BD™ AbSeq on the BD Rhapsody™ system:

Exploration of single-cell gene regulation by simultaneous digital mRNA and protein quantification

Overview of BD AbSeq antibody-oligonucleotide conjugates.

High-throughput sequencing has allowed researchers to examine hundreds to thousands of RNA targets and greatly advanced our understanding of complex biological systems. However, understanding gene regulation and single-cell heterogeneity often requires information about both RNA and protein expression. There are few technologies that allow concurrent examination of both types of molecules in a single experiment with a single readout. BD AbSeq allows simultaneous measurement of protein and RNA expression at the single-cell level, in combination with the BD Rhapsody high-throughput single-cell capture system. BD AbSeq uses oligonucleotide-conjugated antibodies (Ab-oligos) to examine protein expression from high-throughput sequencing data. Each antibody clone in the BD AbSeq portfolio is conjugated to a unique oligonucleotide containing an antibody-specific barcode (ABC). Adjacent to the ABC is a poly(A) sequence on the 3' end (for capture by oligo-dT-based RNA-seq systems) and a 5' universal PCR amplification sequence that can be efficiently amplified during library preparation. Decoding of the ABCs using next-generation sequencing can be used to estimate the abundance of the protein. Using BD AbSeq with 3'-based single-cell analysis systems—such as BD Rhapsody—allows simultaneous interrogation of mRNA and protein in the same sample.

BD AbSeq can be incorporated into the BD Rhapsody system for a complete workflow solution.

Cells are incubated with BD AbSeq Ab-oligos in the same way that cells are labelled with antibodies before typical cytometry workflows. After incubation of labelled cells with BD AbSeq Ab-oligos, cells can be analyzed as bulk populations or taken through the BD Rhapsody workflow to capture transcriptome and BD AbSeq expression at the single-cell level (Figure 1). The BD Rhapsody platform uses a micro-well technology to partition individual cells[1] with BD Rhapsody cell capture beads. Capture of mRNA and BD AbSeq Ab-oligos within the micro-wells allows

BD

cell-specific barcoding of each mRNA and Ab-oligo. After recovery of the oligonucleotide-coated beads, parallel RNA and BD AbSeq sequencing libraries are then generated with BD Rhapsody library amplification components (included in each BD Rhapsody kit). The oligonucleotide portion of the Ab-oligo is carried through to sequencing to determine the identity of the protein detected. After determining the proportion of reads desired from each library, they can be pooled and sequenced using Illumina sequencers.

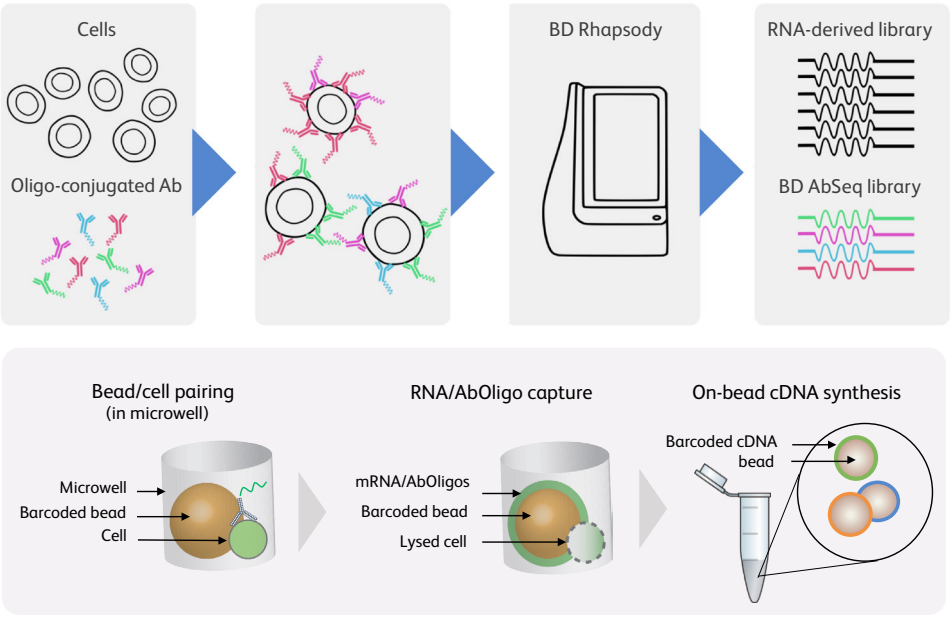


Figure 1. The BD AbSeq workflow is integrated into the BD Rhapsody single-cell analysis system.

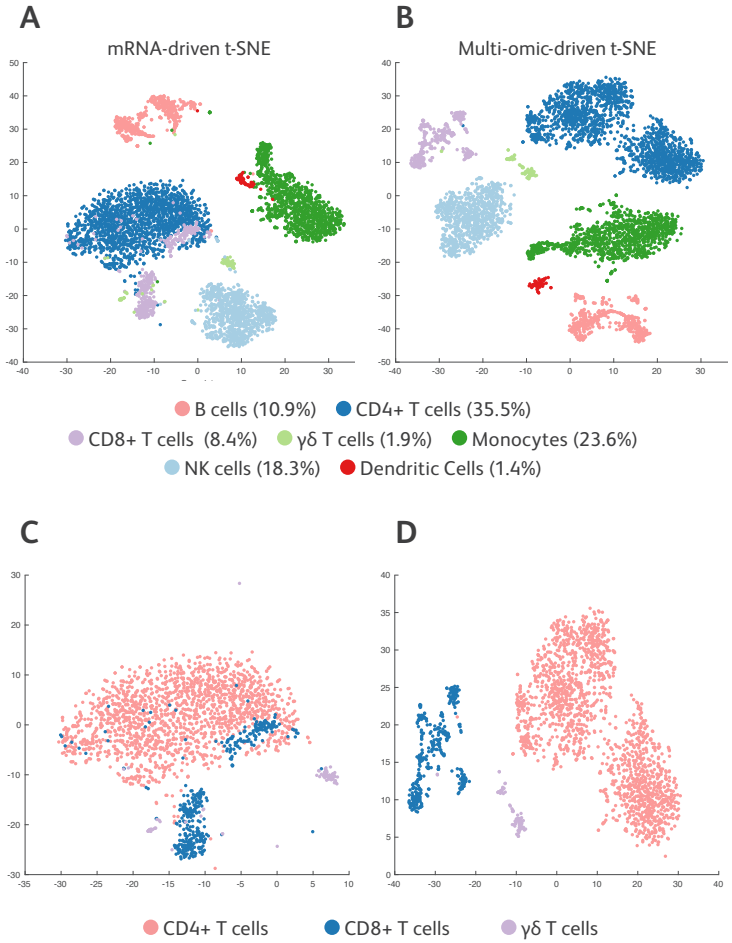


Figure 2. t-SNE clustering of PBMCs using mRNA or multi-omic data.
A. t-SNE coordinates are calculated for all PBMCs based on mRNA data only. Cells are colored based on cell type. **B.** t-SNE coordinates are calculated for all PBMCs based on multi-omic data (mRNA and protein). Cells are colored based on cell type. **C.** Same coordinates as in A, but only T-cell subsets are displayed. **D.** Same coordinates as in B, but only T-cell subsets are displayed. Markers used for cell-type definition (protein data used for all markers except FcγRIIIa, for which mRNA data was used): CD4- CD3+CD4+; CD8- CD3+CD8+; γδT Cells- CD3+TCRγδ+; B Cells- CD19+; Monocytes-CD14+; NK Cells- CD3-CD45RA+ FcγRIIIA-high

Multi-omic analysis provides more defined clustering.

Single-cell analysis allows researchers to uncover new cell signatures; however, this relies on the ability to effectively identify different cell subsets within a complex sample. To determine whether the protein data provided by BD AbSeq enables more specific cell-type identification, peripheral blood mononuclear cells (PBMCs) were analyzed using only mRNA data or mRNA and protein data together. PBMCs were isolated from a healthy individual and incubated with a 30-antibody panel (Appendix 1) before preparing mRNA and BD AbSeq libraries using the BD Rhapsody workflow. Cell subsets within the ~5,000 cells identified were first defined based on canonical protein and mRNA markers retrieved from the sequencing data. After defining these cell types, they were visualized using t-distributed stochastic neighbor embedding (t-SNE). These t-SNE projections collapse multi-dimensional data into two dimensions to show, on arbitrary axes, how different cells are based on gene expression, protein expression, or a combination of the two. t-SNE projections were calculated using only mRNA data (Figure 2A) or mRNA and protein data (Figure 2B). While mRNA-based analysis revealed distinct groups of monocytes and lymphocytes (B cells, T cells, and NK cells), subsets within the CD3+ population were especially difficult to distinguish (Figure 2C). Multi-omic-driven projections revealed cleaner cell separation both between all cell types (Figure 2B) and within the CD3+ population (Figure 2D). These analyses show that multi-omic data enables better identification of different cell subsets within a complex sample. This increased ability to discern different cell types will be crucial for experiments that seek to define novel cell subsets in healthy or diseased models.

Direct detection of protein targets with low-expressed corresponding mRNAs such as PD1.

The ability to examine protein directly, rather than inferring protein information based on mRNA information, could be especially useful in situations where mRNA level is not correlated to protein expression, or when mRNA is difficult to detect due to low expression. To determine whether BD AbSeq data can provide clearer information in cases where mRNA and protein expression are not correlated, we compared mRNA and BD AbSeq data for CD4 and the γδTCR receptor, which are both targets used at the protein level to define different cell types. Examination of *CD4* mRNA showed that it is expressed in both monocytes and CD4+ T cells (Figure 3A, top). In contrast, when BD AbSeq data was used to examine CD4 protein levels, only the CD4+ T cells showed high expression (Figure 3A, bottom). Similarly, examination of the TCR delta constant domain at the mRNA level revealed expression in both NK cells and γδ T cells (Figure 3B, top). When examining γδTCR expression at the protein level, only the smaller γδ T-cell population emerges as high-γδTCR-expressing (Figure 3B, bottom). These results show that protein data can be used to define more precise cell subsets, particularly in cases where mRNA expression and protein expression are not correlated.

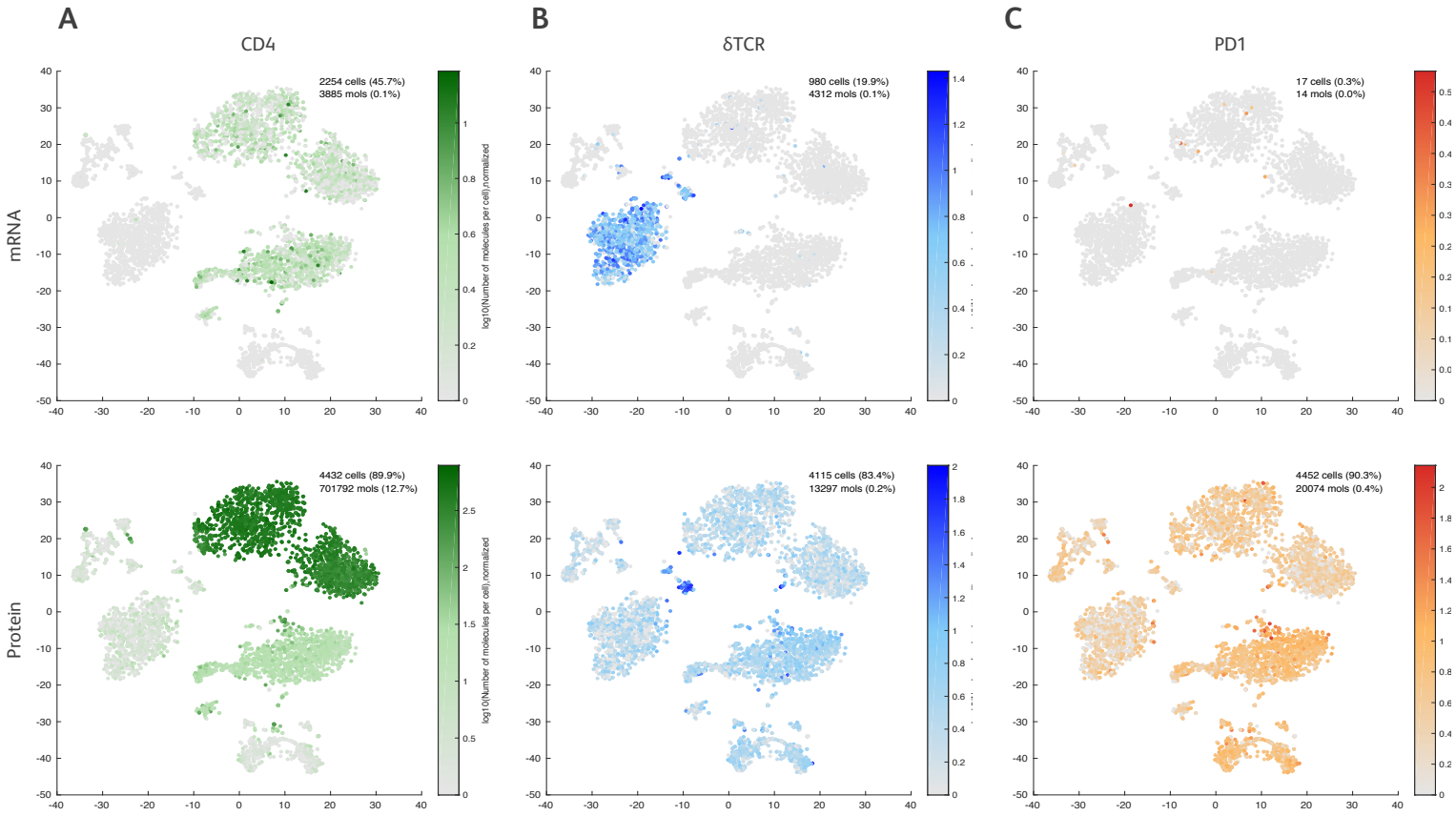


Figure 3. Detection of protein targets associated with low mRNA expression. t-SNE projection and cell types are as in Figure 2B. Cells are colored based on the expression of the mRNA (top) or protein (bottom) for each gene being examined. A. CD4. B. δTCR. C. PD1.

To examine BD AbSeq data in a context where mRNA is difficult to detect due to low expression, we focused on PD1, a T-cell exhaustion marker. Because targeting PD1-high or PD1-low populations can enable immune modulation, this molecule has emerged as an important immunotherapy target.[2,3] To determine if BD AbSeq can be used to identify cells expressing different levels of PD1, we examined PD1 at the mRNA and protein levels in the PBMC population. Although *PD1* mRNA is undetectable due to low expression level (Figure 3C, top), BD AbSeq data revealed that PD1 protein is expressed more highly in certain cell types and in specific individual cells (Figure 3C, bottom). These results suggest that BD AbSeq can be used to identify high- and low-expressors at the protein level, particularly when mRNA expression is low. For assays where cell-surface molecules like PD1 are targeted for drug delivery, the ability to identify and study cells based on protein expression will be vital to understanding drug responses.[3]

Identifying novel differentially expressed genes in naïve and memory T-cell populations that cannot be identified by RNA-seq alone.

Although 3' RNA-seq methods can be used to examine the expression of mRNA, it is difficult to examine distinct isoforms, unless the differences are close to the 3' end of the transcript. One example of this is the CD45 gene, which is spliced at the 5' end to produce CD45RA and CD45RO (markers of naïve and memory lymphocytes, respectively[4]). Although these isoforms cannot be distinguished by 3' RNA-seq, well-established anti-CD45RA and anti-CD45RO antibodies exist that can be used to distinguish naïve and memory T-cell subsets. To determine whether BD AbSeq can also be used to distinguish these subsets, CD45RA and CD45RO expression were examined within the PBMC population. Multi-omic t-SNE projection revealed two distinct subsets of CD4+ T cells (Figure 4A, box), which were further examined for CD45RA and CD45RO expression. This analysis showed that the two distinct subsets were CD4+CD45RA+CD45RO- (Figure 4B, top) and CD4+CD45RA-CD45RO+ (Figure 4B, bottom) populations.

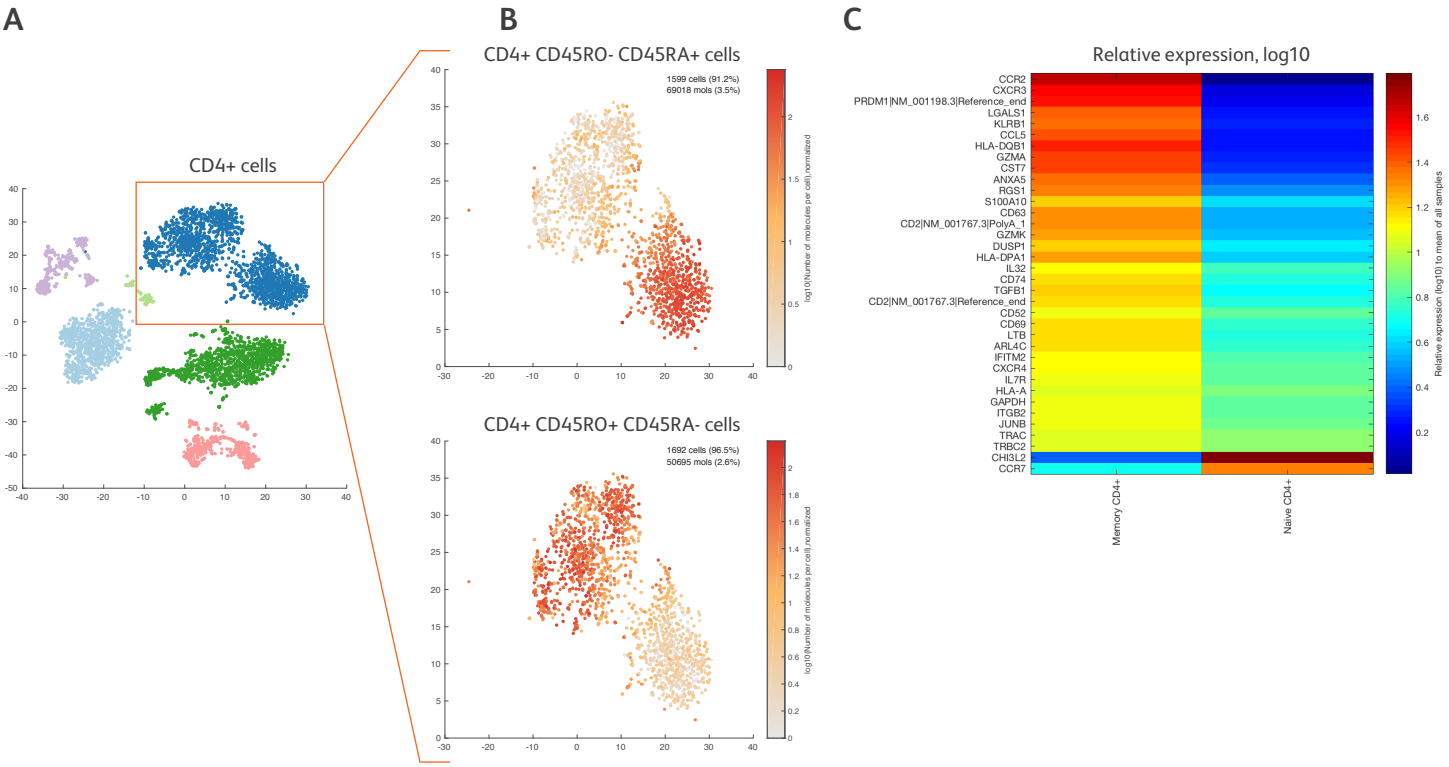


Figure 4. Examination of protein targets by mRNA sequencing. A. All PBMCs projected onto the t-SNE projection calculated in Figure 2B. Cells are colored based on cell type. **B.** CD4+ T cells projected onto the t-SNE projection calculated in Figure 2B. Cells are colored based on protein expression of CD45RA (top) or CD45RO (bottom). **C.** Heat map showing statistically significantly differentially expressed mRNAs between CD4+CD45RA+ and CD4+CD45RA- cells.

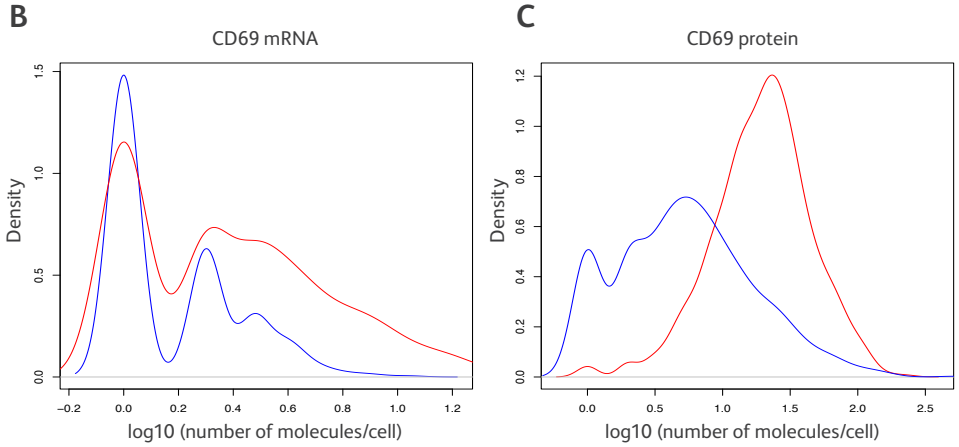
To further leverage this multi-omic dataset, differential mRNA expression was interrogated between the manually gated CD4+CD45RA+CD45RO- and CD4+CD45RA-CD45RO+ T-cell subsets. This differential gene expression analysis identified 36 mRNAs—such as *CCR2* and *CHI3L2*—that are significantly enriched in one of the two populations (Figure 4C). Together, this multi-omics analysis enables comprehensive gene expression analysis and biomarker discovery in heterogeneous cell populations.

Observing temporal and stimulated early immune-cell responses through multi-omic sequencing.

The ability to simultaneously examine protein and mRNA expression could be particularly important when studying responses to cell stimulation, which occur rapidly at both the transcriptional and translational level. To determine whether BD AbSeq can used to simultaneously profile early RNA and protein changes, we examined the early transcriptional response to immune-cell activation. Specifically, PBMCs stimulated with PMA/ionomycin for 0 or 4 hours were examined using BD Rhapsody. After 4 hours of stimulation, we identified significant upregulation of multiple known activation markers[1,5] including *IFNγ* and *TNF* mRNA, and CD69 protein (Figure 5A). Interestingly, although CD69 upregulation detected by BD AbSeq is evident 4 hours after stimulation, *CD69* mRNA remains low (Figure 5B and 5C), highlighting the differences in RNA and protein regulation.

A

Name	Molecule	P value	Function
CD69	Protein	2.13e-144	Cell-surface activation marker
IFNG	RNA	<1e-150	Cytokine
TNF	RNA	<1e-150	Cytokine



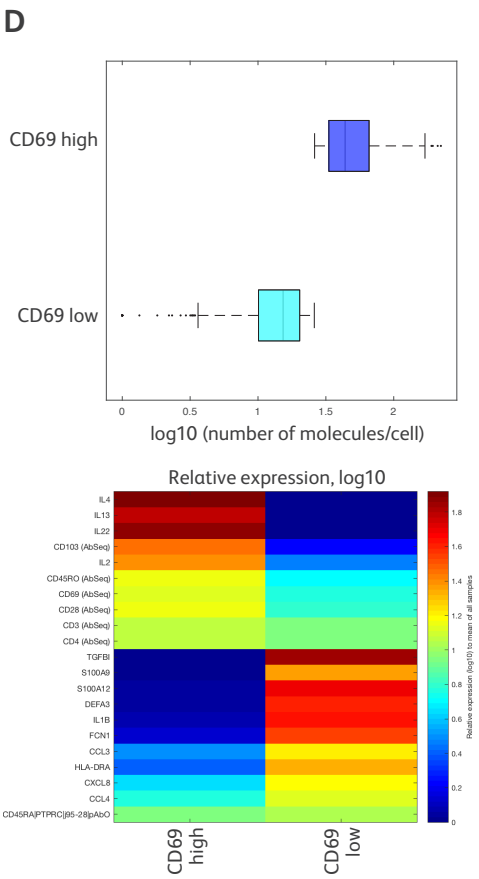


Figure 5. Markers associated with strong cell activation. A. Subset of classical markers of immune cell activation that are significantly upregulated after 4 hours stimulation with PMA/ionomycin. **B.** Distribution of *CD69* mRNA expression after 0 (blue) and 4 (red) hours of PMA/ionomycin stimulation. **C.** Distribution of CD69 protein expression after 0 (blue) and 4 (red) hours of PMA/ionomycin stimulation. **D.** Top: Boxplot showing CD69 protein expression in CD69-high and CD69-low cells (upper and lower 50th percentiles of CD69 expression, respectively). Bottom: Heat map showing significantly differentially expressed mRNAs and proteins in CD69-high vs CD69-low cells.

Next, we examined gene expression heterogeneity between immune cells that were more activated (CD69-high) and less activated (CD69-low) (Figure 5D, top). Differential mRNA and protein expression in the two populations was used to identify mRNA and protein markers that are associated with high CD69 upregulation. This analysis revealed >20 protein and mRNA markers – such as IL4 and TGFBI – associated with either the CD69-high or CD69-low populations (Figure 5D, bottom). These results show that BD AbSeq data can be used to define novel cell populations based on a cellular response, which can then be used for biomarker discovery. Multi-omic analyses like these will be crucial to enable definition and interrogation of cell populations based on rapid and time-sensitive cellular responses.

Interrogation of gene regulation at RNA and protein levels

The single-cell and multi-omic capabilities provided by BD Rhapsody and BD AbSeq enable examination of RNA and protein expression changes in dynamic and heterogeneous samples. To harness these capabilities, we added small molecules to inhibit transcription, translation, and proteasome activity during PBMC activation. Specifically, PMA/ionomycin-stimulated PBMCs were treated with four different chemical conditions: DMSO was used as a control, while actinomycin D, cycloheximide, and MG132 were used to inhibit transcription, translation, and proteasome activity, respectively (Figure 6A). 0 and 4 hours after PMA/ionomycin stimulation, cells from each of the four chemical treatments were pooled using the BD Single-Cell Multiplexing Kit, and BD Rhapsody libraries were prepared.

To determine the level at which different molecules are regulated, *TNF* mRNA and CD69 protein were examined for upregulation in each sample. While *TNF* is expressed uniformly at a low level in all 0-hour time-points, upregulation can be seen in all 4-hour time-points except the actinomycin D-treated cells (Figure 6B, right). This is consistent with TNF mRNA upregulation being controlled at the transcriptional level. Conversely, CD69 upregulation is observed at all 4-hour time-points except the cycloheximide-treated cells, which is consistent with control of CD69 protein production at the level of translation (Figure 6B, left).

To determine whether different cell subsets regulate these molecules with distinct mechanisms, expression in different cell types was analyzed. Within each cell type, CD69 upregulation was examined to determine if upregulation is controlled at the level of translation in all cell types. While T cells showed CD69 upregulation in all 4-hour samples except those treated with cycloheximide (Figure 6C, left), B cells showed a loss of CD69 protein upregulation in actinomycin-treated cells as well as cycloheximide-treated cells (Figure 6C, right).

This suggests that CD69 protein upregulation in B cells relies on transcription of *CD69* mRNA, as well as translation of that mRNA. Together, these analyses show that BD AbSeq on the BD Rhapsody platform can be used to probe gene regulation at multiple levels in diverse cell types within a complex sample.

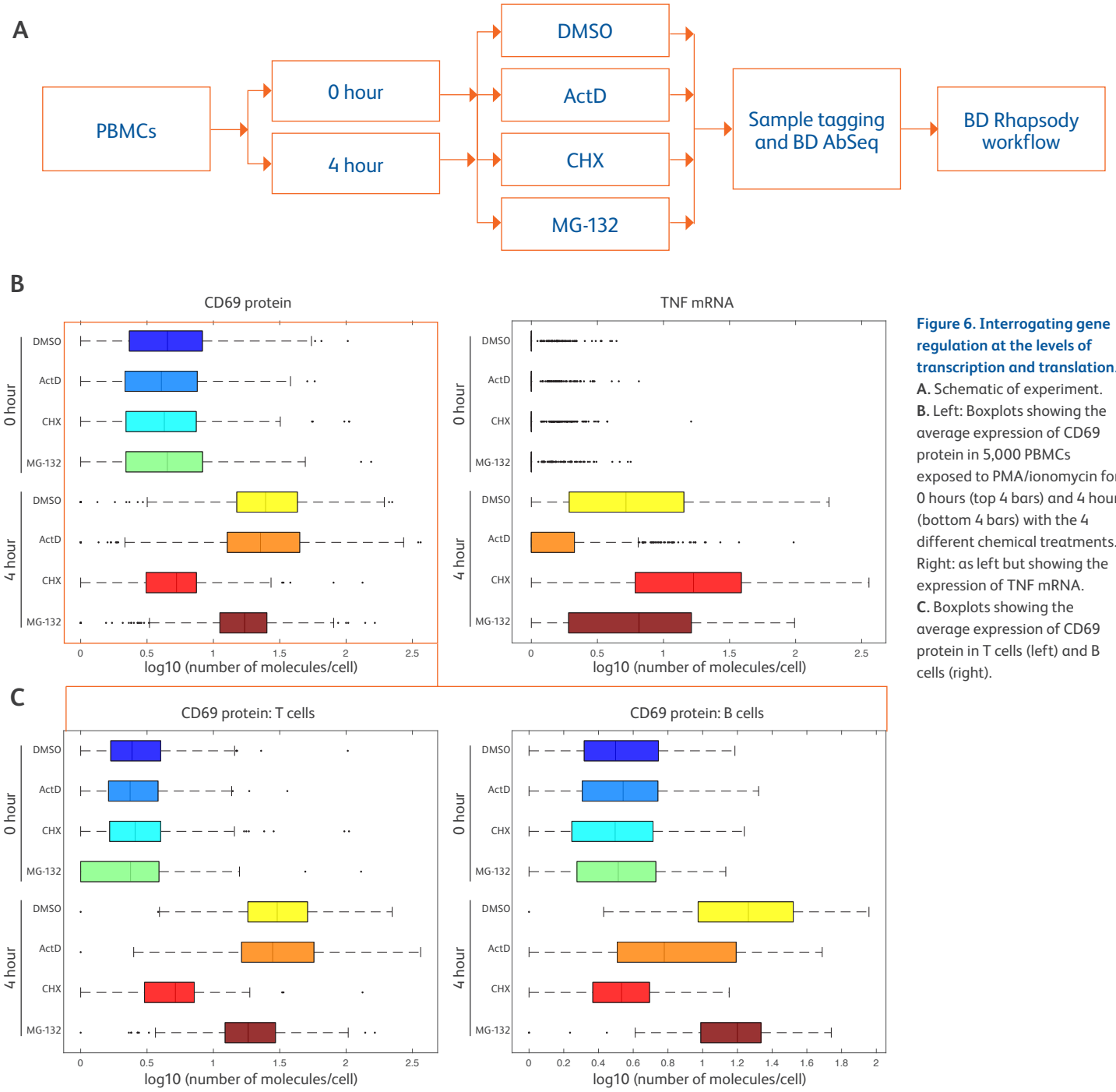


Figure 6. Interrogating gene regulation at the levels of transcription and translation. **A.** Schematic of experiment. **B.** Left: Boxplots showing the average expression of CD69 protein in 5,000 PBMCs exposed to PMA/ionomycin for 0 hours (top 4 bars) and 4 hours (bottom 4 bars) with the 4 different chemical treatments. Right: as left but showing the expression of TNF mRNA. **C.** Boxplots showing the average expression of CD69 protein in T cells (left) and B cells (right).

Conclusion

Single-cell biology is going through a series of technological advances and breakthroughs. While single-cell RNA-seq and flow cytometry have each made significant strides towards understanding the heterogeneity within complex biological samples, the union of these two disciplines under a single solution can provide even greater insight in the burgeoning field of genomic cytometry. In this study, expression of mRNA and protein levels was profiled using BD AbSeq with the BD Rhapsody system for single cells to examine hard-to-detect mRNAs, perform biomarker discovery, and unveil dynamic gene regulation changes during immune-cell activation. Whether you are a researcher using single-cell RNA-seq or flow cytometry, BD is pleased to offer a single workflow—from single-cell capture to informatics analysis—that uses BD AbSeq with the BD Rhapsody system to dive deeper into mRNA and protein expression profiling in the single-cell level.

References

1. Fan, H. C., Fu, G. K. & Fodor, S. P. A. Combinatorial labeling of single cells for gene expression cytometry. *Science (80-.).* **347,** (2015).
2. Haanen, J. B. A. G. & Robert, C. Immune Checkpoint Inhibitors. *Prog. tumor Res.* **42**, 55–66 (2015).
3. Schmid, D. *et al.* T cell-targeting nanoparticles focus delivery of immunotherapy to improve antitumor immunity. *Nat. Commun.* **8**, (2017).
4. Zikherman, J. & Weiss, A. Alternative Splicing of CD45: The Tip of the Iceberg. *Immunity* **29**, 839–841 (2008).
5. Ziegler, S. F., Ramsdell, F. & Alderson, M. R. The activation antigen CD69. Stem Cells **12**, 456–465 (1994).

Appendix 1: List of BD AbSeq antibodies used

CD3	CD123 (IL3Ra)
CD4	CD127
CD8	CD134 (OX40)
CD14	CD137
CD19	CD161
CD25	CD183
CD27	CD185 (CXCR5)
CD28	CD194

CD38	CD196 (CCR6
CD45RA (1)	CD197 (CCR7)
CD45RA (2)	CD278
CD45RO	CD279 (PD1)
CD69	CD366 (Tim3)
CD95	TCRgd
CD103	HLA-DR

BD Life Sciences, San Jose, CA, 95131, USA

23-20841-00

www.bd.com/genomics