI, Antonov A, Kinach R, Lou X, Pavlov S, Vorobiev S, Dick JE, Tanner SD. Mass cytometry: technique for real time single cell multitarget immunoassay based on inductively coupled plasma time-of-flight mass spectrometry. Analytical Chemistry. 2009; 81:6813–6822. [PubMed: 19601617]

Bendall SC, Simonds EF, Qiu P, Amir ED, Krutzik PO, Finck R, Bruggner RV, Melamed R, Trejo A, Ornatsky OI, et al. Single-cell mass cytometry of differential immune and drug responses across a human hematopoietic continuum. Science. 2011; 332:687–696. [PubMed: 21551058]

Bjornson ZB, Nolan GP, Fantl WJ. Single-cell mass cytometry for analysis of immune system functional states. Current Opinion in Immunology. 2013; 25:484–494. [PubMed: 23999316]

Brodin P, Davis MM. Human immune system variation. Nature Reviews. Immunology. 2017; 17:21–29.

Brodin P, Jojic V, Gao T, Bhattacharya S, Angel CJL, Furman D, Shen-Orr S, Dekker CL, Swan GE, Butte AJ, et al. Variation in the human immune system is largely driven by non-heritable influences. Cell. 2015; 160:37–47. [PubMed: 25594173]

Bruggner RV, Bodenmiller B, Dill DL, Tibshirani RJ, Nolan GP. Automated identification of stratifying signatures in cellular subpopulations. Proceedings of the National Academy of Sciences of the United States of America. 2014; 111:E2770–7. [PubMed: 24979804]

Chang S, Kohrt H, Maecker HT. Monitoring the immune competence of cancer patients to predict outcome. Cancer Immunology, Immunotherapy. 2014; 63:713–719. [PubMed: 24487923]

Costantini A, Mancini S, Giuliodoro S, Butini L, Regnery CM, Silvestri G, Montroni M. Effects of cryopreservation on lymphocyte immunophenotype and function. Journal of Immunological Methods. 2003; 278:145–155. [PubMed: 12957403]

Gnjatic S, Bronte V, Brunet LR, Butler MO, Disis ML, Galon J, Hakansson LG, Hanks BA, Karanikas V, Khleif SN, et al. Identifying baseline immune-related biomarkers to predict clinical outcome of immunotherapy. Journal for immunotherapy of cancer. 2017; 5:44. [PubMed: 28515944]

Hiniker SM, Reddy SA, Maecker HT, Subrahmanyam PB, Rosenberg-Hasson Y, Swetter SM, Saha S, Shura L, Knox SJ. A Prospective Clinical Trial Combining Radiation Therapy With Systemic Immunotherapy in Metastatic Melanoma. International Journal of Radiation Oncology, Biology, Physics. 2016; 96:578–588.

Lin D, Gupta S, Maecker HT. Intracellular Cytokine Staining on PBMCs Using CyTOF Mass Cytometry. Bio-protocol. 2015; 5:1370. Available at: http://bio-protocol.org/e1370.

Lovelace P, Maecker HT. Multiparameter intracellular cytokine staining. Methods in Molecular Biology. 2011; 699:165–178. [PubMed: 21116983]

Maecker HT, Harari A. Immune monitoring technology primer: flow and mass cytometry. Journal for immunotherapy of cancer. 2015; 3:44. [PubMed: 26380089]

Maecker HT, Rinfret A, Souza PD', Darden J, Roig E, Landry C, Hayes P, Birungi J, Anzala O, Garcia M, et al. Standardization of cytokine flow cytometry assays. BMC Immunology. 2005; 6:13. [PubMed: 15978127]

Qiu P, Simonds EF, Bendall SC, Gibbs KD, Bruggner RV, Linderman MD, Sachs K, Nolan GP, Plevritis SK. Extracting a cellular hierarchy from high-dimensional cytometry data with SPADE. Nature Biotechnology. 2011; 29:886–891.

Valiathan R, Deeb K, Diamante M, Ashman M, Sachdeva N, Asthana D. Reference ranges of lymphocyte subsets in healthy adults and adolescents with special mention of T cell maturation subsets in adults of South Florida. Immunobiology. 2014; 219:487–496. [PubMed: 24661720]

Significance Statement

Recent advances in immunology have yielded new strategies to treat diseases like cancer, autoimmunity and infectious diseases, by specifically engaging the patient's immune system. However, only some patients benefit from these therapies. The immune system is highly complex and consists of hundreds of different cell subsets with many different functions. Yet, there is currently no consensus on how to measure these subsets and their functions, or which ones are important to monitor. Using mass cytometry, we can simultaneously detect more than 40 markers on individual immune cells, to determine the frequencies of various cell subsets and assess their functionality. This can provide an exploratory method to evaluate the immune competence of patients and guide the selection of immune-based therapies.

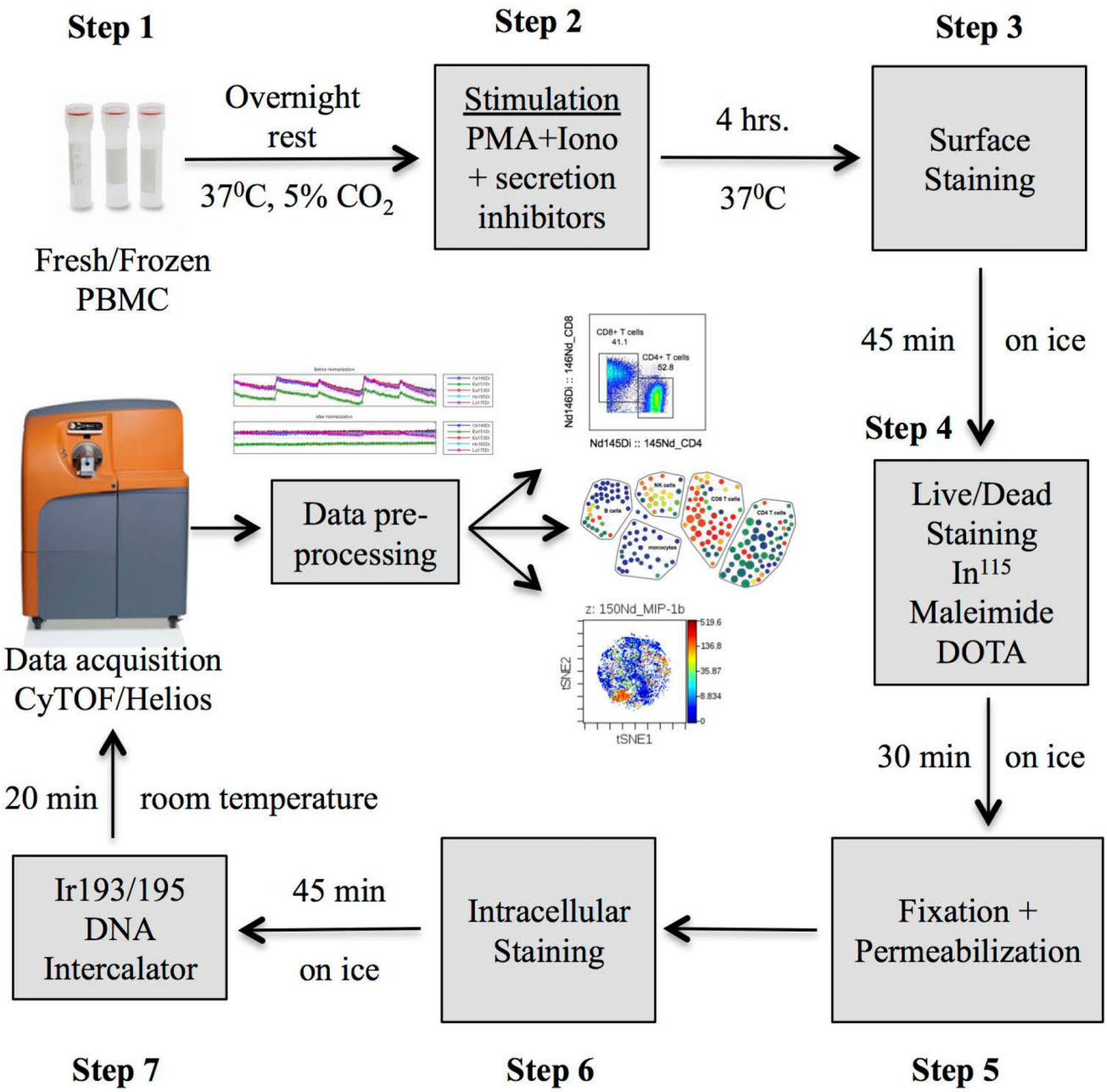

Figure 1.

Schematic of workflow for CyTOF intracellular cytokine staining assay for measurement of immunocompetence. Thaw cells and rest overnight (Step 1). Then stimulate with PMA/Ionomycin in the presence of secretion inhibitors Brefeldin A and Monensin for 4 hours (Step 2). Following stimulation, stain for surface markers (Step 3), then live/dead staining using ^{115}In DOTA Maleimide (Step 4). Fix cells in 2% paraformaldehyde overnight at 4°C and wash twice with permeabilization buffer (Step 5). Then carry out intracellular staining to detect cytokines and cytotoxic markers (Step 6). Finally stain with ^{193}Ir/^{195}Ir DNA intercalator for 20 min and wash with MilliQ grade water prior to data acquisition (Step 7).

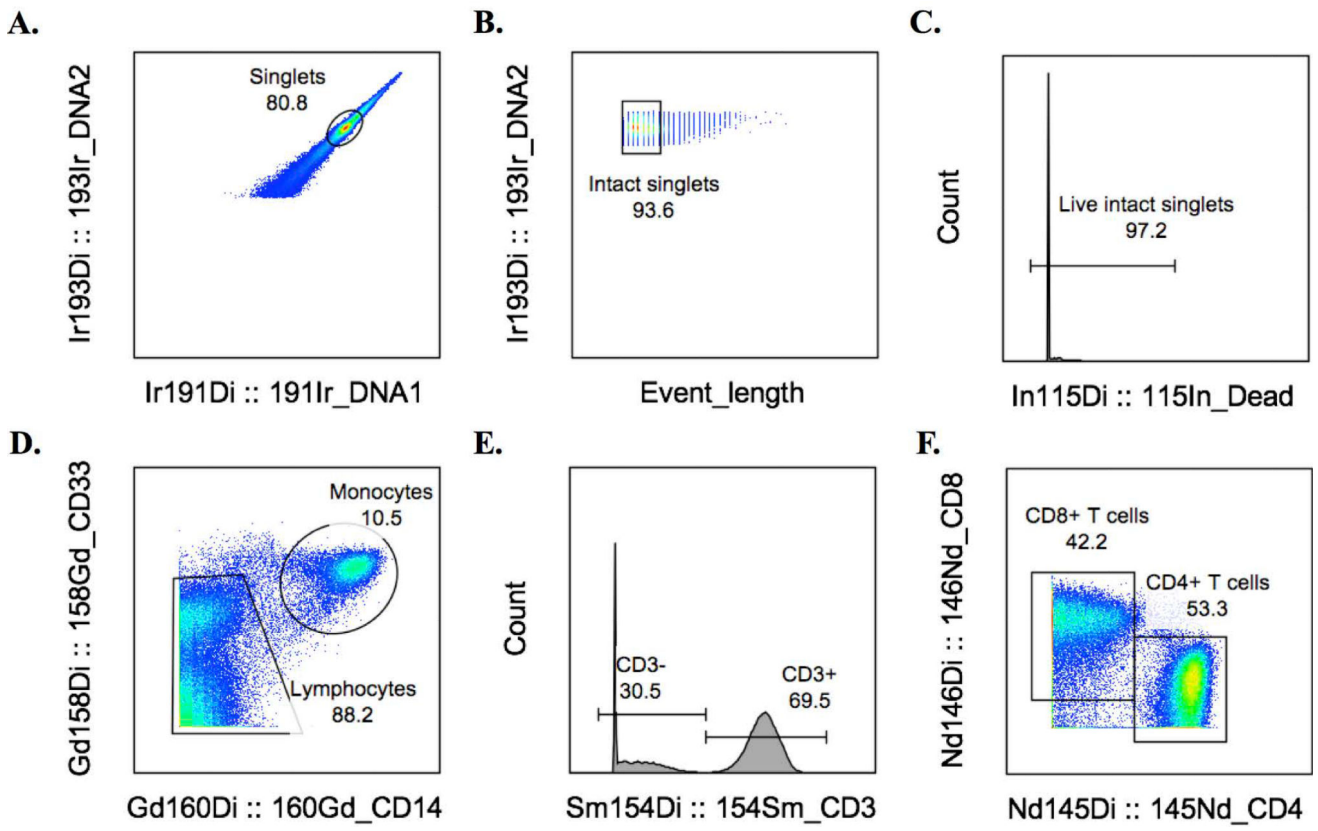

Figure 2.

Gating strategy for CyTOF data. (A) The two DNA markers ^{193}Ir and ^{195}Ir are used to detect singlets. (B) Singlets are further gated on the Event Length parameter to remove any remaining doublets or higher aggregates and detect intact single cell events only. (C) Intact singlets are then gated on ^{115}In DOTA Maleimide live/dead, to remove dead cells; events negative for the stain are gated as live intact singlets. (D) Since CyTOF lacks FSC and SSC as in conventional flow, we use CD14 and CD33 gating on live intact singlets to distinguish between lymphocytes and monocytes. (E) CD3 staining is then used to identify $CD3^+$ T cells and $CD3^-$ cells which consist of other cell types such as B cells and NK cells. (F) Finally, $CD4^+$ and $CD8^+$ T cells are gated from the $CD3^+$ T cells and functional markers such as cytokines and activation/cytotoxicity markers can be detected on various cell subsets.

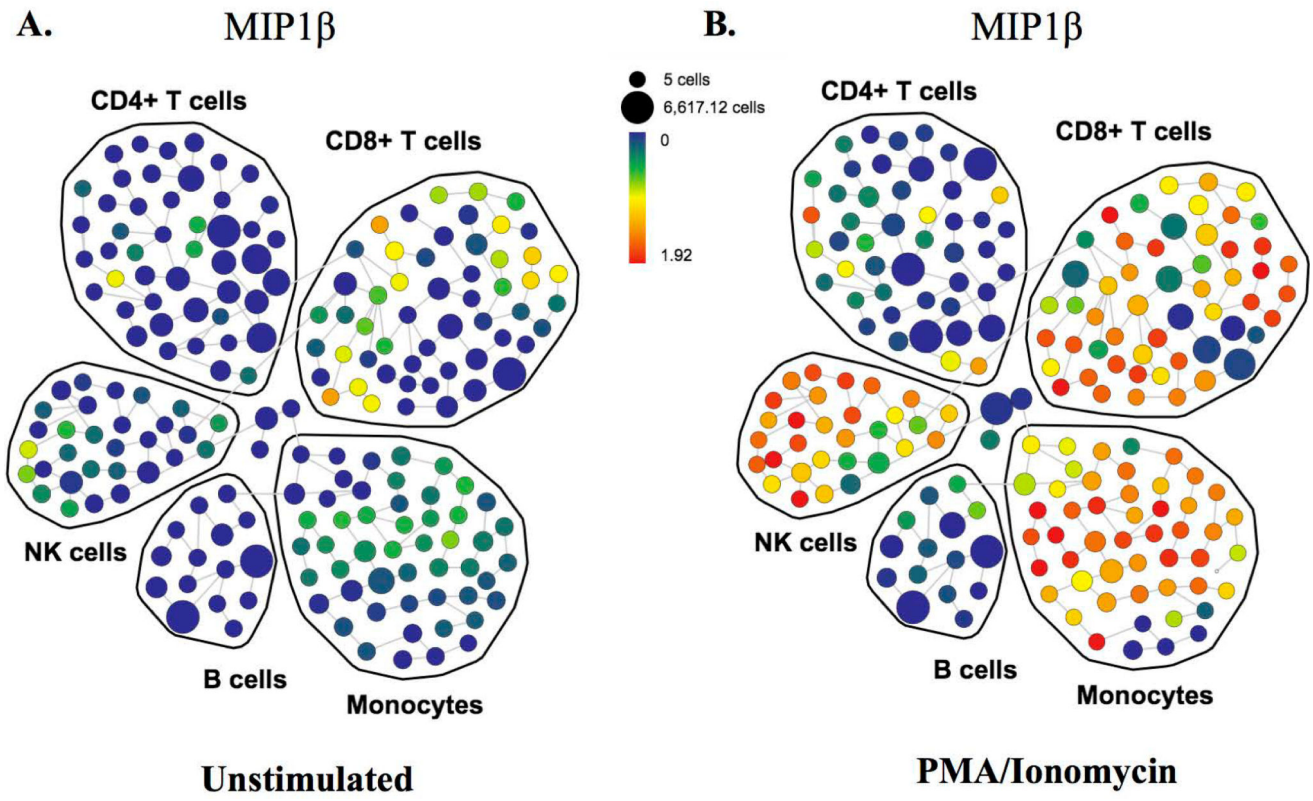

Figure 3.

SPADE analysis CyTOF ICS data. A healthy donor PBMC sample was analyzed by the described immunocompetence assay. Various lineage markers were used to detect basic cell subsets such as T cells ($CD4^+$ and $CD8^+$), B cells, NK cells and monocytes. MIP1β levels were then displayed in (A) unstimulated control and (B) PMA/Ionomycin stimulated samples. The node size represents the frequency of cells and the node color represents the level of expression of the marker under study, in this case MIP1β. (A) The unstimulated control shows mostly blue/green colored nodes, indicating low levels of MIP1β expression. (B) High expression of MIP1β can be seen as orange/red colored nodes. PMA/Ionomycin stimulation *ex vivo* leads to upregulation of MIP1β expression in various cell subsets.

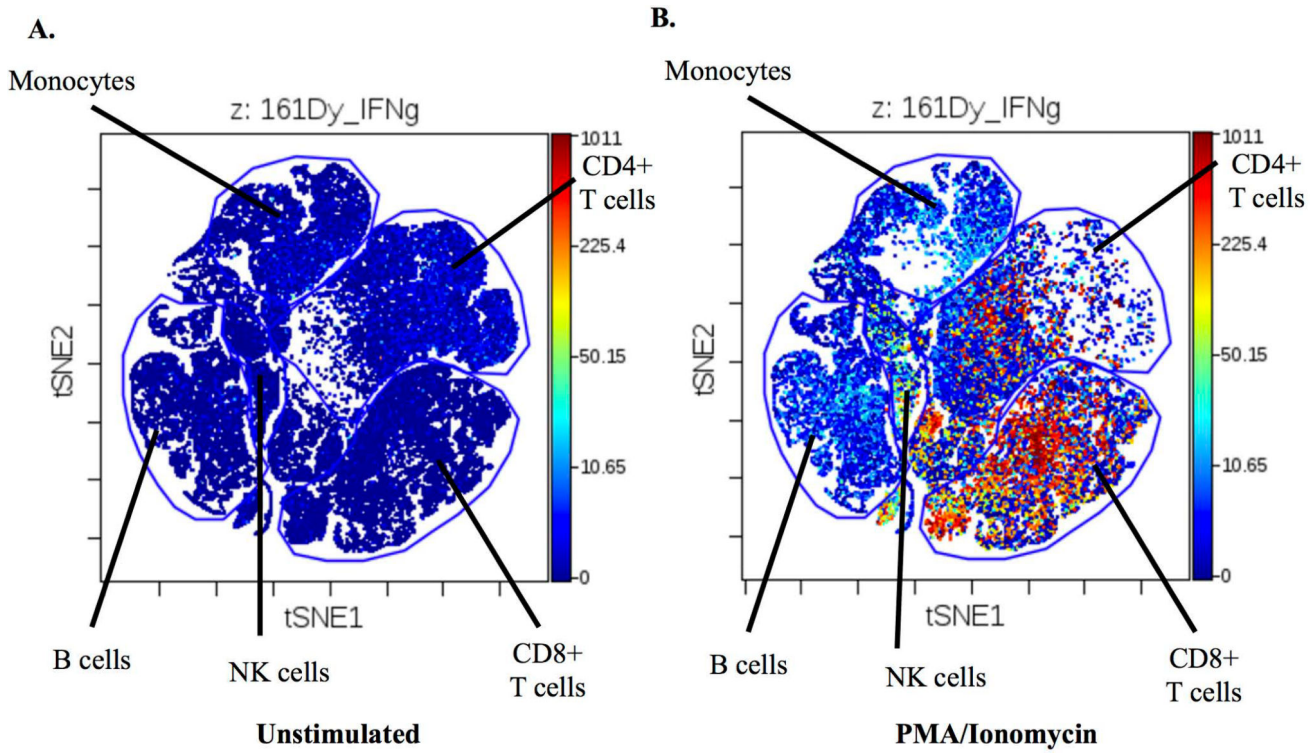

Figure 4.

viSNE analysis of CyTOF ICS data. A healthy donor PBMC sample was analyzed by the described immunocompetence assay. viSNE processes high-dimensional data and represents single cell events in two dimensional space. Manual gating based on lineage markers was used to detect basic cell subsets such as T cells ($CD4^+$ and $CD8^+$), B cells, NK cells and monocytes. IFNγ levels were then determined in (A) unstimulated control and (B) PMA/Ionomycin stimulated samples. (A) The unstimulated control looks mostly blue, indicating low levels of IFNγ expression. (B) After PMA/Ionomycin stimulation, there is increased expression of IFNγ, which is seen in red. As expected, high IFNγ expression is most prominent in $CD8^+$ T cells and to a lesser extent in $CD4^+$ T cells and NK cells.

Table 1

Example of a Staining Panel

Metal label	Specificity	Antibody Clone	Source
140Ce	Beads	-	Fluidigm
141Pr	CD25	MA251, BD	in-house
142Nd	CD19	HIB19	Fluidigm
143Nd	IL-10	JES3-9D7, Biolegend	in-house
144Nd	IL-4	MP4-25D2	Fluidigm
145Nd	CD4	RPA-T4	Fluidigm
146Nd	CD8	RPA-T8	Fluidigm
147Sm	CD20	2H7	Fluidigm
148Nd	CD57	HCD57, Biolegend	in-house
149Sm	CTLA-4	14D3, eBioscience	in-house
150Nd	MIP1β	D21-1351	Fluidigm
151Eu	CD107a	H4A3	Fluidigm
152Sm	TNFα	Mab11	Fluidigm
153Eu	CD45RA	HI100	Fluidigm
154Sm	CD3	UCHT1	Fluidigm
155Gd	CD28	L283, BD	in-house
156Gd	CD38	HB-7, BD	in-house
157Gd	HLA-DR	G46-6, BD	in-house
158Gd	CD33	WM53	Fluidigm
159Tb	GMCSF	BVD2-21C11	Fluidigm
160Gd	CD14	M5E2	Fluidigm
161Dy	IFNγ	4S.B3, eBioscience	in-house
162Dy	CD69	MCA 1442	Fluidigm
163Dy	TCRγδ	B1, Biolegend	in-house
164Dy	IL-17	N49-853	Fluidigm
165Ho	CD127	A019D5	Fluidigm
166Er	IL-2	MQ1-17h12	Fluidigm
167Er	CD27	L128	Fluidigm
168Er	CD154 (CD40L)	24-31	Fluidigm
169Tm	CCR7	150503, R&D Systems	in-house
170Er	PD1	EH12.1, BD	in-house
171Yb	Granzyme B	GB11	Fluidigm
172Yb	PD-L2	24F.10C12	Fluidigm
173Yb	Perforin	B-D48, Abcam	in-house
174Yb	CD16	3G8, Biolegend	in-house
175Lu	PD-L1	29E.2A3	in-house
176Yb	CD56	NCAM16.2	DVS

Table 2

Internet Resources

Resource	Purpose	Link
Antibodies	Metal-tagged antibodies for CyTOF	https://www.fluidigm.com/reagents/mass-cytometry
Antibody labeling kits	MaxPar antibody labeling kit for in-house metal tagging of antibodies	https://www.fluidigm.com/reagents/mass-cytometry
Panel Builder	Staining panel design tool	https://www.fluidigm.com/
Normalizer	Normalization of CyTOF data using calibration beads	https://github.com/nolanlab/bead-normalization/releases
SPADE, viSNE, Citrus, gating	High-dimensional data analysis tools	https://www.cytobank.org/

Table 3

Stimulation of Cells

Reagent	Intermediate Stock Dilution	Final Concentration (Unstimulated)	Final Concentration (PMA+Ionomycin)
Brefeldin A (5mg/ml in DMSO)	1:10 in CyPBS	5 μg/ml 1:100 of intermediate stock	5 μg/ml 1:100 of intermediate stock
Monensin (1000×)	1:10 in CyPBS	1× 1:100 of intermediate stock	1× 1:100 of intermediate stock
PMA (1mg/ml in DMSO)	1:1000 in CyPBS	-	10 ng/ml 1:100 of intermediate stock
Ionomycin (1mg/ml in DMSO)	1:10 in CyPBS	-	1 μg/ml 1:100 of intermediate stock