Correlating gene and protein expression at the single-cell level using index sorting

Downstream genomics applications for the BD FACSMelody™ cell sorter

Features

- Sort cells easily with streamlined and automated workflow
- Efficiently sort single cells for downstream mRNA expression analysis
- Sequence only the cells of interest from a heterogeneous population
- Perform single-cell whole transcriptome or targeted gene expression analysis
- Correlate gene expression with protein expression at the single-cell level

Conventional bulk approaches to cell analysis have been a mainstay of biological research and discovery, but they can only average information across the cells in a sample. The resulting *average proteome* or *average transcriptome* can mask differences at the single-cell level and limit the ability to characterize rare cell populations.

Single-cell analysis is therefore critical to understand the biology of heterogeneous samples. Single-cell RNA sequencing (scRNA-Seq) can profile the transcriptome of every cell in a sample. However, this approach is not feasible when the cells of interest are rare in a heterogeneous sample. The use of fluorescence-activated cell sorting (FACS) upstream from genomic analysis can overcome this limitation by enabling you to isolate and interrogate only the cells of interest, virtually devoid of unwanted contaminants, and to correlate protein and gene expression for individual cells.

BD

Figure 1

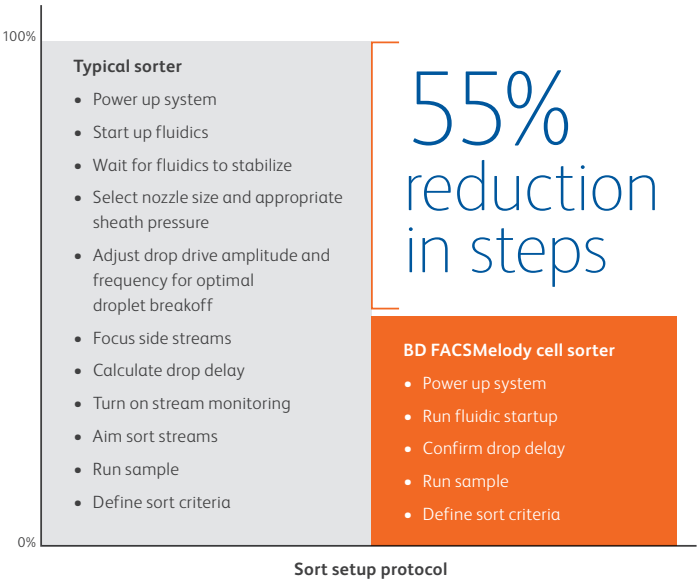

Figure 1. Automation in BD FACSMelody cell sorter makes sorting simple and saves time
Operating the BD FACSMelody cell sorter is easy and requires minimal training for effective operation. BD FACSChorus software simplifies the workflow with smart automation. Typically the system is ready in less than 17 minutes. Researchers are guided throughout the entire cell sorting process using advanced automation technology.

Figure 2

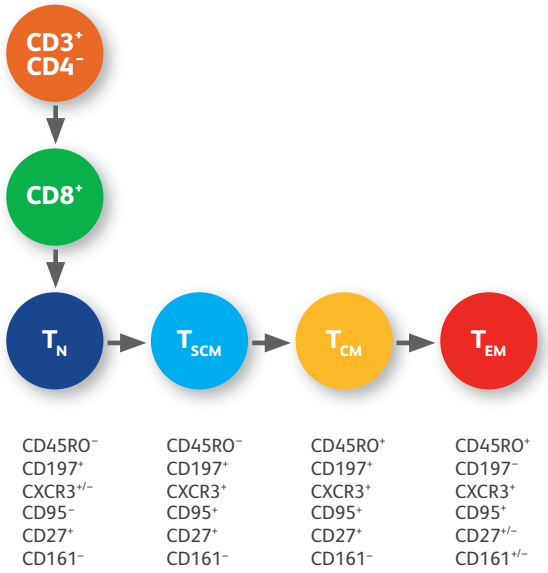

Figure 2. Population hierarchy and multicolor panel design
Upon antigen presentation, naïve T cells (T_N) progressively differentiate into stem cell memory (T_{SCM}), central memory (T_{CM}) and effector memory (T_{EM}) T cells prior to terminal differentiation into effector T cells. Each subset can be identified and isolated based on the expression of specific surface markers.

Combining automation and ease of operation with high sensitivity and resolution, the BD FACSMelody™ cell sorter can precisely and efficiently sort and deposit single cells for downstream analysis (Figure 1). The plate sorting capability of the BD FACSMelody records the phenotype for each sorted cell. Further, with a feature called *index sorting*, BD FACSChorus™ software records the final location for each event (for example, well location in a 96-well plate). This allows you to later go back and correlate the cellular phenotype with downstream sequence data for any cell.

This data sheet describes an experiment that pairs upstream index sorting with downstream scRNA-Seq to perform a targeted gene expression analysis on different subsets of $CD8^+$ T cells. We particularly wanted to investigate a recently identified subset of rare stem-cell-like, self-renewing, *stem cell memory* T cells (T_{SCM}) that has transformed our understanding of memory T-cell development.[1] These cells, which may prove critical for vaccine development as well as cell therapy applications, have a naïve-like protein expression profile and are defined by the expression of CD95, IL-2rb, CXCR3 and LFA-1. The rarity of T_{SCM} cells makes them desirable for single-cell index sorting and transcriptome analysis.

The cells were acquired on the BD FACSMelody and immunophenotyped into increasingly differentiated $CD8^+$ T-cell subsets—from naïve (T_N) to stem cell memory (T_{SCM}), central memory (T_{CM}) and effector memory (T_{EM})—based on the expression of surface markers (Figure 2). The multicolor panel was designed and optimized to account for antigen co-expression, fluorochrome brightness and antigen density (Table 1).

Marker	Fluorochrome
Viability	7-AAD
CD4/CD14/CD19	PerCP-Cy™5.5
CD3	FITC
CD8	APC-H7
CD45RO	PE-Cy™7
CD197 (CCR7)	Alexa® Fluor 647
CD183 (CXCR3)	BV421
CD95	PE
CD27	BV786
CD161	BV510

Table 1. 10-color T-cell panel for index sorting

Figure 3A

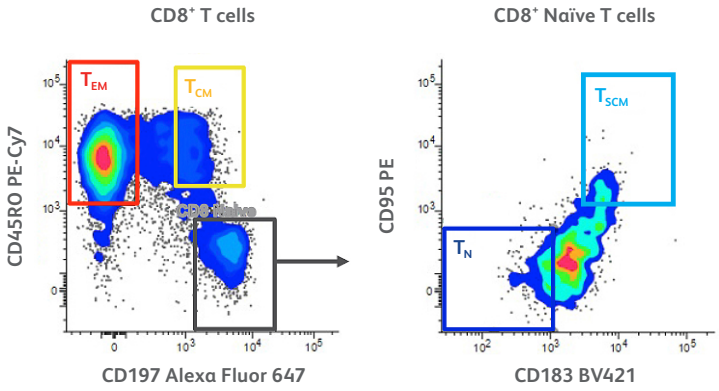

Figure 3B

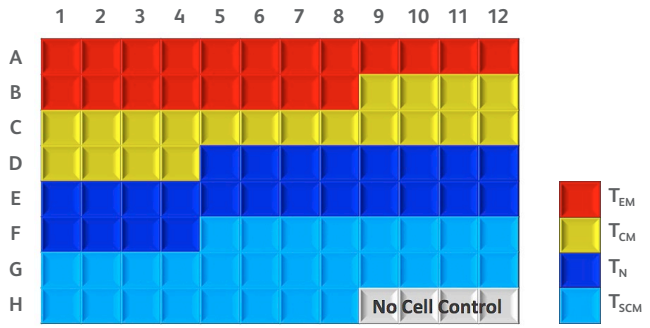

Figure 3. Gating and single-cell deposition strategy
Human peripheral blood mononuclear cells were isolated from a healthy donor and stained with antibodies listed in the ordering information table. Cells were resuspended in BD FACS™ Pre-Sort Buffer and acquired on the BD FACSMelody. Lymphocyte singlets were identified using forward and side scatter. Viable $CD3^+$ T cells were identified based on CD4, CD14, CD19 and 7-AAD exclusion, and $CD8^+$ T-cells were identified and gated based on CD3 and CD8 expression (not shown). **A.** T-cell subsets were identified based on expression of CD197 (CCR7), CD45RO, CD183 (CXCR3) and CD95. Cells were categorized as T_N, T_{SCM}, T_{CM} or T_{EM}. **B**. Cells were sorted individually onto 96-well plates according to the grid shown.

Using the gating strategy in Figure 3A, the cells were then sorted as single cells into a 96-well BD Precise™ RNA quantification assay plate according to the scheme in Figure 3B. Each well of the plate contains specialized reagents that lyse the cell, extract the cellular RNA, and enable preparation of a sequencing-ready library. Each well contains unique barcoding primers so that all the single cells in a plate can be pooled for downstream library preparation, and the sample origin of each transcript can be identified upon sequencing.

A total of 368 cells were sorted individually into four plates for downstream gene expression analysis. The mRNA from these sorted cells was subjected to cDNA synthesis and library preparation using a targeted T-cell panel that contains primers for 220 different genes.

Figure 4 shows a t-stochastic neighbor embedding (tSNE) projection of the 368 cells after sequencing, in which cells with similar gene expression profiles cluster together. Notice how T_N cells (dark blue) are clustered together at the upper left and T_{EM} cells (red) at the lower right. The transcriptome profiles from these two cell types are quite homogeneous, though very different from each other.

T_{SCM} (light blue) and T_{CM} (yellow) cells, however, demonstrate much higher levels of heterogeneity. For these two heterogeneous cell types, some of the gene expression signatures are more like T_N cells, while others are more like T_{EM} cells. The tSNE analysis indicates high heterogeneity and suggests that additional markers might further distinguish less from more differentiated T cells. This finding validates the use of scRNA-Seq, since bulk sequencing would only have provided an average expression level for each cell type.

Using the index sorting feature of the BD FACSMelody, we then correlated phenotypic and gene expression signatures. Figure 5 shows the results for a representative plate, from which we selected 26 T_N, 26 T_{SCM}, 20 T_{CM} and 20 T_{EM} cells in BD FACSChorus™ software. The software color-codes the selected cells in the plots for easy identification.

Figure 4

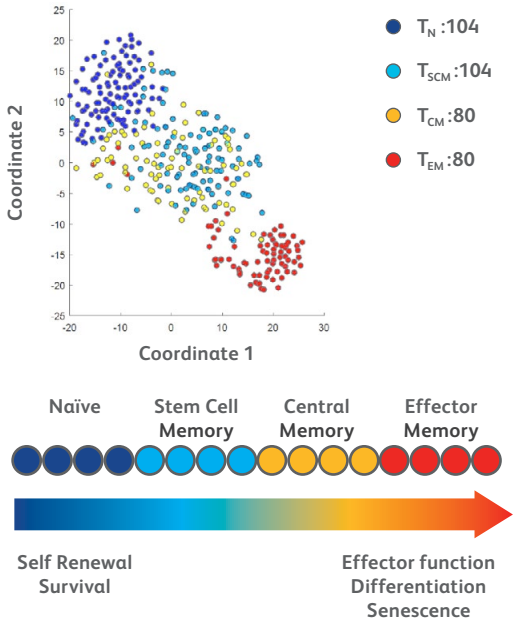

Figure 4. Single-cell RNA-Seq analysis of $CD8^+$ T-cell subsets
mRNA from the single cells sorted into BD Precise plates was subjected to cDNA synthesis and library preparation using a targeted T-cell panel that contains primers for 220 different genes. Sequencing was performed using Illumina® sequencers. **Results:** tSNE projection of 368 single cells (104 T_N, 104 T_{SCM}, 80 T_{CM}, and 80 T_{EM}) combined from four 96-well BD Precise plates. Cells are annotated based on originating sorted cell type.

Figure 5A confirms that the sorted T_N, T_{CM}, T_{SCM} and T_{EM} cells are located within the CD183 vs CD95 and CD197 vs CD45RO gates used to identify and sort them. In Figure 5B, we used index sorting to assess how expression of CD27 and CD161, which are known to be differentially expressed by different T-cell subsets,[2–4] changed at the single-cell level as the cells differentiated. Although these two markers were not included in the sorting gating strategy, their expression can be retrospectively analyzed via index sorting to assess correlation with mRNA expression.

Figure 5A

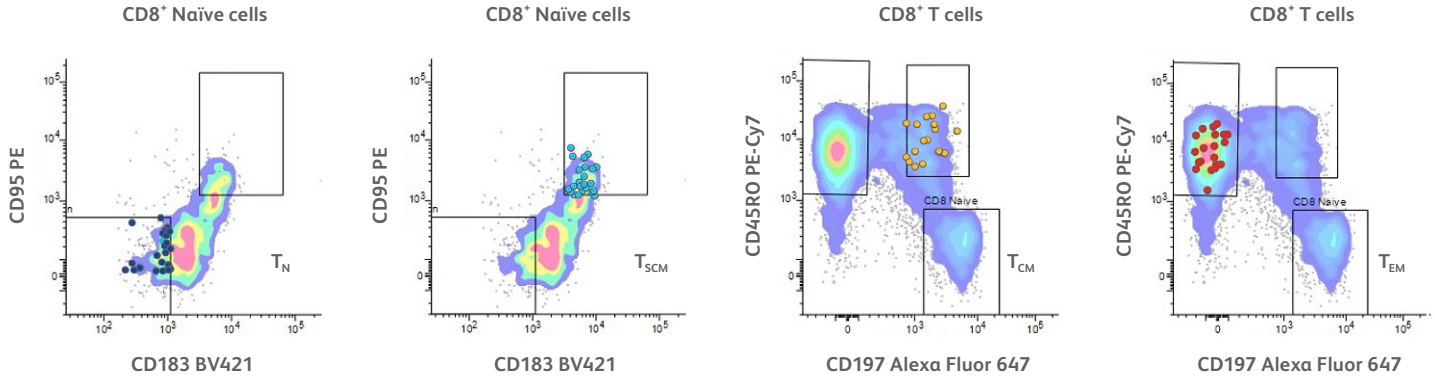

Figure 5B

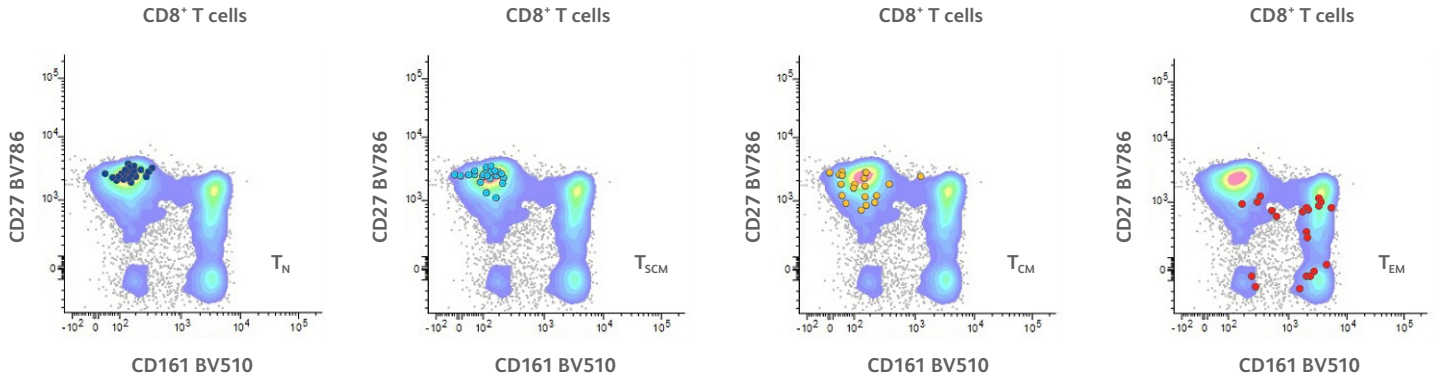

Figure 5C

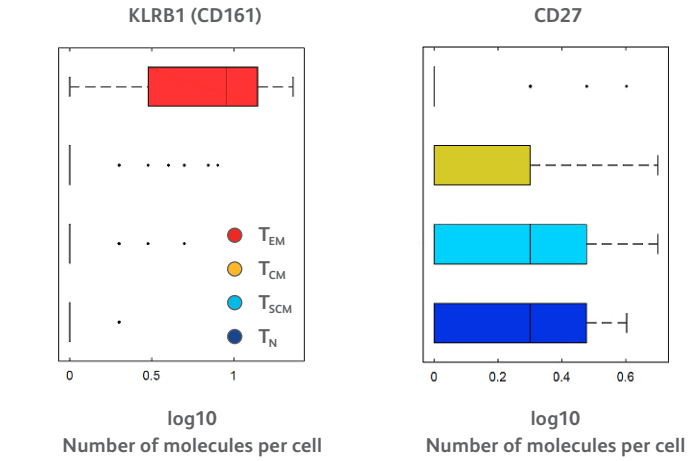

Figure 5D

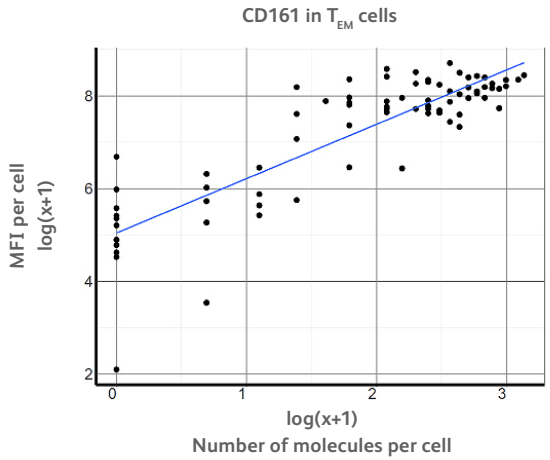

Figure 5. Correlation of protein levels with downstream molecular signatures
The index sorting feature of the BD FACSMelody enables researchers to select individual cells from the plate and correlate their data from downstream applications with their original cell phenotype—including markers that were not part of the sorting gate. **A**. Index sort data for a single 96-well plate showing individual $CD8^+$ T cells within the gates used for cell sorting. **B**. Index sort data showing differential expression of CD27 and CD161 by individual $CD8^+$ T cells. **C**. Box plots show KLRB1 (CD161) and CD27 mRNA levels in the $CD8^+$ T cell subsets. **D**. Correlation of mRNA and protein levels of KLRB1 (CD161) in T_{EM} cells. (MFI: mean fluorescence intensity)

The RNA-Seq results in Figure 5C show that, consistent with protein expression, only the T_{EM} subset expressed CD161 mRNA. Conversely, T_{EM} cells did not express CD27 mRNA at all, while the other subsets expressed it at mixed levels. Finally, Figure 5D plots protein expression levels vs mRNA levels for T_{EM} cells and shows a moderate ($R^2 = 0.74$) correlation between protein abundance and mRNA abundance. It is known that mRNA transcript levels only partially correlate with protein levels due to post-transcriptional regulatory mechanisms.[5]

Combining index sorting on the BD FACSMelody with single-cell RNA-Seq can provide deep insights into cell function and development. The ability to correlate protein and mRNA signatures on a single-cell level offers a rich opportunity for discovery. The BD FACSMelody can also improve assay efficiency, saving cost and time by enabling sequencing of only rare subsets of interest.

Genomic analysis is only one of many downstream applications of the BD FACSMelody across immunology, stem cell research, genomics, bioprocessing and cancer biology. With up to three lasers, nine fluorescence channels, and one- or two-way sorting into plates, slides or tubes, the easy-to-learn, easy-to-use BD FACSMelody makes sorting accessible to more researchers and labs.

References

1. Gattinoni L, Lugli E, Ji Y, Pos Z, et al. A human memory T cell subset with stem cell-like properties. *Nat Med.* 2011;17:1290-7.
2. Fergusson JR, Hühn MH, Swadling L, et al. $CD161^{int}$ $CD8^+$ T cells: a novel population of highly functional, memory $CD8^+$ T cells enriched within the gut. *Mucosal Immunol.* 2016;9:401-13.
3. Romero P, Zippelius A, Kurth I, et al. Four functionally distinct populations of human effector-memory $CD8^+$ T lymphocytes. *J Immunol.* 2007;178:4112-9.
4. Takahashi T, Dejbakhsh-Jones S, Strober S. Expression of CD161 (NKR-P1A) defines subsets of human CD4 and CD8 T cells with different functional activities. *J Immunol.* 2006;176:211-216.
5. Vogel C, Marcotte EM. Insights into the regulation of protein abundance from proteomic and transcriptomic analyses. *Nat Rev Genet.* 2012;13:227-32.

Additional Resources

Fergusson JR, Smith KE, Fleming VM, et al. CD161 defines a transcriptional and functional phenotype across distinct human T cell lineages. *Cell Rep.* 2014;9:1075-88.

Ussher JE, Bilton M, Attwod E, et al. $CD161^{++}$ $CD8^+$ T cells, including the MAIT cell subset, are specifically activated by IL-12^+IL-18 in a TCR-independent manner. Eur J Immunol. 2014;44:195-203.

Systems and software

Description
BD FACSMelody™ 9-Color Cell Sorter Blue, Red, and Violet Laser Configuration, with Plate Sorting
BD FACSChorus™ software v1.1 (or later)

Ordering information: Reagents

Description	Cat. No.
BD Pharmingen™ PerCP-Cy™5.5 Mouse Anti-Human CD4	566316
BD Pharmingen™ PerCP-Cy™5.5 Mouse Anti-Human CD14	562692
BD Pharmingen™ PerCP-Cy™5.5 Mouse Anti-Human CD19	561295
BD Pharmingen™ FITC Mouse Anti-Human CD3	555332
BD Pharmingen™ APC-H7 Mouse Anti-Human CD8	560179
BD Horizon™ BV510 Mouse Anti-Human CD161	563212
BD OptiBuild™ BV786 Mouse Anti-Human CD27	740972
BD Pharmingen™ PE-Cy™7 Mouse Anti-Human CD45RO	560608
BD Pharmingen™ Alexa Fluor® 647 Mouse Anti-Human CD197	560816
BD Pharmingen™ PE Mouse Anti-Human CD95	561976
BD Horizon™ BV421 Mouse Anti-Human CD183	562558
BD Pharmingen™ 7-AAD	559925
BD FACS™ Pre-Sort Buffer	563503

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