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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.

1. 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 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, 2014a, 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.

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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.

2. Materials and methods

2.1. 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 non-steroidal anti-inflammatory drugs within one month of enrollment. Biopsy of discarded surgical skin samples from healthy donors was obtained as approved without identifiers.

2.2. Preparation of blood and skin cells

Human peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll-Paque (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 ($0.3–1.4 \times 10^6$ cells) was filtered through a cell strainer (40 μm, BD Falcon®) and nylon mesh to remove debris. Immune cells from the blood and skin were used on the day of isolation.

2.3. 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.

2.4. Cell subset identification and statistical analysis

Multidimensional data generated by CyTOF was assessed using SPADE on the Cytobank platform (Chen and Kotecha, 2014; Qiu et al., 2011). Gating of cell subsets followed exclusion of debris ($Iridium^-$; DNA^-), cell doublets ($Iridium^{hi}$; 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 ($CD45^+CD3^-CD19^-CD20^-CD14^-HLA\text{-}DR^-CD38^+CD16^+$), monocytes ($CD45^+CD3^-CD19^-CD20^-CD14^+HLA\text{-}DR^+$), myeloid DC (mDC, $CD45^+CD3^-CD19^-CD20^-CD14^-HLA\text{-}DR^+CD11c^+CD123^-$), and plasmacytoid DC (pDC, $CD45^+CD3^-CD19^-CD20^-CD14^-HLA\text{-}DR^+CD11c^-CD123^+$). 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 ranges of the "gold standard" starting concentration of 1×10^6 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 value 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).

3. Results

To determine the minimal cell number detectable by CyTOF, we isolated PBMCs from healthy donors and labeled cells with a

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	159 Tb	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.