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# CyTOF supports efficient detection of immune cell subsets from small samples

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## Abstract

Analysis of immune cell states is paramount to our understanding of the pathogenesis of a broad range of human diseases. Immunologists rely on fluorescence cytometry for cellular analysis, and while detection of 8 markers is now well established, the overlap of fluorescent signals limits efficiency. Mass cytometry or CyTOF (Cytometry by Time-Of-Flight) is a new technology for multiparameter single cell analysis that overcomes many limitations of fluorescence-based flow cytometry and can routinely detect as many as 40 markers per sample. This technology provides tremendous detail for cellular analysis of multiple cell populations simultaneously and is a powerful technique for translational investigations. Here we present reproducible detection of immune cell subsets starting with as few as 10,000 cells. Our study provides methods to employ CyTOF for small samples, which is especially relevant for investigation of limited patient biopsies in translational and clinical research.

### Keywords

CyTOF; multidimensional single cell analysis; translational research; mass cytometry; translational immunology; leukocytes

## INTRODUCTION

Efficient and reproducible detection of immune cells and their functional states is paramount to our understanding of the pathogenesis of a broad range of human diseases. Currently, immunologists rely on fluorescence cytometry for analysis of the immune system on a



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cellular level. While detection of 8 markers in a sample is now well established for flow cytometry, a frequent difficulty is the overlap of emission spectra of fluorescent antibody labels. Mass cytometry or CyTOF (Cytometry by Time-Of-Flight) is a new technology for multiparameter single cell analysis, which uses heavy metal ions as antibody labels and thus overcomes many of the limitations of fluorescence-based flow cytometry. Due to the precision of distinct mass resolution, CyTOF has virtually no bleed through between channels, and essentially no background, as the rare earth metal tags are absent from cells. Thus CyTOF studies can combine ~ 40 labels in a sample. CyTOF has recently been employed to characterize peripheral blood cells in detail (Bendall et al., 2011) as well as NK cells (Horowitz et al., 2013), γδ cells in Celiac disease (Han et al., 2013), responding phenotypes in cancer (Irish and Doxie, 2014), and even holds the promise of examining solid tumors (Giesen et al., 2014).

In our studies of individual immune variations associated with viral susceptibility, we employ panels of antibodies to profile immune cell status from subjects in stratified cohorts of disease severity (Qian et al., 2013; Qian et al., 2014a; Qian et al., 2014b). Using fluorescence cytometry, a sample can be reproducibly labeled for 8 distinct markers, while using CyTOF, we can increase the detection to 40 markers. Thus, a single sample can provide functional results of multiple cell lineages simultaneously, which greatly increases the efficiency of the experiment. However, the detection efficiency of CyTOF for low cell numbers –such as from pediatric subjects or where sample is limited– is unclear. We have undertaken the current study to determine the limits of CyTOF detection for reproducible characterization of a small number of immune cells.

## MATERIALS AND METHODS

### Human Subjects

Heparinized blood from healthy volunteers was obtained after written informed consent under the guidelines of the Human Investigations Committee of Yale University School of Medicine. Donors had no acute illness, and took no antibiotics or nonsteroidal anti-inflammatory drugs within one month of enrollment. Biopsy of discarded surgical skin samples from healthy donors was obtained as approved without identifiers.

### Preparation of blood and skin cells

Human peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll-Hypaque (GE Healthcare, NJ) as described previously (Qian et al., 2012). Immune cell subsets were purified from sterile skin biopsies (1-5 $cm^2$) as described (Angel et al., 2007) with some modifications. After removing subcutaneous fat and epidermis, the dermis was minced into small pieces (less than 2 mm in thickness) and incubated for 20 min at 37 °C with 0.3% trypsin (Worthington, Lakewood, NJ) followed by grinding with glass slides. The cell suspension (0.3-1.4 × $10^6$ cells) was filtered through a cell strainer (45 μM, BD Falcon®) and nylon mesh to remove debris. Immune cells from blood and skin were used on the day of isolation.

### CyTOF marker labeling and detection

Labeling of PBMCs and skin cell suspensions was conducted by two independent lab members according to established conditions for CyTOF (Horowitz et al., 2013). Briefly, viability of cells in 400 μl RPMI in wells of a 96-deepwell plate (Thermo Fisher Scientific, Waltham, MA) was identified by incubation with 50 μM cisplatin (Sigma-Aldrich, St. Louis, MO) for 1 min at RT and quenched with 500 μl fetal bovine serum. Next, cells were incubated for 30 min at 4°C with a 50 μl cocktail of metal conjugated antibodies selected from the MaxPar® Human Peripheral Blood Phenotyping Panel Kit (Fluidigm/DVS Science, Sunnyvale, CA). The metal content of the antibodies used is listed in Table 1; note that batch variation in metal content may be a relevant limitation to detection. Cells were washed, fixed and permeabilized (BD Pharm Lyse™ lysing solution, BD FACS Permeabilizing Solution 2, BD Biosciences, San Jose, CA) for 10 min each at RT. Total cells were identified by DNA intercalation (0.125 μM Iridium-191/193 or MaxPar® Intercalator-Ir, Fluidigm/DVS Science) in 2% PFA at 4°C overnight. Labeled samples were assessed by the CyTOF2 instrument (Fluidigm) using a flow rate of 0.045 ml/min.

### Cell subset identification and statistical analysis

Multidimensional data generated by CyTOF was assessed using SPADE on the Cytobank platform (Qiu et al., 2011; Chen and Kotecha, 2014). Gating of cell subsets followed exclusion of debris (Iridium-; $DNA^{-}$), cell doublets (Iridium high; $DNA^{hi}$) and dead cells ($cisplatin^{+}$). To assess the ability to detect specific PBMC subsets, we compared detection as a function of input cell number for cell subsets defined as in Table 1: T cells ($CD45^{+}CD3^{+}$), B cells ($CD45^{+}CD19^{+}CD20^{+}$), NK cells ($CD45^{+}CD3^{-}CD19^{-}CD20^{-}CD14^{-}$HLA-$DR^{-}CD38^{+}CD16^{+}$), monocytes ($CD45^{+}CD3^{-}CD19^{-}$ $CD20^{-}CD14^{+}$HLA-$DR^{+}$), myeloid DC (mDC, $CD45^{+}CD3^{-}CD19^{-}CD20^{-}CD14^{-}HLADR^{+}CD11c^{+}CD123^{-}$), and plasmacytoid DC (pDC, $CD45^{+}CD3^{-}CD19^{-}CD20^{-}CD14^{-}HLADR^{+}CD11c^{-}CD123^{+}$). For equivalence testing of recovery in PBMC samples over the sample dilutions, Schuirmann's Two One-sided tests (TOST) approach was used (Schuirmann, 1987). The upper and lower bounds were defined as the lower and upper range of the “gold standard” starting concentration of $1 \times 10^{6}$ PBMCs, with each subsequent dilution compared with this. A right one-sided test was applied to the lower bound and a left to the upper bound using alpha = 0.1 or an 80% confidence limit. The larger of the two p-values was retained as the p-value of the equivalence test. For all calculations and tests, the lower confidence limit was truncated at zero percent. All analyses were performed with SAS v9.3 (SAS Institute©, Cary, SC, USA).

## RESULTS

To determine the minimal cell number detectable by CyTOF, we isolated PBMCs from healthy donors and labeled cells with a cocktail of markers for human immunophenotyping (Table 1), which includes markers for major peripheral blood cell subsets, e.g., T cells, B cells, NK cells, monocytes, and myeloid and plasmacytoid dendritic cells (mDC and pDC). Freshly isolated PBMCs ($1 \times 10^{6}$) were labeled for analysis by CyTOF and cell lineages were identified using SPADE, a clustering program designed for CyTOF data sets to visually and quantitatively gauge the phenotypic diversity between cell types and donors

(Qiu et al., 2011). Detection of the cell lineage markers was efficient from samples of $1 \times 10^6$ PBMCs and identified major cell subsets as expected (Fig. 1A).

To determine the lower practical limits of detection, we reduced the number of PBMCs by serial dilution prior to labeling. We were able to detect the main cell lineages in PBMC samples from starting PBMC numbers of $1 \times 10^6$ to as low as $1 \times 10^4$, and the more abundant lineages such as T and B cells were detected from starting samples of $1 \times 10^3$ PBMCs (Fig. 1B). The percentage of each cell lineage detected remained relatively constant from $1 \times 10^6$-$1 \times 10^4$ for lymphocytes (Fig. 1C) and statistical analysis of the samples revealed that detection was equivalent from $1 \times 10^6$-$1 \times 10^4$ cells for T and B lymphocytes (Table 2). Remarkably, mDC (~1% in PBMC) and pDC (~0.2% in PBMC), considerably more rare cell lineages, were detected from PBMC starting numbers as low as $3 \times 10^3$ (Fig. 1B), and detection for NK cells and mDCs, which had limited variability among the samples, remained statistically equivalent from as few as $1 \times 10^3$ starting cells (Table 2). Surprisingly, we noted lower efficiency of detection of monocytes – despite higher proportions in PBMCs than mDCs – which may reflect limitations of the antibodies used here (CD45, HLA-DR, and CD14) to capture the many classes of monocytes in circulation (Wong et al., 2011). Indeed, our study was not designed to identify the full spectrum of cells in each subset, but rather to determine the number of input cells necessary to identify cells labeling with a particular defined set of markers. Notably, addition of an unrelated commercially available and readily cultivatable cell line (HL-60 cells) as carrier cells, labeled with a distinct marker (Qdot-CD45), did not improve the detection of PBMC subsets (data not shown). These studies demonstrate quantitative cell subset identification in small samples approaching cell numbers that may reasonably be obtained in translational settings or from tissue biopsies.

As further demonstration of CyTOF detection in the range relevant for translational studies, we labeled immune cells from biopsies of skin from healthy human subjects. From starting total cell numbers of $0.6\text{-}1.4 \times 10^6$ per biopsy, we detected multiple lineages of $CD45^+$ immune cells relevant for studies of immune infiltrates (Fig. 2) as well as $HLA\text{-}DR^+$ non-immune ($CD45^-$) cells that may be of endothelial origin (Angel et al., 2007). These findings provide excellent support for the use of CyTOF in studies of immune infiltrates in skin such as would be relevant for pathologic specimen in lupus and other types of systemic or localized diseases affecting skin such as melanoma and psoriasis (Nestle et al., 2009; Kirchhof and Dutz, 2014).

## CONCLUSIONS

Translational studies are critical to investigate relevant cellular responses in human disease, as conclusions drawn from animal models may not reflect pathophysiological responses (Seok et al., 2013). Yet in many cases the limited amount of donated blood or tissues from human subjects is limiting for in depth investigation. The recent introduction of CyTOF offers tremendous opportunities for high-dimensional analysis of cellular samples including detection of up to 5 times more markers from each sample. However, to be most useful for translational investigators, CyTOF detection would extend to detection from very limited size samples, such as 1-2 ml of blood, or a small tissue core biopsy. The studies presented

here demonstrate reproducible detection of multiple immune cell lineages starting from only 10,000 cells (and in some cases even fewer) in samples from healthy donors. These detection limits should be confirmed in samples from individuals with the relevant disease or distinct immune condition, as they may show greater variability over the course of infection or progression of disease. In addition, samples should include metal-labeled calibration beads to normalize over instrument fluctuations, which might be particularly relevant at the lower limits of detection (Finck et al., 2013). Nevertheless, with these precautions in place, our results support in depth investigation of clinically relevant but quite limited samples.

CyTOF detection of multiparameter antibody panels provides tremendous detail for cellular analysis of immune subsets. Further, CyTOF analysis can be combined with other recent advances in technology, such as intracellular signaling pathways with high-resolution digital imaging (Qian and Montgomery, 2012) and transcriptional profiling (Blankley et al., 2014), both quantitative assays that are feasible to perform from < 5 ml of blood. Such in depth phenotyping and functional assessments from small samples ushers in new efficiency for translational studies. These data can be compiled for detailed analysis of complex interactions to generate a systems level understanding of disease susceptibility or pathogenesis, and can provide valuable insight into mechanisms that underlie cellular function, such as immune dysregulation in specialized cohorts, following response to treatment, or to highlight key mechanisms in cells from therapy responders and nonresponders.

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## Highlights

- Mass cytometry or CyTOF is a new technology for multiparameter single cell analysis
- CyTOF can detect as many as 40 markers per sample with minimal background or overlap
- CyTOF can show reproducible detection of cells starting from as few as 10,000 cells
- CyTOF detection of very small samples may be relevant for studying patient biopsies

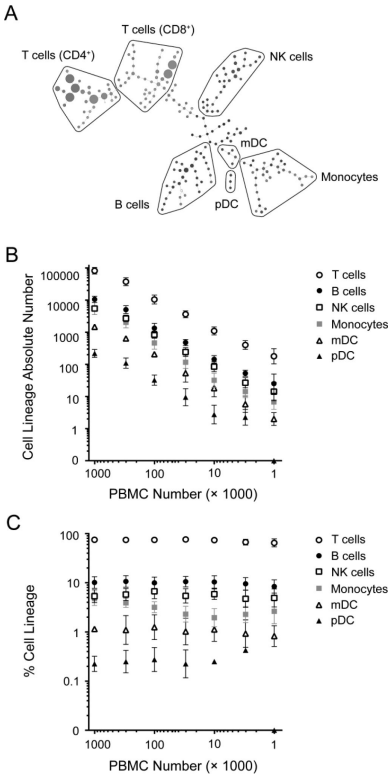


**Figure 1. Detection of cell lineages in different starting numbers of PBMCs**

PBMCs were labeled with markers of immune cell lineages and detected by CyTOF. Representative data from $1 \times 10^6$ PBMCs shown by SPADE tree outline of cell lineages. The node size represents the number of cells from $10^6$ PBMCs and shading intensity represents CD3 expression level (A). The means ± sem for absolute cell numbers (B) and percentages of each cell lineage in PBMCs (C) detected from indicated starting numbers of PBMCs are represented; n = 4 independent donors.

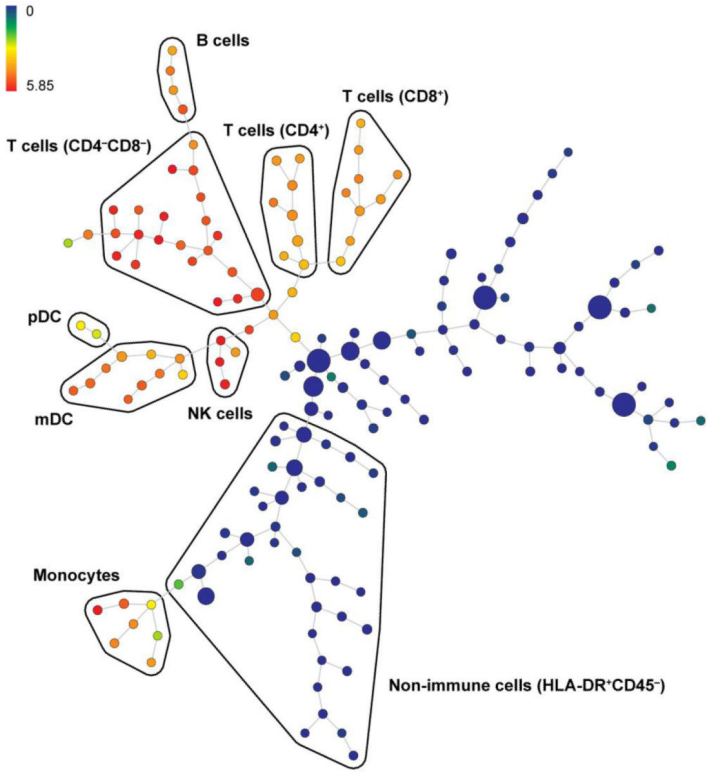


**Figure 2. Multidimensional analysis of immune cells from skin**

Cells harvested from healthy skin were labeled with markers for immune cell lineages and detected by CyTOF. Cell subset representation uses analysis platform SPADE (Qiu et al., 2011); n = 3 independent donors.

## Table 1

Characteristics of metal-conjugated antibodies and cell subsets identified

| Antibody marker | Metal | Metal Atoms/Antibody | T cells | B cells | NK cells | Monocytes | mDCs | pDCs | Non-immune Skin |
|---|---|---|---|---|---|---|---|---|---|
| CD3 | 170Er | 101 | X | | | | | | |
| CD4 | 145Nd | 103.2 | X | | | | | | |
| CD8a | 146Nd | 92.1 | X | | | | | | |
| CD11c | 159Tb | 106.4 | | | | | X | | |
| CD14 | 160Gd | 96.6 | | | | X | | | |
| CD16 | 148Nd | 83.6 | | | X | | | | |
| CD19 | 142Nd | 209.8 | | X | | | | | |
| CD20 | 147Sm | 103 | | X | | | | | |
| CD38 | 172Yb | 97.5 | | | X | | | | |
| CD45 | 154Sm | 147.16 | X | X | X | X | X | X | |
| CD123 | 151Eu | 44.4 | | | | | | X | |
| HLA-DR | 174Yb | 108.9 | | | | X | X | X | X |

Table shows cell lineage markers with the metal conjugate and metal atoms per antibody for the lot number used. Cell type gating strategy is as in Materials and Methods.

## Table 2

Equivalence testing comparing starting concentrations of PBMCs.

| P value of difference compared to $1 \times 10^6$ cells* | | | | | | |
|---|---|---|---|---|---|---|
| **Starting cell number (× 1000)** | **T cells** | **B cells** | **NK cells** | **Monocytes** | **mDC** | **pDC** |
| 300 | 0.002 | <.001 | 0.005 | 0.049 | 0.003 | 0.123 |
| 100 | 0.053 | <.001 | 0.184 | 0.304 | 0.011 | 0.249 |
| 30 | 0.089 | <.001 | 0.019 | 0.828 | 0.012 | 0.114 |
| 10 | 0.061 | 0.002 | 0.029 | 0.916 | 0.051 | 0.259 |
| 3 | 0.815 | 0.025 | 0.005 | 0.853 | 0.014 | 0.711 |
| 1 | 0.802 | 0.135 | 0.049 | 0.747 | 0.025 | 0.907 |

*Schuirmann's Two One-s ided tests (TOST) for equivalence testing of recovery in PBMC samples with each dilution compared to $1 \times 10^6$ starting cells (n= 4 healthy donors). P value cutoff of 0.1 was used to establish equivalence; non-equivalent values are shaded.