3D Flow™ Analysis: A simple and integrated workflow for deep proteomic and transcriptomic analysis of sorted cell populations

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Application Highlights

- Integrated flow cytometry cell sorting with RNA and protein analysis on the nCounter® system
- Simultaneous analysis of 770-plex RNA and 30-plex protein from as few as 5,000 cells (500 cells protein only)
- No PCR or amplification required
- Simple analysis solution with nSolver™ software

Introduction

High parameter flow and mass cytometry and the recent advances in single cell RNAseq have dramatically increased our capacity to identify previously unknown cell sub-types and further characterize known cell populations. Despite these advances, our understanding remains incomplete due to the immense heterogeneity in immune cell phenotype and function.

Understanding the role of the immune system across a wide spectrum of diseases requires increasingly sophisticated analytical approaches to discover novel biology, potentially leading to the discovery of new therapeutic targets and biomarker signatures.

Here, we highlight the development of 3D Flow Analysis, a streamlined workflow that seamlessly integrates standard immune cell FACS with nCounter analysis of both RNA and protein from rare cell populations (Figure 1). The NanoString nCounter system enables the highly multiplexed, digital detection of both RNA and protein from a single biological specimen. Specifically, the Vantage 3D RNA:Protein Immune Cell Profiling Assay contains curated content to assess key aspects of T cell biology, characterizing the immune response in oncology, infectious disease, and autoimmune disorders, 30 cell surface proteins focus on deep characterization of multiple T cells subtypes and 770 RNA targets cover genes across different immune cell types, common checkpoint inhibitors, CT antigens, and the adaptive and innate immune response (Table 1).

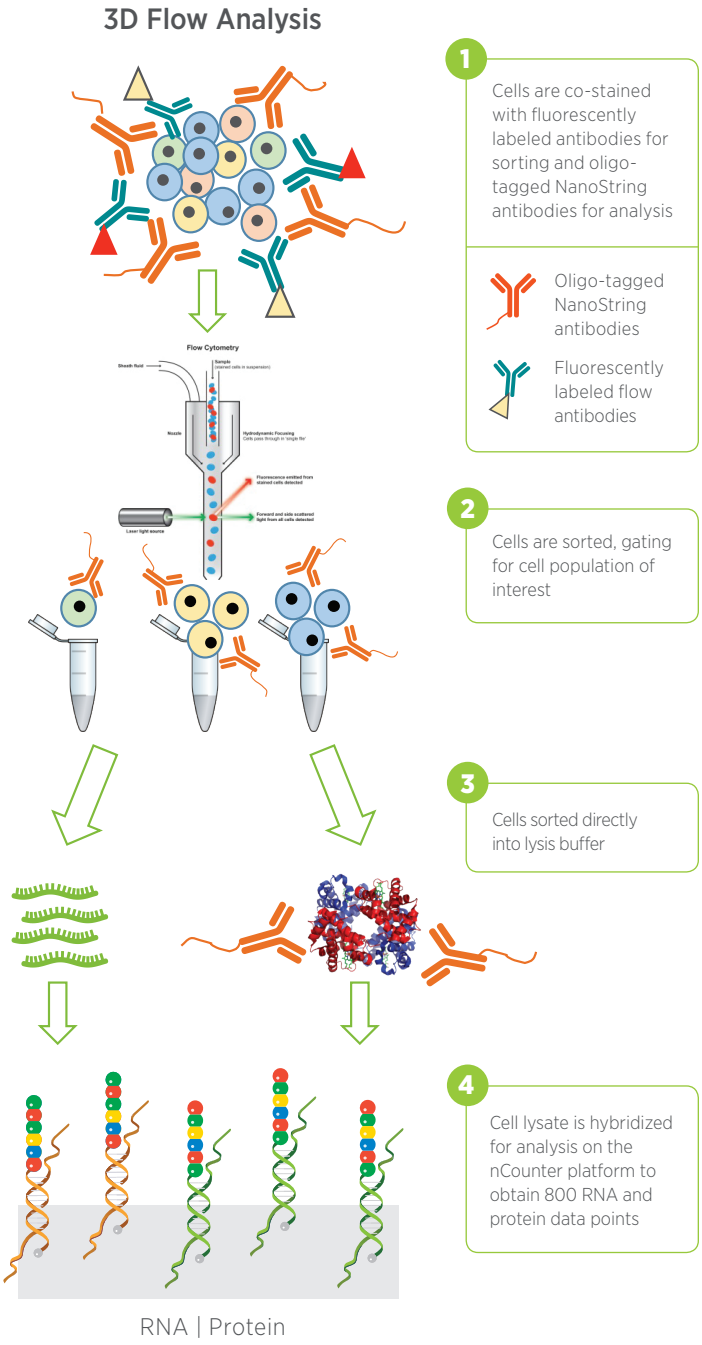

FIGURE 1 Workflow for the isolation and deep proteomic and genomic analysis of defined sorted cell populations

3D Flow Analysis

As an example of 3D Flow Analysis, PBMC were co-stained with fluorophore-labeled antibodies for flow cytometry sorting and 30 DNA-barcoded NanoString antibodies using the Vantage 3D RNA:Protein Immune Cell Profiling Assay. Live CD3+ and CD4+ T cells were isolated for RNA:Protein analysis. To achieve optimal results, fluorophore-conjugated antibodies for cell sorting were selected to minimize competition or cross-blocking activity against the NanoString antibodies. To facilitate selection of flow cytometry sorting antibodies that are compatible with NanoString oligo-tagged antibodies, NanoString has tested pre-defined BioLegend antibody panels that minimize interactions (Table 2). These flow cytometry panels are designed to be used in cytometers equipped with blue and red lasers, or blue, green, yellow-green, red laser combination. These panels also leave the PE channel empty, to accommodate additional, user-defined antibodies conjugated to that fluorophore.

Flow cytometry is often used to analyze cells that are a small fraction of the sorting sample input. In this experiment, isolated CD4+ cells were directly sorted into lysis buffer and analyzed on the nCounter system, which requires no molecular biology, such as RNA purification or downstream sequencing library construction. This workflow enables simplified downstream sample handling and ensures minimal post-sort cell loss. To demonstrate the capabilities of 3D Flow Analysis in profiling rare cell populations, the number of CD4+ cells were titrated to determine the sensitivity of the workflow (Figure 2). Quantitative protein data was obtained with as few as 500 cells, showing high correlation to data obtained from 5,000 cells.

To obtain, RNA and protein data from a single sorted population, 5,000 stimulated and unstimulated CD3+ T cells were sorted, lysed and analyzed using the Vantage 3D RNA:Protein Immune Cell Profiling Assay. The volcano plot reveals both genes and proteins whose expression is altered by stimulation with CD3 and CD28 (Figure 3). The data was analyzed with nSolver software, allowing a workflow that moves from heterogenous sample, to data on pure cell populations in only 2 days.

The work described here demonstrates the ability of 3D Flow Analysis to provide a comprehensive solution for deep profiling of unique immune cell populations. 3D Flow Analysis seamlessly integrates with current flow sorting protocols to enable collection of high parameter, multi-omic data with minimal modifications to current FACS workflows. The ability to obtain quantitative data from minimal cell numbers allows new questions to be addressed and provides a unique solution for deep interrogation of precious cells of interest.

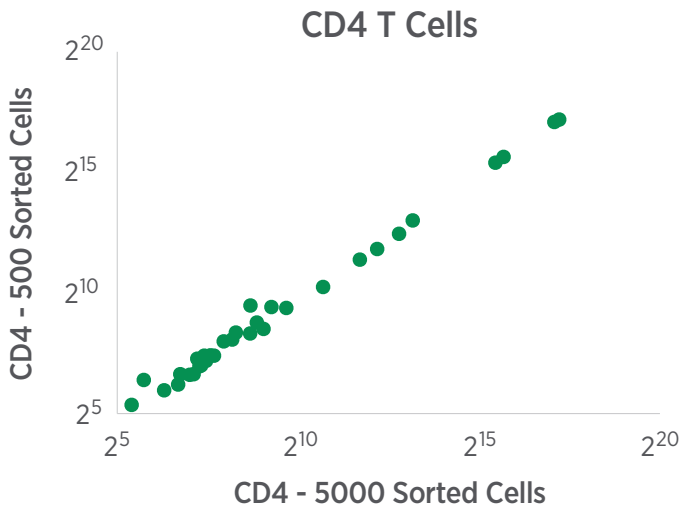

FIGURE 2 Correlation of the expression of 30 protein markers in CD4+ T cells across different cell inputs (500 vs. 5,000). Values on X and Y axis are normalized digital NanoString counts

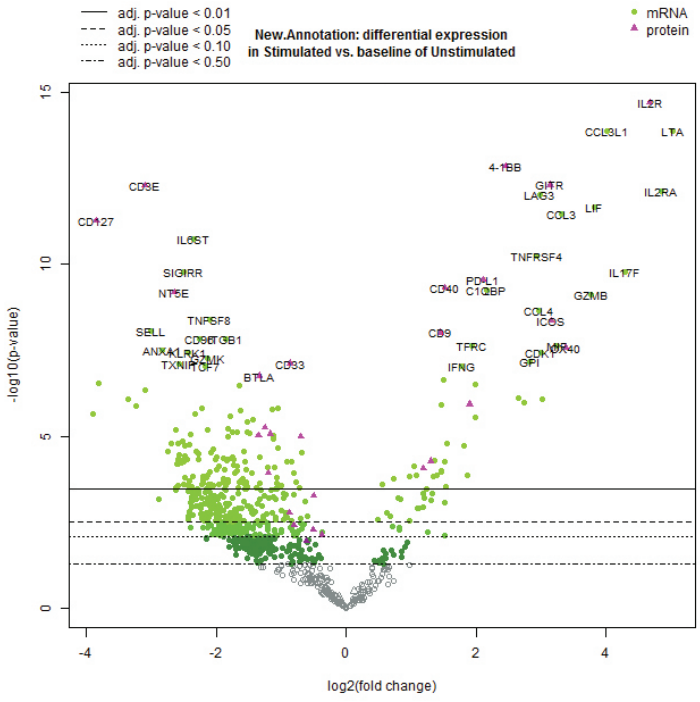

FIGURE 3 Volcano plot showing stimulation induced changes in protein and gene expression from 5,000 CD3+ cells

Ordering Information

Biological question (What)	Insight (Why)	Markers (How)
Elucidate T cell biology	• Deeply analyze extracellular markers across T cell subtypes • Characterize different T cell populations	4-1BB, BTLA, CD27, CD28, CD3, CD40, CD40L, CD45RO, CD8, CTLA-4, GITR, ICOS, IL2RA, NT5E, OX40, PD-1, PD-L1, CD4
Quantify T cell activation	• Profile the reactivity of specific T cell populations • Analyze levels of activation with T cell specific activation markers	4-1BB, CD27, CD28, CD40, CD40L, CD45RO, CD8, ICOS, IL2RA, KIR3DL1, OX40
Characterize T cell inhibition	• Measure immune checkpoints and exhaustion markers on T cells • Relative expression of inhibition/activation markers	CTLA-4, PD-1, PD-L1, PD-L2, NT5E, BTLA, GITR
Analyze immune cell populations	• No sort is perfect- quantify the presence of a wide variety of possible contaminating cells	CD127, CD14, CD163, CD33, CD3, CD68, HLA-DRA, NCAM, NKP46, KIR3DL1
Verify with internal controls	• Check data quality- ensure immune content is present and consistent across samples • Immune cell population level information	CD3, CD4, CD8, CD9, CD45

TABLE 1 Protein targets in the Vantage 3D RNA:Protein Immune Cell Profiling Assay

Product	Product Description	Catalog number
BioLegend: Human CD4 Treg ID Panel	Flow cytometry antibody panel to sort human regulatory T cells Contains: anti-CD127, anti-CD25, anti-CD3, anti-CD4 and live/dead stain	BioLegend - 362251
BioLegend: Human Activated CD8 T Cell ID Panel	Flow cytometry antibody panel to sort activated human CD8+ T cells Contains: anti-CD25, anti-CD3, anti-CD8a, anti-CD45RO and live/dead stain	BioLegend - 362252
NanoString: Vantage 3D RNA:Protein Immune Cell Profiling Assay	770 immune focused mRNA and 30 extracellular proteins to profile immune cells with 3D Flow Analysis	NanoString - VRPC-HIPS2-12

TABLE 2 Description and ordering information on the products referenced

References

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3. Abey, SK, Yuana, Y, et al. (2016) Lysozyme association with circulating RNA, extracellular vesicles, and chronic stress. BBA Clin. Dec 20;7:23-35.

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