Adapting a broad 30-color spectral flow cytometry panel into a targeted 12-color panel for routine assessment of T-cell biology using conventional flow cytometry

Ali Rezvan[1], Moen Sen[1], Xiaoshan Shi[1], Darci Gorgone Chavez[1], Rodrigo Pestana Lopes[1], Stephanie Widmann[2], Aaron Tyznik[2]

[1]BD Biosciences, Milpitas, CA 95035, [2]BD Biosciences, San Diego, CA 92130

Abstract

Large flow cytometry panels comprising numerous markers and fluorochromes are applied for investigation and discovery. These panels require expertise on panel design (more fluorochromes), sample preparation (more pipetting), instrument setup (more parameters) and data analysis (more dimensions). Adapting large panels suitable for discovery, into smaller panels suitable for routine assessments might be beneficial when data collection needs scaling and reproduction. In this study, we illustrate the transformation of a comprehensive 30-color immunophenotyping panel originally designed for profiling T cells, B cells, dendritic cells (DCs), and natural killer (NK) cells using spectral flow cytometry (BD FACSymphony™ A5 SE Cell Analyzer) into a targeted panel suitable for routine use on a conventional flow cytometer (BD FACSLyric™ Clinical Cell Analyzer). The goal was to streamline the panel selecting 12 relevant markers for T cell characterization. Following the selection of markers based on their biological relevance, antibodies were reassigned to fluorochromes compatible with the BD FACSLyric™ System instrument configuration. Fluorochrome assignment was optimized to ensure clear resolution of target cell populations while minimizing spillover. The consistency in the frequencies of different cell subsets across both platforms confirmed the reliability of the adapted panel. Our study exemplifies the successful transformation of a large, complex, discovery panel into a smaller, targeted panel suitable for routine assessments. This adjusted approach not only simplifies complexity and accelerates sample processing time but also preserves the high resolution essential for effective cell characterization.

Methods

Fresh human peripheral blood mononuclear cells (PBMCs) were derived from whole blood obtained from healthy donors. PBMCs were isolated by Ficoll™ gradient centrifugation and resuspended in BSA wash buffer (1XPBS+0.5%BSA+0.09%NaN3). The staining solution comprised a cocktail of antibodies as per the specified test volumes (Table 1) suspended in BD Horizon™ Brilliant Stain Buffer Plus. Cells were stained by first prestaining the freshly isolated PBMCs with antibodies against TCRγδ, CD185 and CD197 for 10 min at 37 °C. The antibody cocktail was then added to the pre-stained PBMCs, and the mixture was incubated for 30 min at room temperature in the dark. Single-stained controls were processed in parallel with the full panel. Cells were washed twice using cold BSA wash buffer. Cells were resuspended in wash buffer and stored on ice, protected from light, until acquisition. Optimized gain settings were derived for the BD FACSymphony™ A5 SE Cell Analyzer to generate Application Settings. Application Settings were then applied to maintain consistent fluorescence intensity values across experiments. For BD FACSLyric™ Cell Analyzer, a customized reference setting with optimized voltages was applied.
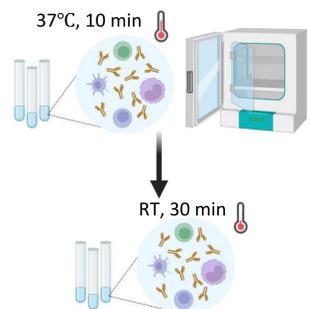
Cell preparation
- SepMate™ PBMC isolation
- FcR blocking

Healthy donor x3 → PBMC isolation from fresh whole blood
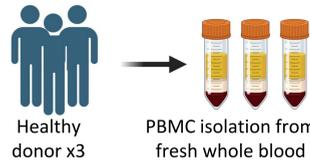
Cell staining
- Consecutive incubation steps:
  1. Chemokine receptors & TCR markers
  2. Remaining surface markers
- Three washes before cell suspension

37°C, 10 min
RT, 30 min

Sample acquisition
Experimental controls for unmixing/compensation:
- SpectraComp® particles (Slingshot Bio) for:
  1. CD158
  2. CD303
  3. CD123
  4. IgG
- PBMC single stain controls for all other markers

BD FACSymphony™ A5 SE Cell Analyzer

BD FACSLyric™ Cell Analyzer

Results:

Figure 1: Adapting a broad 30C spectral panel from BD FACSymphony™ A5 SE Cell Analyzer into a T-cell targeted 12C panel for a BD FACSLyric™ Clinical Cell Analyzer
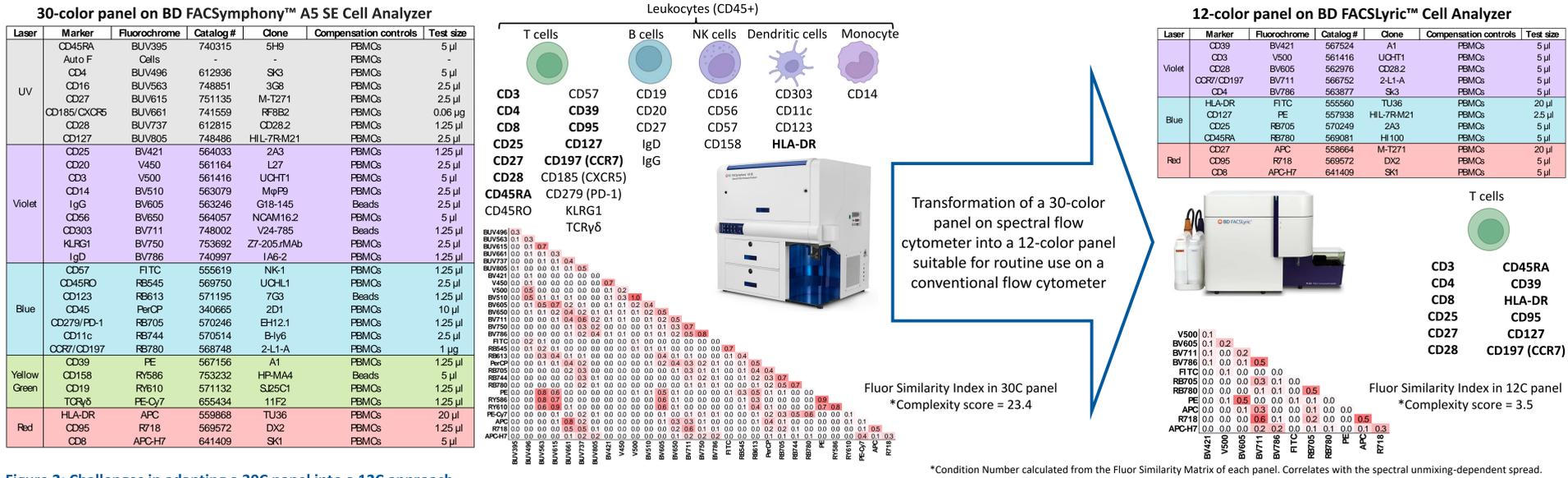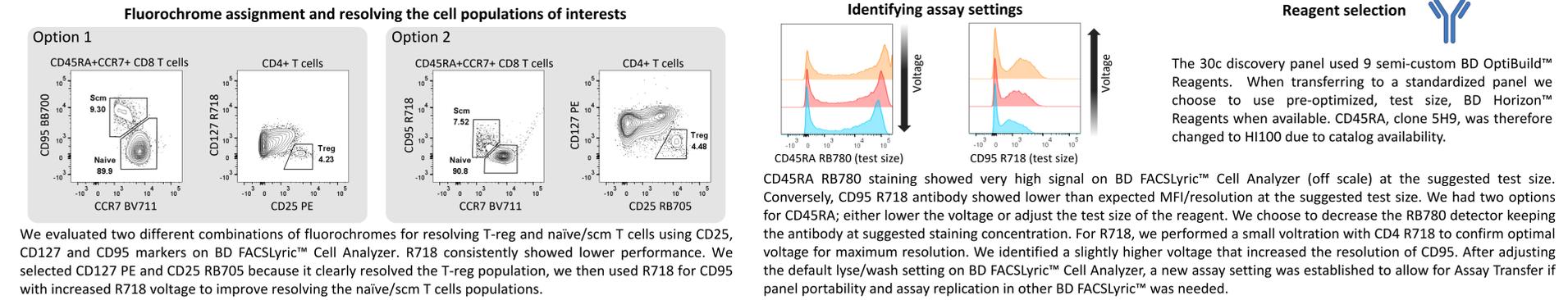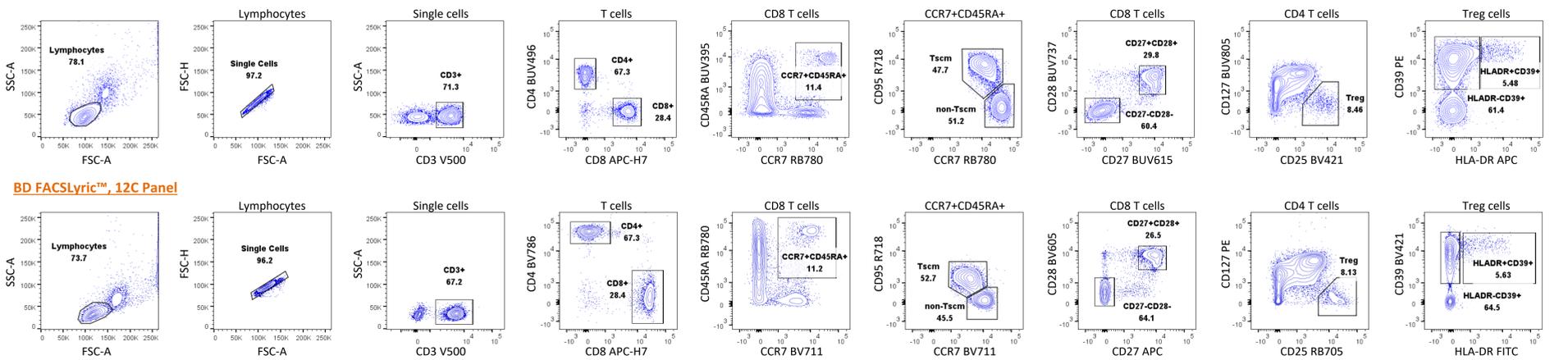

Transformation of a 30-color panel on spectral flow cytometer into a 12-color panel suitable for routine use on a conventional flow cytometer

Fluor Similarity Index in 30C panel
*Complexity score = 23.4

Fluor Similarity Index in 12C panel
*Complexity score = 3.5

*Condition Number calculated from the Fluor Similarity Matrix of each panel. Correlates with the spectral unmixing-dependent spread.

Figure 2: Challenges in adapting a 30C panel into a 12C approach

Fluorochrome assignment and resolving the cell populations of interests

We evaluated two different combinations of fluorochromes for resolving T-reg and naïve/scm T cells using CD25, CD127 and CD95 markers on BD FACSLyric™ Cell Analyzer. R718 consistently showed lower performance. We selected CD127 PE and CD25 RB705 because it clearly resolved the T-reg population, we then used R718 for CD95 with increased R718 voltage to improve resolving the naïve/scm T cells populations.

Identifying assay settings

CD45RA RB780 (test size)   CD95 R718 (test size)

CD45RA RB780 staining showed very high signal on BD FACSLyric™ Cell Analyzer (off scale) at the suggested test size. Conversely, CD95 R718 antibody showed lower than expected MFI/resolution at the suggested test size. We had two options for CD45RA; either lower the voltage or adjust the test size of the reagent. We choose to decrease the RB780 detector keeping the antibody at suggested staining concentration. For R718, we performed a small voltration with CD4 R718 to confirm optimal voltage for maximum resolution. We identified a slightly higher voltage that increased the resolution of CD95. After adjusting the default lyse/wash setting on BD FACSLyric™ Cell Analyzer, a new assay setting was established to allow for Assay Transfer if panel portability and assay replication in other BD FACSLyric™ was needed.

Reagent selection

The 30c discovery panel used 9 semi-custom BD OptiBuild™ Reagents. When transferring to a standardized panel we choose to use pre-optimized, test size, BD Horizon™ Reagents when available. CD45RA, clone 5H9, was therefore changed to HI100 due to catalog availability.

Figure 3: Evaluation of cell populations across panels and instruments

BD FACSymphony™ A5 SE, 30C Panel (gating with the shared 12 markers for consistency)

BD FACSLyric™, 12C Panel

Contour plots show the capability of both panels for resolving the populations of interest. The same gating strategy was used for consistency.

Histograms (gated on lymphocytes) show the positive population frequency across different markers for both instruments despite higher unmixing-dependent spread of negative populations on 30c panel (complexity score).

The bar plot illustrates the frequency of different cell populations across both instruments using the 12 markers that are shared between the two panels.

The consistency in the frequencies of different cell subsets across both platforms confirms the reliability of the adapted panel from the BD FACSymphony™ A5 SE to the BD FACSLyric™.

BD FACSymphony™ A5 SE (gated on lymphocytes)

BD FACSLyric™ (gated on lymphocytes)

Conclusions

- The consistency in the frequencies of different cell subsets across both platforms shows the reliability of the adapted panel from BD FACSymphony™ A5 SE to BD FACSLyric™.
- Our study exemplifies the successful transformation of a large, complex discovery panel into a smaller, targeted panel suitable for routine assessments, simplifying complexity, accelerating sample processing time, improving or preserving the high resolution essential for effective cell characterization.

BD Biosciences