

Abstract

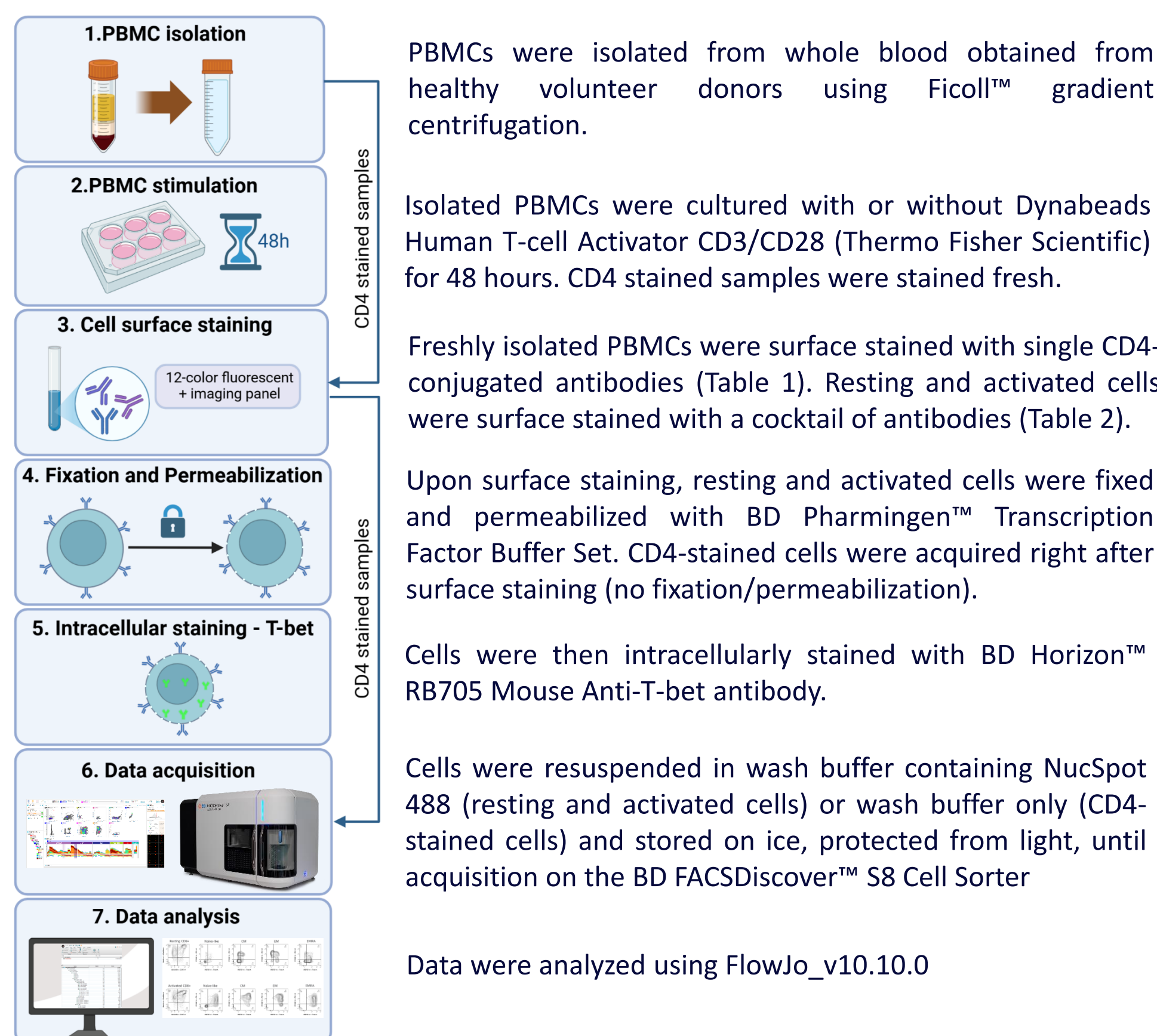
Imaging flow cytometry combines capabilities of flow cytometry with single-cell imaging acquisition. As a result, rich data sets are generated by measuring many features derived from fluorescent, spatial and morphological parameters.

The BD FACSDiscover™ S8 Cell Sorter features BD CellView™ Image Technology, a novel high-speed cell imaging system that enhances cell sorting by enabling real-time analysis with image-based parameters. Since its introduction, there has been a growing interest in integrating imaging capabilities with fluorescent parameters to better characterize and sort cell populations. However, comprehensive guidelines on setting up BD CellView™ parameters and experimental controls to ensure successful data generation are still lacking.

In this work, we evaluated the resolution and spillover of 46 catalogue fluorochromes including BD Horizon RealBlue™ and RealYellow™ reagents and 9 early candidates from the BD new dye technology team for performance in both imaging and fluorescent cell analysis. With these results we created a guideline for assigning the best fluorochromes for BD CellView™ applications. In addition to fluorescence spillover, imaging settings can impact data analysis, resulting in erroneous data interpretation. We show how to confirm the default imaging gain settings are optimal for the cell population of interest. To demonstrate the capabilities of BD CellView™ we describe how to design and optimize a 12-color spectral + 2-color imaging flow cytometry panel, including fluorochrome selection, critical biological controls and imaging parameters optimization.

Methods

Figure 1. Experimental Workflow



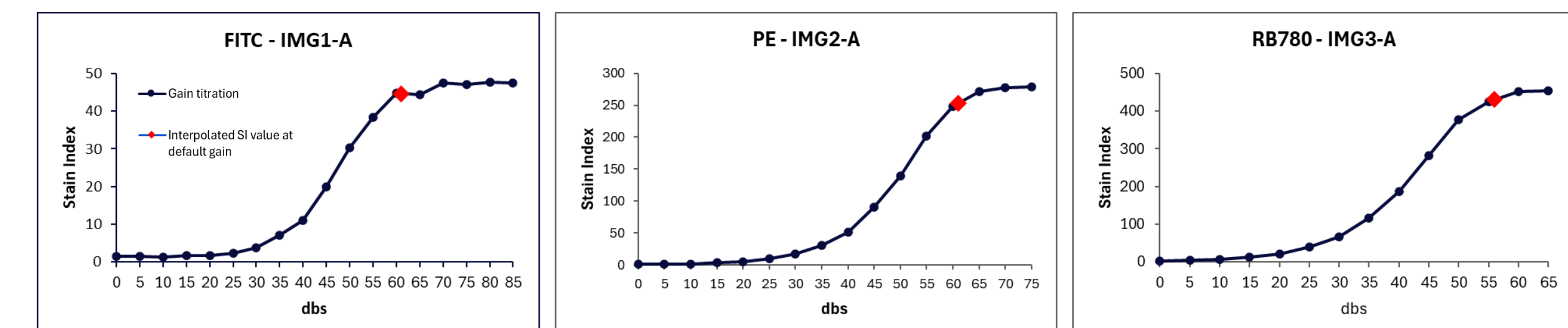
Conclusions

- Gain titration of imaging channels demonstrate that default gains are the optimal gains for best Stain Index
- Performance (Stain Index) of a given fluorochrome is likely to be different in the spectral and in the imaging detectors
- It is crucial to titrate reagents to ensure best resolution and to evaluate spillover
- FMOs are critical to assess spillover impact into imaging channels
- Spillover should be assessed using data plots, since signal visualization on the wall depends on post-acquisition imaging adjustments
- We were able to visualize T-bet translocation upon PBMCs stimulation by applying best practices in panel design and lessons learnt in Imaging Cytometry
- T-bet translocation was successfully visualized both on the Cell Wall and using imaging parameters (Correlation)
- When adding imaging to spectral cytometry more subsets can be visualized

BD Flow cytometers are class I Laser Products. For Research Use Only. Not for use in diagnostic or therapeutic procedures. Trademarks are the property of their respective owners. © 2025 BD, BD, the BD Logo and all other trademarks are property of Becton, Dickinson and Company. BD-147548 (v1.0) 0425

Results (1) Understanding Fluorochrome Performance and Gain Setup on Imaging Detectors

1A. Gain Titration and Interpolation of Default Gains



A gain titration of the three imaging channels was performed using freshly isolated PBMCs single stained with different CD4-conjugates (FITC for IMG1, PE for IMG2 and RB780 for IMG3). Samples with corresponding CD4-conjugates were acquired at intervals of 5db until reaching detector saturation. Stain index at each gain was calculated and gain titration curve generated for each imaging channel. Corresponding stain indices at default gains were calculated by interpolation (red dot).

1B. MFI Ranking and Spillover Assessment on Imaging Channels

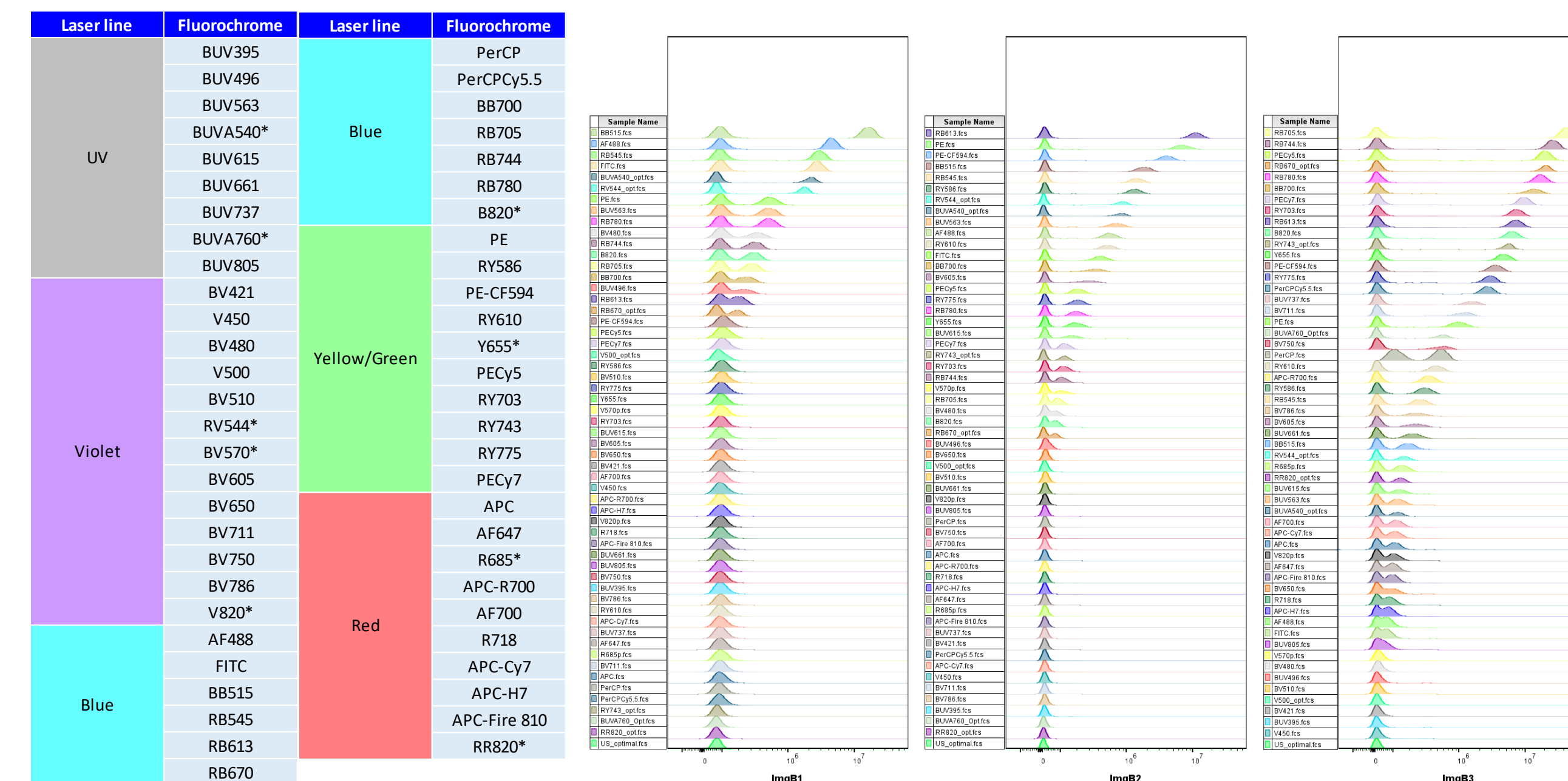
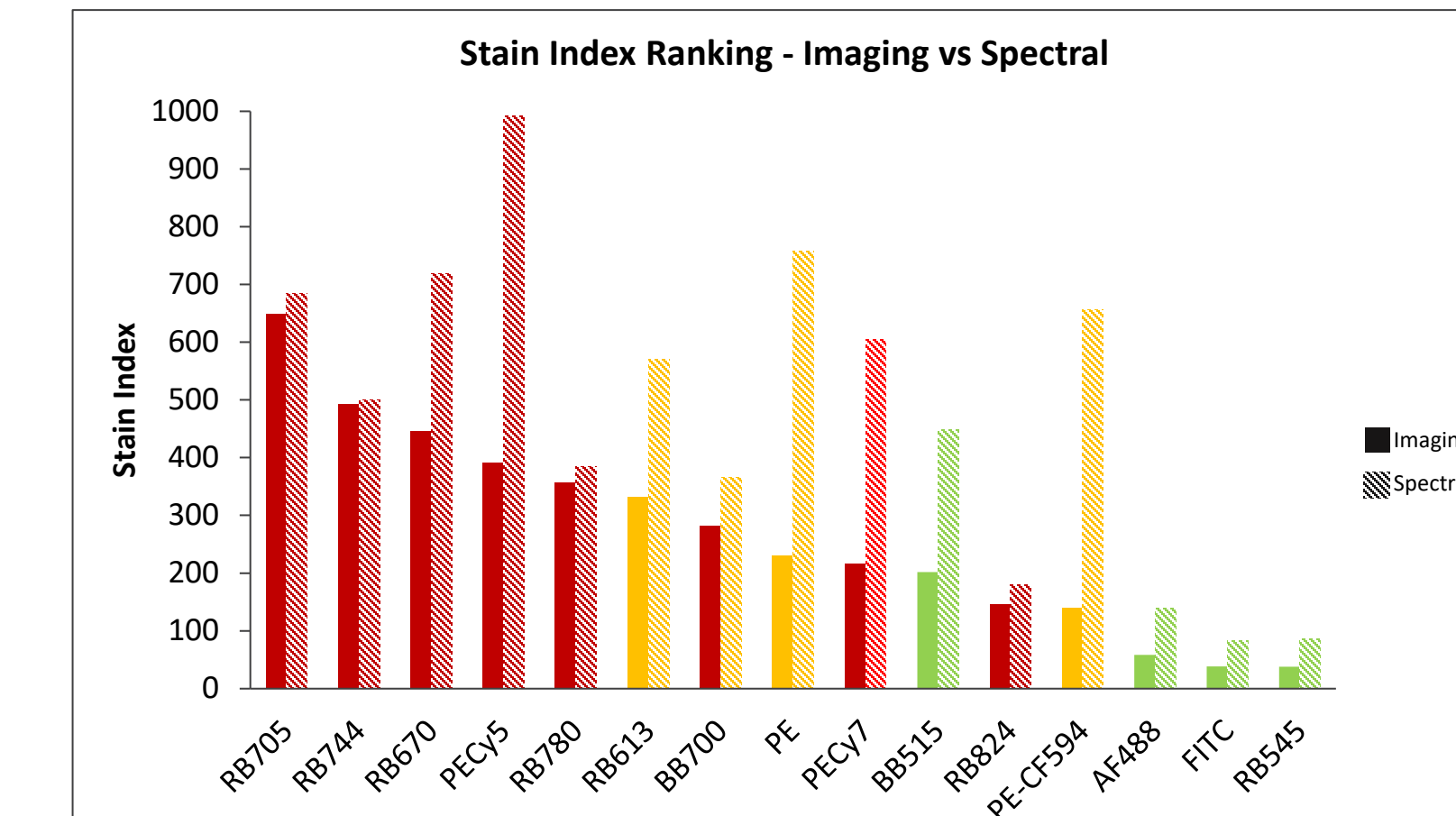


Table 1. CD4 – conjugated antibodies. Freshly isolated PBMCs were single stained with CD4-conjugated antibodies (indicated in table 2). Samples were acquired on the FACSDiscover™ S8 set up at its default optimal gains. Histogram overlays show Mean Fluorescence Intensity (MFI) of each fluorochrome in each imaging channel. Fluorochromes were ranked from highest to lowest MFI. Fluorophores marked with asterisk (*) are early candidates from the BD new dye technology team.

1C. Stain Index Ranking for Imaging Fluorochromes



Imaging (filled) and Spectral (striped) Stain Index Rankings of imaging fluorochromes (fluorochromes excited by blue laser). The same fluorochrome for imaging will also be part of the spectral analysis and its performance (SI) is likely to be different between both detectors.

1D. Imaging Spillover Reference Table



Results (2) Design and Optimization of a 12-color spectral + 2-color Imaging Flow Cytometry Panel

2A. Spectral + Imaging Panel Design

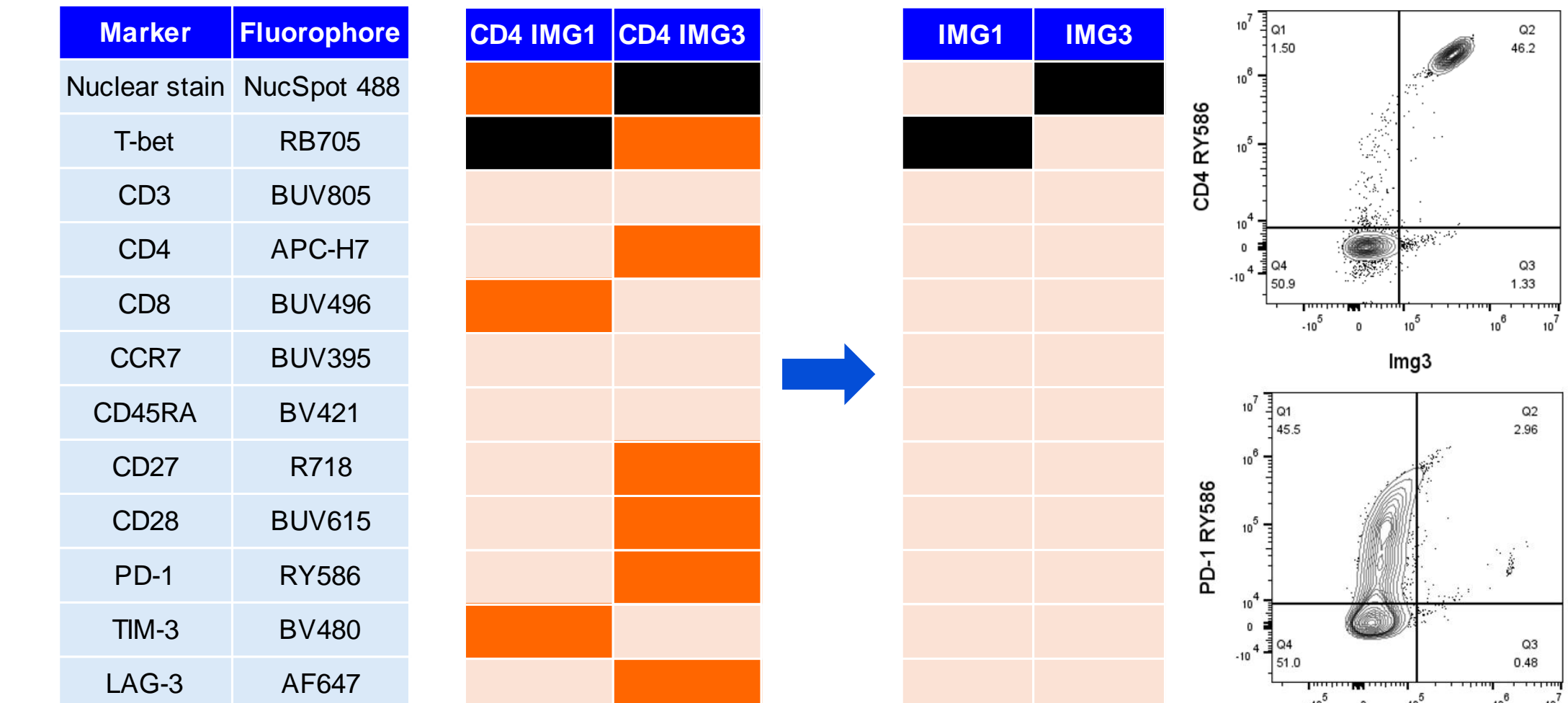
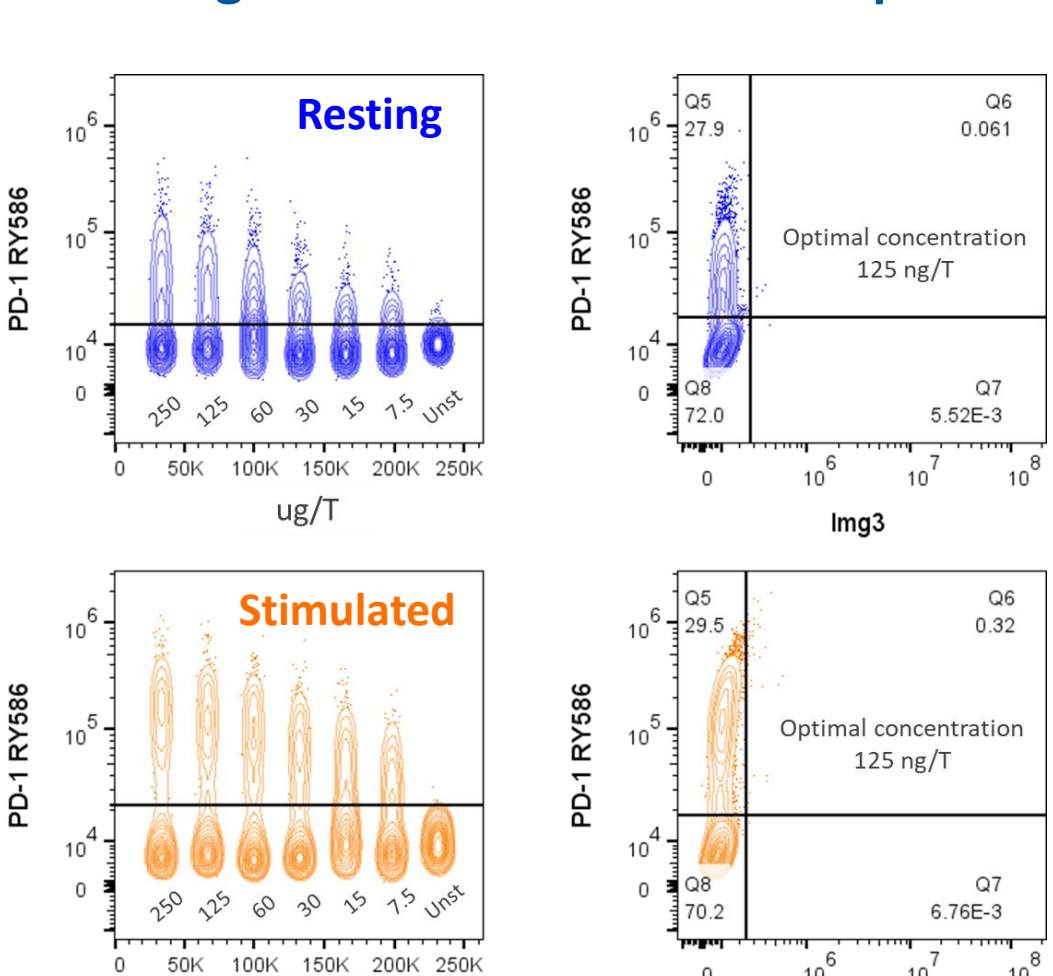


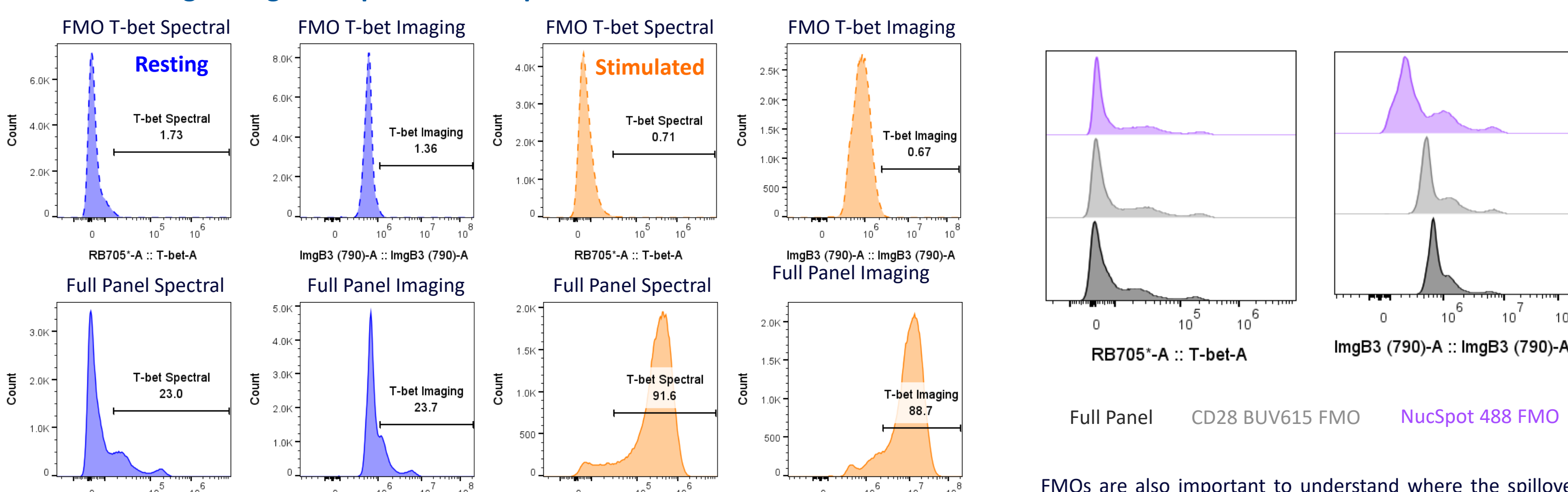
Table 2. 12-color fluorescent + 2-color imaging panel to localize T-bet in activated lymphocytes. Panel was designed by selecting fluorochromes with no or mild spillover into the imaging channels based on CD4 stain (Results 1D). Fluorochromes with mild spillover into imaging channels were assigned to lower density antigens. Table on the right represents imaging spillover expectation after antigen matching (expression level vs CD4) and reagents titration.

2B. Reagent Titration to Evaluate Spillover



Reagents titration is critical for determining optimal concentration for best resolution and to minimize spillover. It is important to assess spillover in all the experimental conditions since marker expression may change.

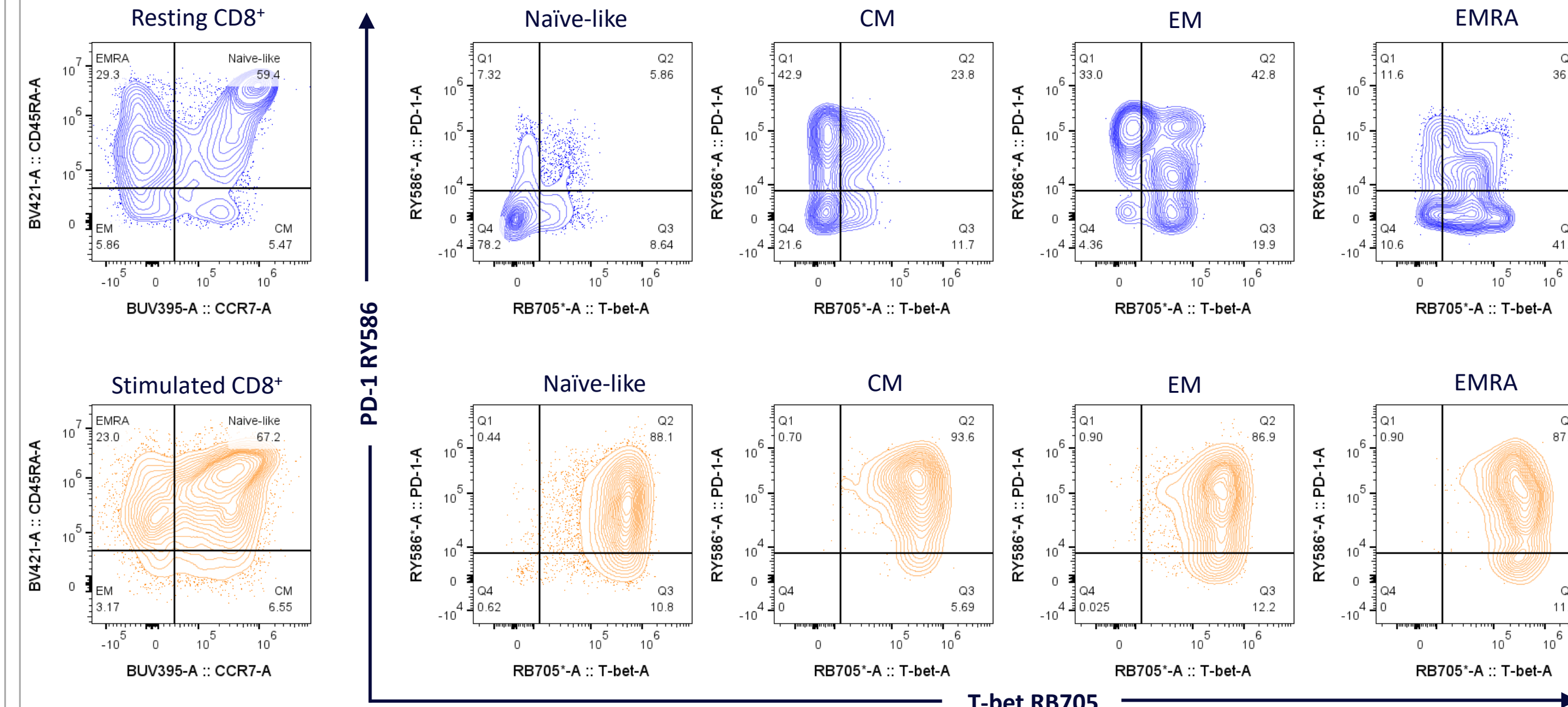
2C. Evaluating Biological Impact Due to Spillover



FMOs are critical to assess spillover impact into imaging channels. FMOs are used to set gates for T-bet, both spectral and imaging detectors. Spectral data are unmixed and represent the biological standard. We can see spillover in IMG3 as shown by the high negative population. However, based on FMO gates, we can still resolve a dim and bright population. This data confirm we were able to minimize spillover and maintain resolution.

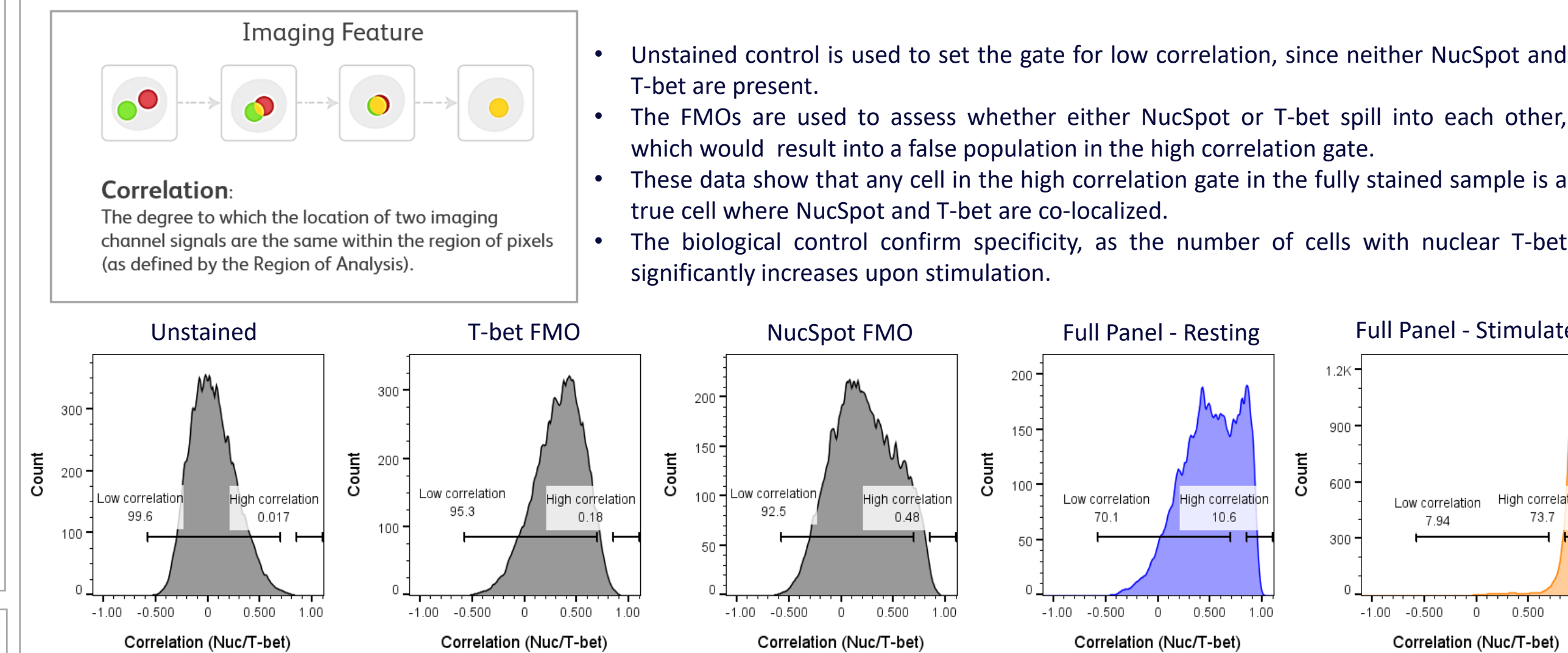
Results (3) Integrating Spatial T-bet Protein Expression with Flow Cytometry

3A. T-bet and PD-1 Expression in CD8+ T cells

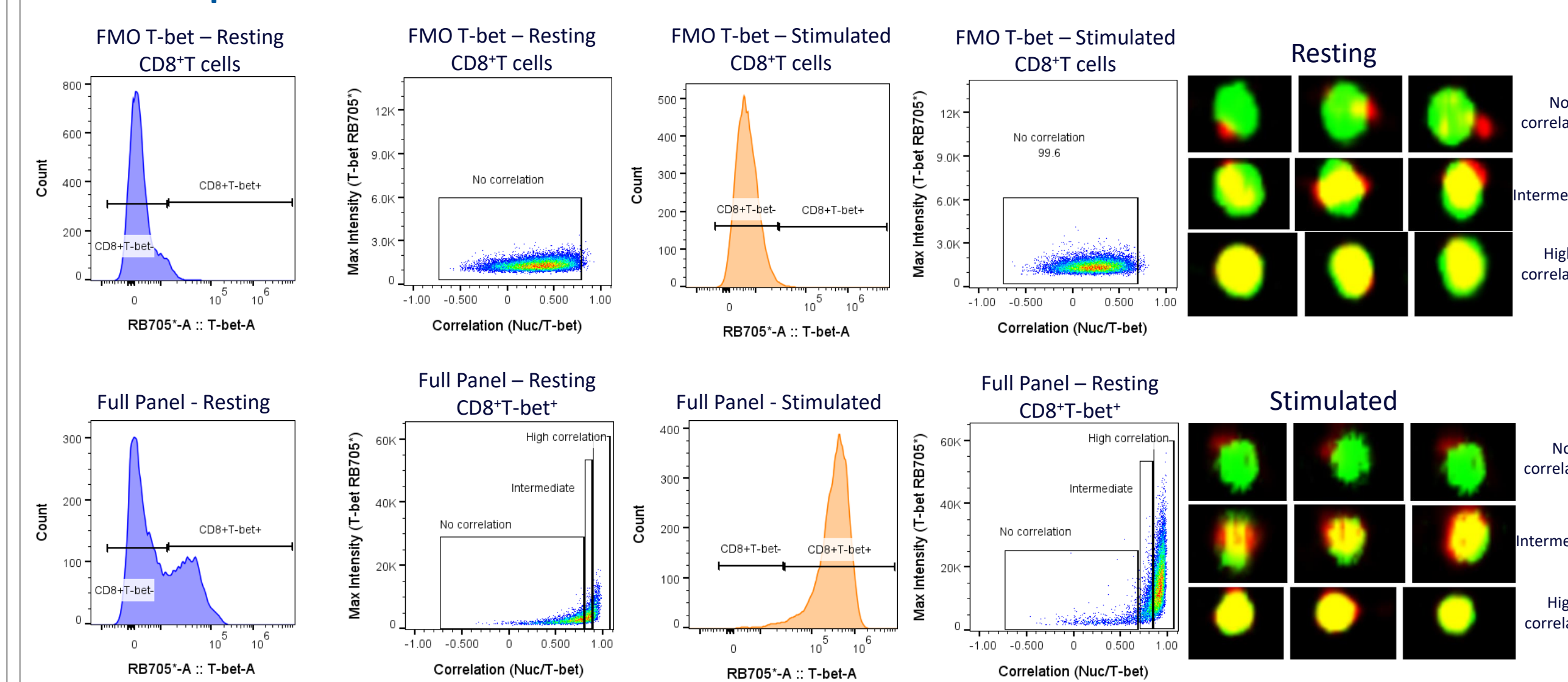


The panel successfully indicates the differential expression levels of PD-1 and T-bet in all evaluated CD8+ T-cell subsets. Upon activation, CD8+ T cells present upregulation of both PD-1 and T-bet increasing the % of double-positive subsets.

3B. Measurement of the "Correlation" Imaging Feature

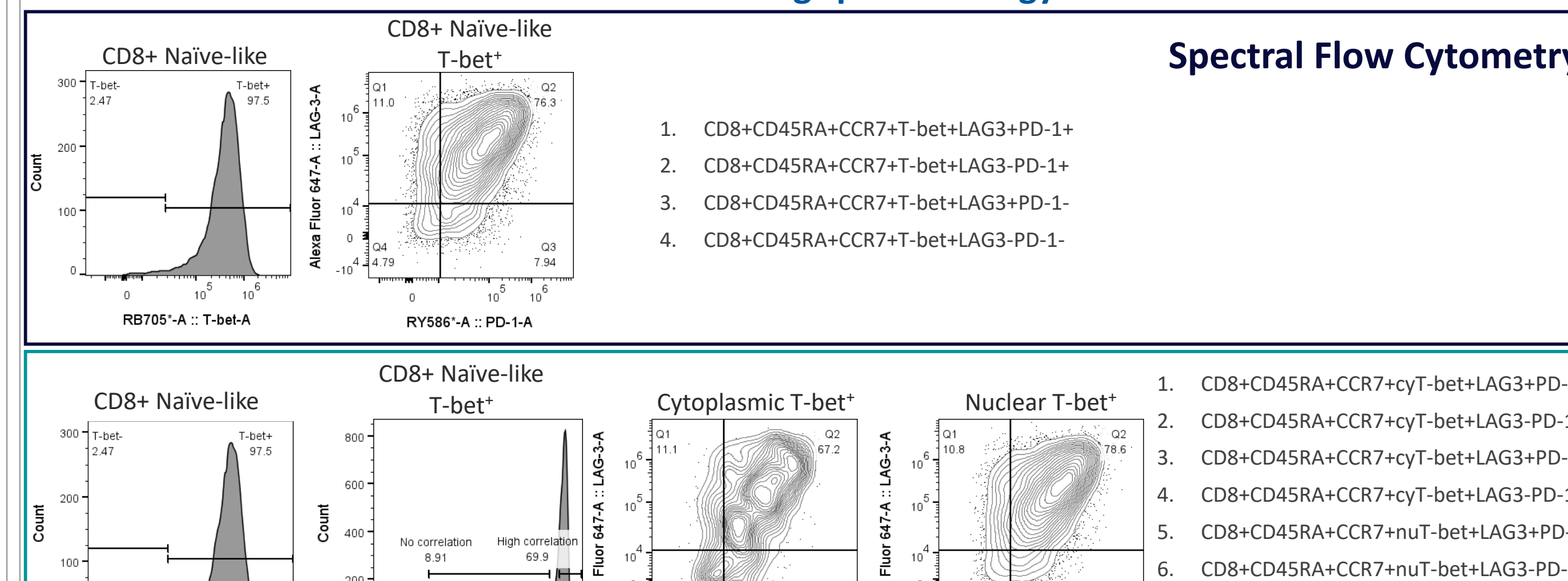


3C. T-bet Spatial localization within CD8+ T cells



Cell Wall images confirm T-bet localization based on Correlation (Nuc/T-bet). No correlation gate shows T-bet localization within the cytoplasm; Intermediate correlation gate shows T-bet localization both in the cytoplasm and in the nucleus; High correlation gate shows T-bet localization within the nucleus.

3D. More subsets can be detected when adding spatial biology



Real-Time Imaging + Spectral Flow Cytometry