Optimizing Imaging Flow Cytometry with BD CellView™ Technology for Higher-Parameter Data Generation

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Abstract

Background: Imaging flow cytometry merges quantitative flow cytometry with high-resolution single-cell imaging. BD FACSDiscover™ S8 and A8 systems, featuring BD CellView™ Image Technology, enable real-time imaging that expands data dimensionality beyond fluorescence. BD CellView™ captures spatial and morphological features, supporting subcellular classification and higher-parameter analysis. However, standardized optimization guidelines remain limited.

Objective: To optimize fluorochrome selection and imaging parameters using BD CellView™ technology, demonstrated through a 12-color spectral panel with 2-color imaging for enhanced T-cell analysis.

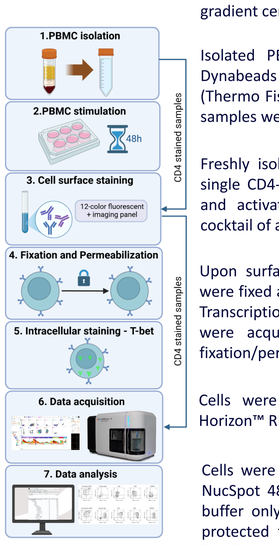
Methods: PBMCs were stained with 55 CD4-conjugated fluorochromes and acquired on a BD FACSDiscover™ S8 using default imaging gains to rank brightness and spillover across three channels. A 12-color panel targeting surface and intracellular markers (NucSpot 488, T-bet-RB705, CD3-BUV805, CD4-APC-H7, CD8-BUV496, CCR7-BUV395, CD45RA-BV421, CD27-R718, CD28-BUV615, PD-1-RY586, TIM-3-BV480, LAG-3-AF647) was used to stain resting and stimulated PBMCs (CD3/CD28 beads, 48h). Cells were fixed, permeabilized, and resuspended in wash buffer prior to acquisition. FMO controls validated imaging detection of NucSpot and T-bet, and confirmed "Correlation" as a parameter for T-bet trafficking.

Results: Stimulation increased PD-1 and T-bet co-expression in CD8+ T-cell subsets: Naïve (5.86%→88.1%), CM (23.8%→93.6%), EM (42.8%→86.9%), EMRA (36.1%→87.5%). Imaging revealed T-bet localization patterns—cytoplasmic, nuclear, or both—with nuclear T-bet predominating post-stimulation (73.7%). Imaging-enhanced analysis identified 48 functional T-cell subsets, tripling the resolution of traditional analysis without imaging using the same panel.

Conclusion: This study provides a practical framework for optimizing BD CellView™ imaging and fluorochrome assignment. Integrating spectral and image-derived parameters enables deeper functional classification and higher-parameter data generation, advancing cytometric analysis.

Methods

PBMCs were isolated from whole blood obtained from healthy volunteer donors using Ficoll™ gradient centrifugation.



Isolated PBMCs were cultured with or without Dynabeads Human T-cell Activator CD3/CD28 (Thermo Fisher Scientific) for 48 hours. CD4 stained samples were stained fresh.

Freshly isolated PBMCs were surface stained with single CD4-conjugated antibodies (Table 1). Resting and activated cells were surface stained with a cocktail of antibodies (Table 2).

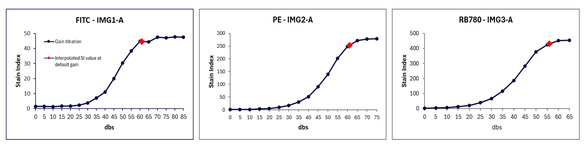
Upon surface staining, resting and activated cells were fixed and permeabilized with BD Pharmingen™ Transcription Factor Buffer Set. CD4-stained cells were acquired right after surface staining (no fixation/permeabilization).

Cells were then intracellularly stained with BD Horizon™ RB705 Mouse Anti-T-bet antibody.

Cells were resuspended in wash buffer containing NucSpot 488 (resting and activated cells) or wash buffer only (CD4-stained cells) and stored on ice, protected from light, until acquisition on the BD FACSDiscover™ S8 Cell Sorter

Data were analyzed using FlowJo_v10.10.0

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).

Results

1) Understanding Fluorochrome Performance on Imaging Detectors

MFI Ranking and Spillover Assessment on Imaging Channels

Laser line	Fluorochrome	Laser line	Fluorochrome
UV	BUV395	Blue	PerCP
	BUV496		PerCPCy5.5
	BUV563		BB700
	BUVA540*		RB705
	BUV615		RB744
	BUV661		RB780
	BUV737		B820*
	BUVA760*	PE	
	BUV805	Yellow/Green	RY586
Violet	BV421		PE-CF594
	V450		RY610
	BV480		Y655*
	V500		PECy5
	BV510		RY703
	RV544*		RY743
	BV570*		RY775
	BV605		PECy7
	BV650	Red	APC
	BV711		AF647
	BV750		R685*
	BV786		APC-R700
	V820*		AF700
Blue	AF488		R718
	FITC		APC-Cy7
	BB515		APC-H7
	RB545		APC-Fire 810
	RB613		RR820*
	RB670		

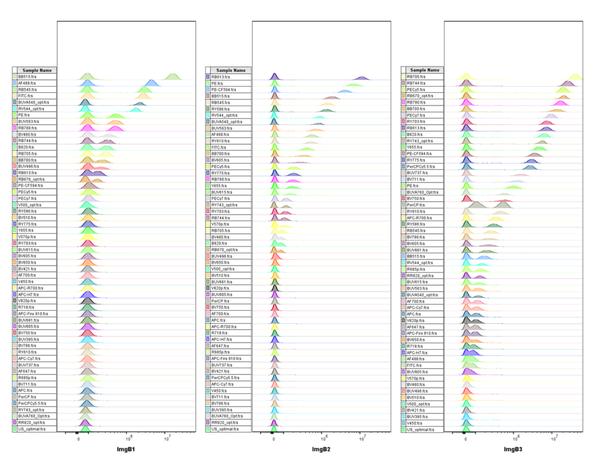
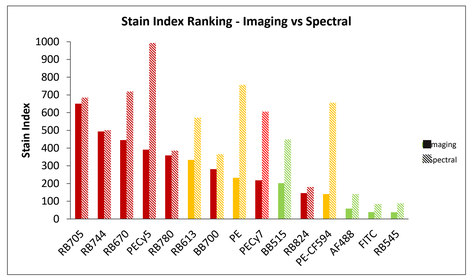


Table 1. CD4 – conjugated antibodies. Freshly isolated PBMCs were single stained with CD4-conjugated antibodies (indicated in table 2). Samples were acquired on the FACSDiscover™ S8 set up at its default optimal gains. Histogram overlays show Mean Fluorescence Intensity (MFI) of each fluorochrome in 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.

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.

Imaging Spillover Reference Table

Fluorochrome	IMG1	IMG2	IMG3	Fluorochrome	IMG1	IMG2	IMG3
AF488				BV480			
FITC				V500			
BB515				BV510			
RB545				RV544*			
RB613				BV570*			
RB670				BV605			
PerCP				BV650			
PerCP-Cy5.5				BV711			
BB700				BV750			
RB705				BV786			
RB744				RB828			
RB780				RY586			
RB824				RY610			
PE				RY655			
PE-CF594				RY703			
PECy5				RY743			
PECy7				RY775			
BUV395				AF647			
BUV496				RR688			
BUVA540*				APC			
BUV563				AF700			
BUV615				APC-R700			
BUV661				R718			
BUVA760*				APC-Cy7			
BUV737				APC-H7			
BUV805				APC-Fire810			
BV421				RR820*			
V450							

Primary IMG channel | Spillover >50% | Spillover 10 - 50 % | Spillover 0-10%

This table can be used as universal reference based on CD4 stain, when default settings are used.

If using one IMG detector, fluorochromes with no spillover into the IMG detector of choice are safe and should be prioritized.

Fluorochromes with spillover may be used if no safe options are available, if panel design rules and strict controls are used.

In the absence of compensation, IMG1 and IMG3 are the recommended detectors to be used together

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

Spectral + Imaging Panel Design

Marker	Fluorophore
Nuclear stain	NucSpot 488
T-bet	RB705
CD3	BUV805
CD4	APC-H7
CD8	BUV496
CCR7	BUV395
CD45RA	BV421
CD27	R718
CD28	BUV615
PD-1	RY586
TIM-3	BV480
LAG-3	AF647

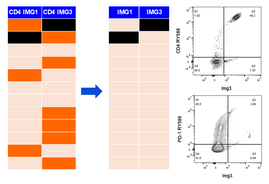
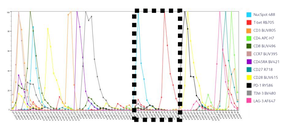
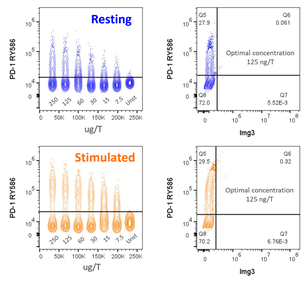


Table 2. 12-color fluorescent + 2-color imaging panel to localize T-bet in activated lymphocytes. Panel was designed by selecting fluorochromes with no or mild spillover into the imaging channels based on CD4 stain (Results 1D). Fluorochromes with mild spillover into imaging channels were assigned to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

Reagent Titration and Spillover

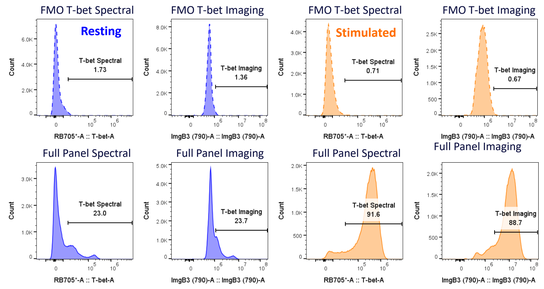


The spectral signatures of single controls in the 12-color spectral + 2-color imaging panel were overlapped. The signal was normalized to positive values only. Spectral blue detectors were highlighted in (dotted rectangle) to indicate that only imaging fluorochromes emit in these channels, with minimal to no spillover from other spectral fluorochromes in the panel.

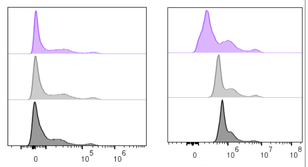


Reagents titration is critical for determining optimal concentration for best resolution and to minimize spillover. It is important to assess spillover in all the experimental conditions since marker expression may change.

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 showed 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.

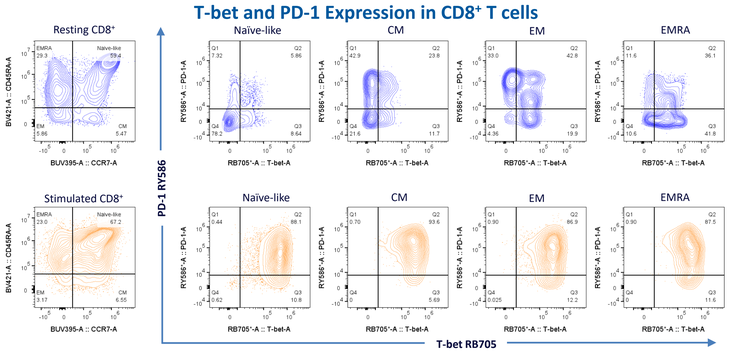


Full Panel CD28 BUV615 FMO NucSpot 488 FMO

FMOs are also important to understand where the spillover is coming from. These data show that NucSpot is responsible for the shift of the negative population. Further probe dilution can be performed to reduce the spillover

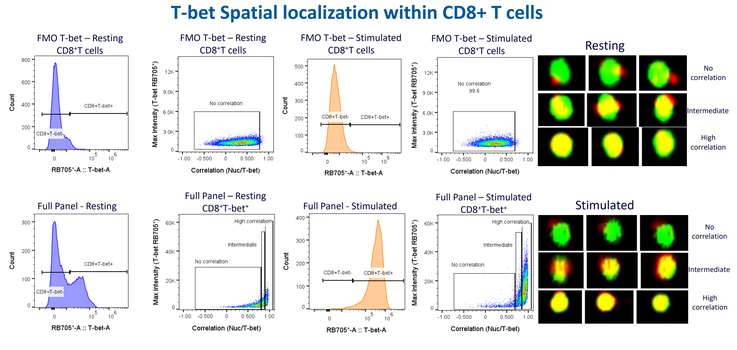
3) Integrating Spatial Localization and Expression Levels of T-bet with Flow Cytometry

T-bet and PD-1 Expression in CD8⁺ T cells



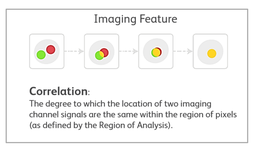
The panel successfully indicates the differential expression levels of PD-1 and T-bet in all evaluated CD8+ T-cell subsets. Upon activation, CD8+ T cells present upregulation of both PD-1 and T-bet increasing the % of double-positive subsets.

T-bet Spatial localization within CD8+ T cells

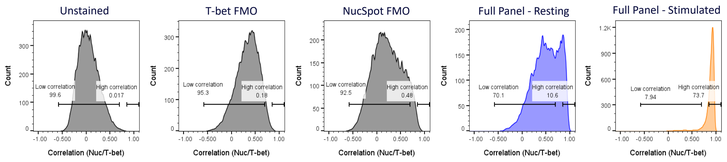


Cell Wall images confirm T-bet localization based on Correlation (Nuc/T-bet). No correlation gate shows T-bet localization within the cytoplasm; Intermediate correlation gate shows T-bet localization both in the cytoplasm and in the nucleus; High correlation gate shows T-bet localization within the nucleus.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

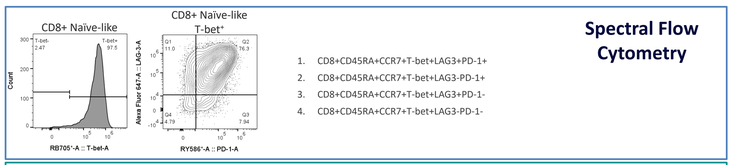
Measurement of the "Correlation" Imaging Feature

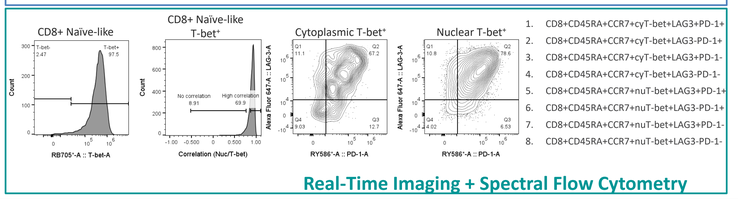


- Unstained control is used to set the gate for low correlation, since neither NucSpot and T-bet are present.
- The FMOs are used to assess whether either NucSpot or T-bet spill into each other, which would result into a false population in the high correlation gate.
- These data show that any cell in the high correlation gate in the fully stained sample is a true cell where NucSpot and T-bet are co-localized.
- The biological control confirm specificity, as the number of cells with nuclear T-bet significantly increases upon stimulation.



More subsets can be detected when adding spatial biology





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.