Profiling Immune Cell Populations and Functional State with Simultaneous, Multiplexed Detection of RNA and Protein on the nCounter® Platform

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Abstract

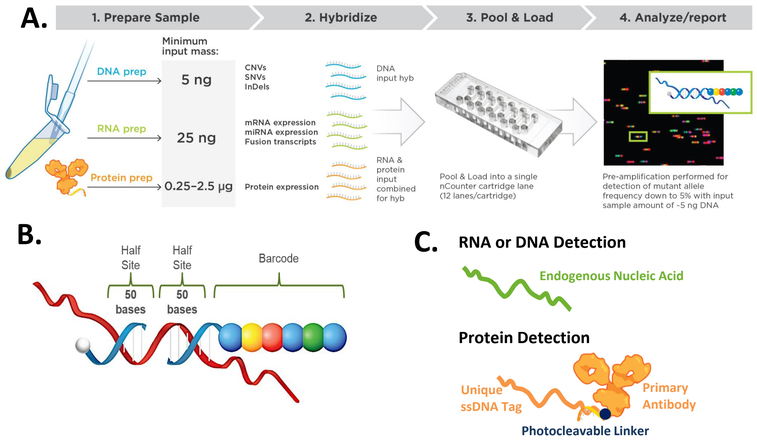
Background:
One of the biggest challenges facing the field of immuno-oncology is development of a comprehensive understanding of how the immune system responds to a tumor. Multi-analyte profiling (DNA, RNA, and protein) from limited sample is crucial to furthering our understanding of tumor immunity. NanoString's nCounter® technology has become an important platform for quantification of transcriptional responses by enabling direct digital quantification of up to 800 targets from a single sample. Now the nCounter technology can simultaneously quantify 770 RNA transcripts plus 30 proteins from as few as 20,000 cells (up to 50,000 primary cells such as PBMCs).

Methods:
NanoString has developed content and methods to allow digital quantification of both cell surface and intracellular protein targets that are essential to immuno-oncology research. These key targets include immune cell population markers, immune checkpoint proteins, transcription factors, chemokines, and cytokines. Protein detection is enabled via primary antibodies, which are covalently linked to single-stranded DNA indexing oligos. Cells are stained with a multiplexed cocktail of barcoded antibodies, and DNA oligos are subsequently released via cell lysis for hybridization to optical barcodes in the standard NanoString workflow. This technique enables quantitative, multiplexed protein detection with over 5 logs of dynamic range.

Results:
As proof of concept, RNA and protein were measured simultaneously from just 50,000 PBMCs treated with phorbol 12-myristate 13-acetate (PMA) and ionomycin, TNFα, IFNγ, or anti-CD3/CD28. NanoString's multiplex RNA and protein detection provides a thorough evaluation of the immunological response in these experiments and demonstrates the value of multi-analyte profiling from the same sample by characterizing the breadth of the response (via 770 RNA measurements) and providing depth to the analysis (via tandem measurements of RNA and protein for key targets). As independent confirmation, the findings were validated with flow cytometry.

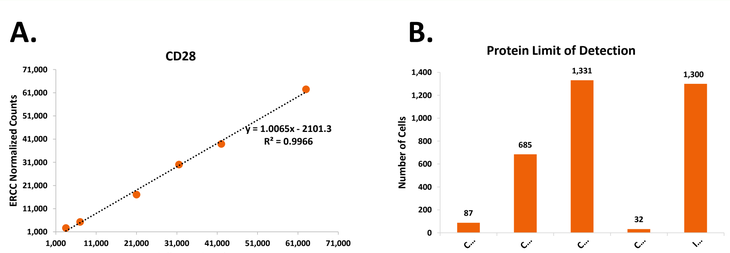
Conclusions:
This advance in multi-analyte, multiplexed digital molecular profiling with low sample input will accelerate immuno-oncology research and may enable the discovery and development of novel immunotherapies and their associated companion diagnostics.

3D Biology™ Technology on the nCounter Platform



A. The nCounter platform can be used to digitally count DNA, RNA, and proteins from a single sample by using fluorescent optical barcodes that attach to a probe, which then binds directly to the analyte of interest. After binding, barcodes are released from the complex and quantitated on the nCounter platform. Results are presented as raw counts of the barcodes detected. **B.** Using this approach, up to 800 targets can be profiled from a small amount of starting material. **C.** Recently, our barcodes have been adapted to detect proteins using oligo conjugated antibodies.

Assay Performance



To assess limit of detection, titration scatter plots were generated. **A.** An example plot for CD28 counts is shown, and the limit of detection was determined based on linear regression. **B.** The limit of detection for additional panel targets was calculated as described and expressed as number of cells.

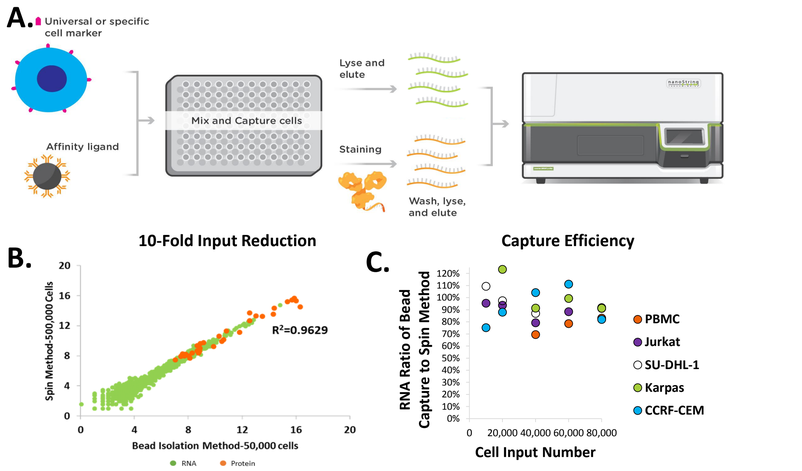
Immune Cell Protein Targets

Immune Cell Profiling Protein	
Stage of Cancer Immunity Cycle	Associated Proteins
(2) Antigen presentation	CD4, CD40, CD40L
(3) Priming and activation	PD-1, PD-L1, PD-L2, IL2R, NCAM, GITR, OX40, CD27, CD28, CD127, CD137
(4–5) Trafficking and infiltration	CD9
(6–7) Recognition of and killing cancer cells	PD-1, PD-L1, PD-L2, BTLA, HLA-DRA
Immune modulation	ICOS, KIR3DL1, NKp46, CTLA-4, CD3E, CD8A, CD14, CD19, CD33, CD68, CD163, CD45RO, NT5E

Immune Cell Signaling Protein	
Immunologic Role	Associated Proteins
Chemokines	CCL5, CXCL5, CXCL8
Cytokines	IL-1β, IL-10, IL-12p40, IL-2, IL-6, TGFβ
Transcription factors	Aiolos, BaF, FoxP3, GATA3, Helios, Ikaros, STAT1, STAT3, T-bet, Th-Pok, IRF4, Bcl-6
Additional immune functions	IDO, GM-CSF, IFNγ, Perforin, IL-15RA

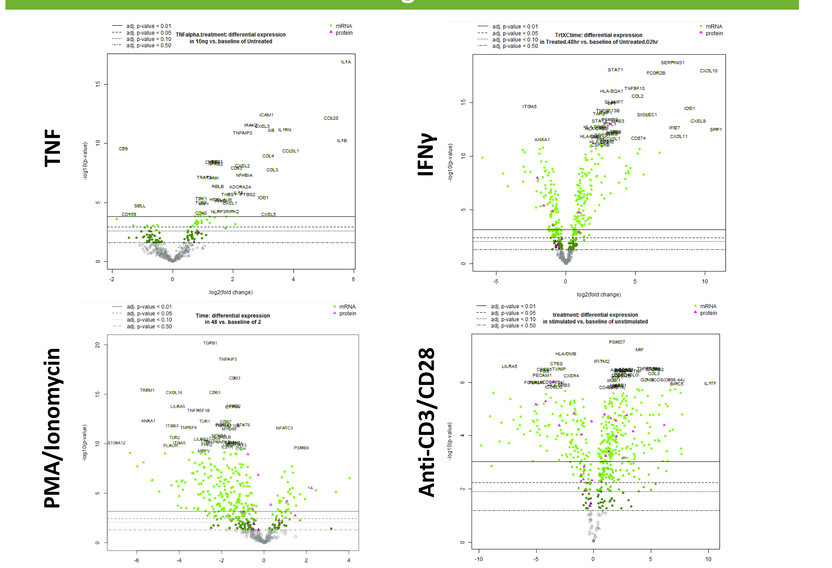
The Immune Cell Profiling panel was designed to profile key cell surface markers that map to the seven stages of the cancer immunity cycle. To further enable characterization of immune response, we developed the Immune Cell Signaling panel, which contains chemokines, cytokines, cytosolic, and nuclear protein targets that regulate key immune pathways, including cell trafficking, inflammation, STAT signaling, cell differentiation, and immune effector functions.

Low Input Protein Preparation



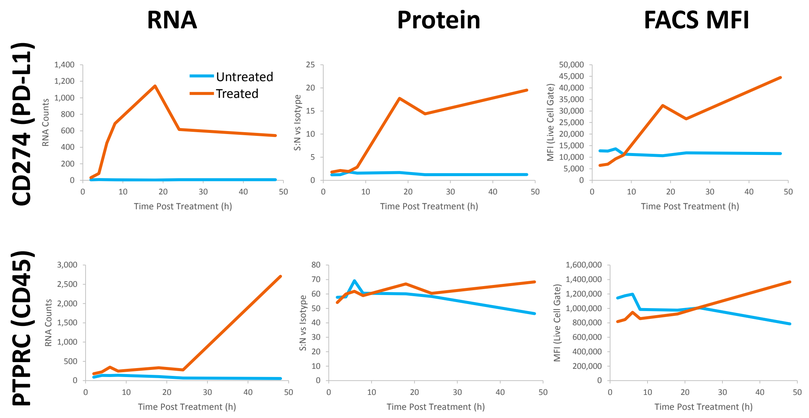
A. To enable low sample input, the RNA:Protein workflow was modified to include Universal Cell Capture technology, which utilizes anti-β_2M antibody coupled to magnetic beads to immobilize nucleated cells for quantification. After cell capture, RNA and protein can be profiled directly using the NanoString barcode chemistry. **B.** This new workflow enabled a 10-fold reduction in cell input, just 50,000 PMBCs, with no change in assay sensitivity. **C.** Additionally, across multiple cell lines, capture efficiency compared to centrifugation was >85% with just 20,000 cells. For PBMCs, capture efficiency was >75% with just 50,000 PBMCs, which was the sample input used for RNA and protein analysis in subsequent studies.

Differential Expression Profiling with nSolver™ Analysis Following Stimuli



In each treatment condition, large and distinct expression changes were observed after treatment of PBMCs for 48 hours. Expression profiling was performed using the 770 RNA transcripts included in the nCounter® Vantage 3D™ RNA:Protein Immune Assays (also known as the PanCancer Immune Profiling panel) and 30 proteins. All multi-analyte volcano plots were generated using nSolver analysis.

Dynamic Immunoprofiling: RNA and Protein Concordance and Disconcordance



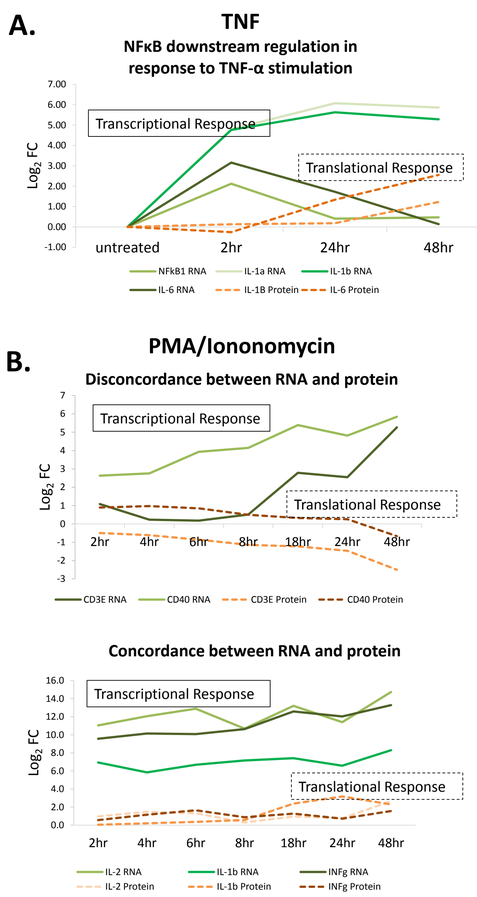
In this experiment, PBMCs were stimulated with PMA-Ionomycin for 2 to 48 hours. Samples were collected periodically and profiled with the RNA:Protein Immune Cell Profiling and Signaling assays to profile cell surface and intracellular protein targets, respectively. Cells were analyzed by flow cytometry to confirm expression patterns on the nCounter platform, and data are presented as MFI. These data show the high concordance between NSTG protein and FACS analysis. PD-L1 shows increased RNA and protein expression following PMA stimulation. However, disconcordance between RNA and protein for CD45 reveal potential post-transcriptional regulation.

Characterizing Differential Regulation of RNA and Protein after Stimulation

PBMCs were profiled post TNF or PMA/Ionomycin stimulation using the nCounter Vantage 3D RNA:Protein Immune assays to investigate 770 RNA targets and up to 30 proteins. Each time course experiment reveals insight into the transcriptional and translation regulation of key targets.

A. Following TNF stimulation, RNA expression for NFκB and IL-6 peak at 12 hours followed by a decrease, whereas IL-1α and IL-1β show sustained upregulation, pointing to potential transcriptional regulation. Furthermore, protein expression of IL-6 and IL-1β show a delayed increase, indicative of translational regulation. IL-6 protein expression peaks sooner than IL-1β, suggesting that translational rates might differ or miRNA regulation might have an influence on protein expression. Together this data set can generate potential hypotheses about regulation of two cytokines transcriptionally controlled by NFκB.

B. Similarly, stimulation with PMA/Ionomycin provides examples of expression profiles where RNA and protein show disconcordance (CD3E and CD40) and other cases where the two analytes concord (IL-2, IL-1β, INFγ).



CONCLUSION

- NanoString's new nCounter Vantage 3D™ RNA:Protein technology, coupled with the Universal Cell Capture workflow, enables RNA and protein profiling from as few as 20,000 cells.
- This low sample input is achieved without compromising cell capture, precision, or LOD.
- NanoString's protein data show high correlation to flow cytometry.
- Measuring RNA and protein simultaneously reveals concordance and disconcordance between RNA and protein expression, uncovering key biological regulation of immune cell function.
- To learn more about 3D Biology technology, visit 3d.nanostring.com.

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