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Application Highlights

- Integrated flow cytometry cell sorting with multiplex 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 if protein only)
- No PCR or amplification required
- Integrated analysis solution with nSolver™ software

High parameter flow and mass cytometry and the recent advances in single cell RNAseq have dramatically increased the number of known immune cell sub-types. Despite these advances, our understanding remains incomplete due to the immense heterogeneity in immune cell phenotype/function and corresponding role in health and disease. This complex challenge requires increasingly sophisticated analytical approaches to discover novel biology, potentially leading to the discovery of new therapeutic targets and biomarker signatures.

This application note highlights the development of a streamlined workflow, 3D Flow Analysis, that seamlessly integrates standard immune cell flow sorting with downstream 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 nCounter Vantage 3D™ RNA:Protein Immune Cell Profiling Assay, which interrogates 30+ cell surface proteins and 770 immune-related RNA is especially relevant to researchers characterizing the immune response in oncology, infectious disease and autoimmune disorders.

The Vantage 3D RNA:Protein Immune Cell Profiling Assay contains curated content to assess key aspects of T cell biology. 30 cell surface protein focused on deep characterization of multiple T cells subtypes (Table 1). 770 RNA targets cover genes across different immune cell types, common checkpoint inhibitors, CT antigens, and the adaptive and innate immune response.

As an example of 3D Flow Analysis capabilities, PBMC were co-stained with fluorescently-labeled antibodies for flow sorting, as well as 30 DNA-barcoded NanoString antibodies, plus three controls, from the Vantage 3D RNA:Protein Immune Cell Profiling Assay. Live, CD3+, CD4+ T cells were isolated by standard flow cytometry methods. To ensure that there was not competition between the fluorescently-labeled and the NanoString DNA barcoded antibody for the same target, clones against different epitopes of the target protein were utilized.

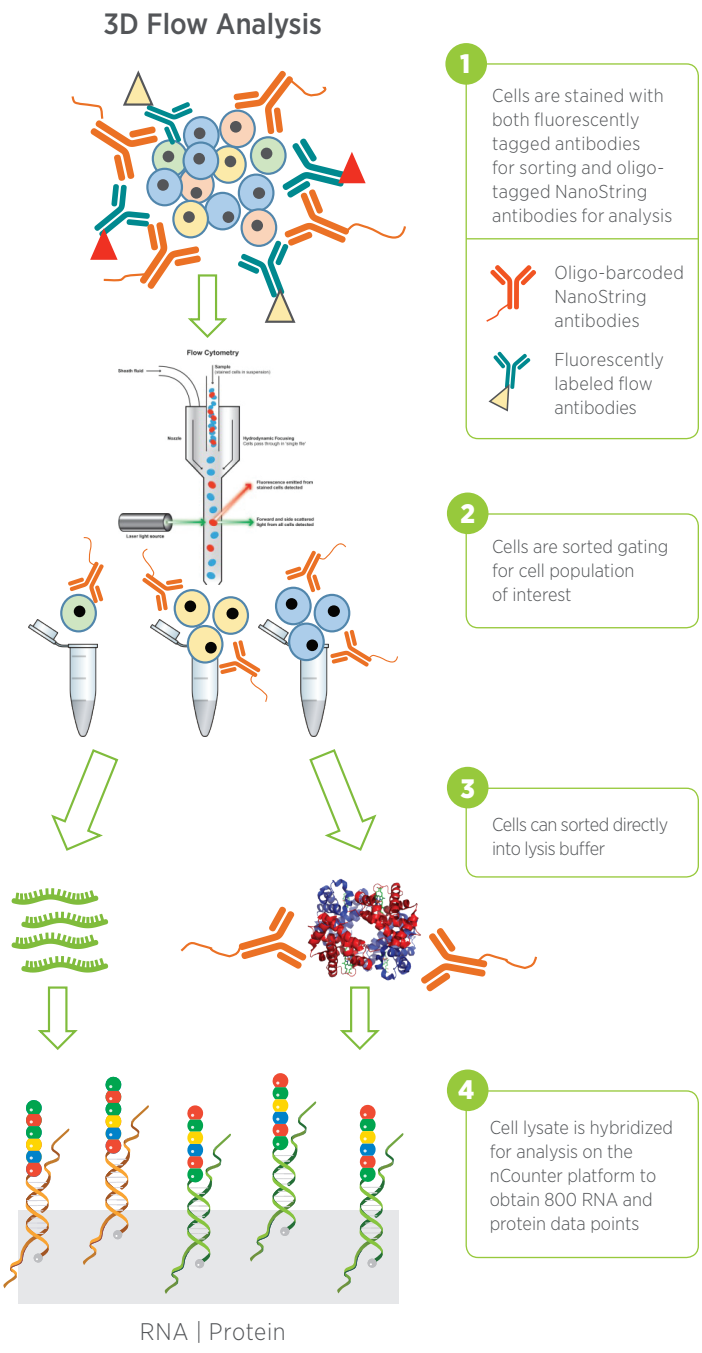

FIGURE 1 Workflow for the isolation and deep proteomic and genomic analysis of defined cell sorted populations.

The isolated CD4+ cells were directly sorted into lysis buffer and analyzed on the nCounter system without the need for additional molecular biology methods, such as RNA purification, cDNA generation or downstream sequencing library construction. By utilizing a direct to lysis buffer sorting protocol, 3D Flow Analysis enables simplified downstream sample handling and ensures minimal post-sort cell loss.

Flow cytometry is often used to analyze cells that are a small fraction of the sorting sample input. To demonstrate the capabilities of 3D Flow Analysis in profiling rare cell populations, the number of target cells were titrated to determine the sensitivity of the workflow (Figure 2). Quantitative protein data was obtained with as few as 500 cells, highlighting the sensitivity of the 3D Flow assay. To obtain multi-omic, RNA and protein, data from a single sorted population, 5,000 stimulated and unstimulated CD3+ T cells were each sorted, lysed and analyzed using the Vantage 3D RNA:Protein Immune Cell Profiling Assay for both RNA and protein expression.

The volcano plot (Figure 3) reveals both genes and proteins whose expression is altered by stimulation with CD3 and CD28. 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 3D Flow Analysis capabilities 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 workflows. The ability to obtain quantitative data from minimal cell numbers allows new questions to be addressed and provides a unique solution to deeply interrogate precious cells of interest.

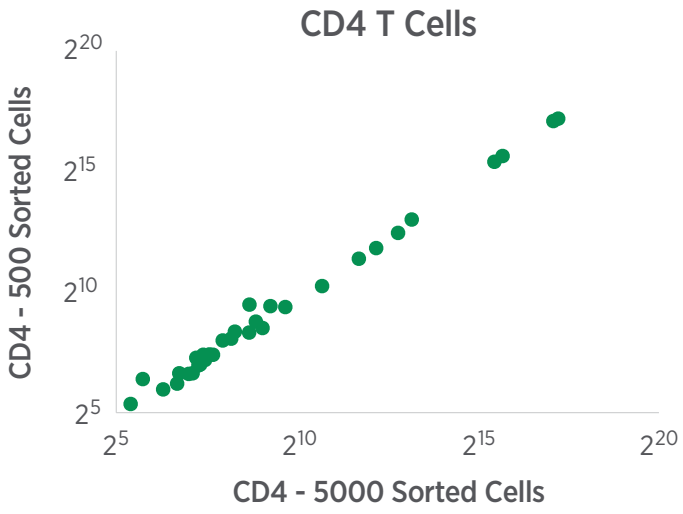

FIGURE 2 Correlation of the expression of 30 protein markers in CD4+ T cells across different cell inputs (500 vs. 5,000). Values on X and Y axis are normalized digital NanoString counts.

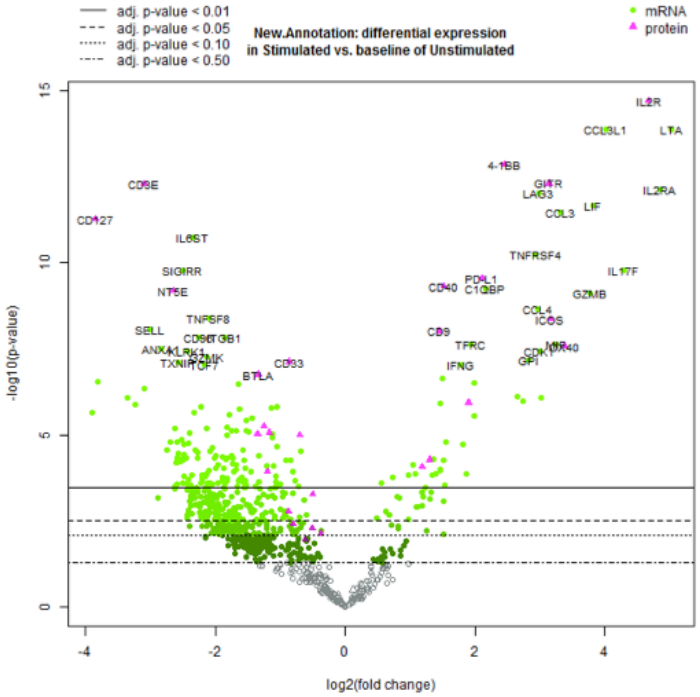

FIGURE 3 Volcano plot showing stimulation induced changes in protein and gene expression from 5000 cells

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 enable deep profiling of rare T cell populations

References

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