**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).

**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

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

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**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 3D DNA-barcode 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.
Elucidate T cell biology
- Deeply analyze extracellular markers across T cell subtypes
- Characterize different T cell populations

Markers (How)
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

Markers (How)
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

Markers (How)
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

Markers (How)
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

Markers (How)
CD3, CD4, CD8, CD9, CD45

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

**Ordering Information**

<table>
<thead>
<tr>
<th>Product</th>
<th>Product Description</th>
<th>Catalog number</th>
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<tbody>
<tr>
<td>BioLegend: Human Activated CD8 T Cell ID Panel</td>
<td>Flow cytometry antibody panel to sort activated human CD8+ T cells Contains: anti-CD25, anti-CD3, anti-CD8a, anti-CD45RO and live/dead stain</td>
<td>BioLegend - 362252</td>
</tr>
<tr>
<td>NanoString: Vantage 3D RNA:Protein Immune Cell Profiling Assay</td>
<td>770 immune focused mRNA and 30 extracellular proteins to profile immune cells with 3D Flow Analysis</td>
<td>NanoString - VRPC-HIPS2-12</td>
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**TABLE 2** Description and ordering information on the products referenced
References


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