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Comparative Analysis of DURAClone IM T-Cell Subsets Antibody Panel: Conventional vs. Spectral Flow Cytometry on CytoFLEX mosaic Spectral Detection Module

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Introduction

Conventional flow cytometry uses dichroic mirrors and discrete bandpass filters to measure specific wavelengths and employs a one-detector-per-fluorochrome approach, whereas spectral flow cytometry captures a variety of points across the entire emission spectrum of each fluorochrome. Consequently, spectral flow cytometers often incorporate a greater number of detectors than fluorochromes, in contrast to traditional bandpass flow cytometry. The CytoFLEX mosaic Spectral Detection Module enables users to operate their conventional CytoFLEX LX/S* Flow Cytometer in spectral mode, in addition to the existing conventional mode. Its modular design provides a sustainable solution, enabling both existing and future customers to upgrade and future-proof their CytoFLEX instruments.

This application note describes the process of comparative analysis of the DURAClone IM T-cell subsets antibody panel using the conventional flow mode on a CytoFLEX LX Flow Cytometer and the CytoFLEX mosaic 88 Spectral Detection Module. The comparison between these two modes provides valuable insights into the advantages of each. We utilized the dry DURAClone IM T-cell panel—a 10-color, 10-monoclonal antibody reagent panel that facilitates the detection of prevalent extracellular markers of T-cell subsets.[1] The DURAClone IM T-cell panel indicates the maturation phases of T lymphocytes, including naïve, effector, memory, and terminal differentiation stages. It utilizes dry reagent technology to streamline workflows by providing pre-formulated antibody panels in a unitized assay. This approach eliminates the need for antibody cocktailing, pipetting, and time-consuming titration of different antibody lots, thereby reducing potential errors and simplifying the procedure.

While both conventional and spectral flow cytometry modes yielded consistent population subset identification, spectral flow cytometry exhibited superior sensitivity, particularly in detecting dim markers and low-abundance populations, resulting in improved signal-to-noise ratios. The modular approach of the CytoFLEX mosaic Spectral Module allowed for the integration of both conventional and spectral flow capabilities, providing flexibility for a range of experimental applications. This versatility positions the system as a practical solution for researchers seeking to address diverse flow cytometry needs.

Background

T-cell-mediated immunity remains a central focus of research into the immune response. Upon maturation in the thymus, naïve T lymphocytes expressing CD197 (CCR7) and other lymph node homing receptors circulate through the blood and lymphatic systems. Exposure to foreign antigens through interaction with specialized antigen-presenting cells in secondary lymphoid organs triggers the differentiation of T-cells into antigen-specific effector cells, accompanied by a decrease in levels of CD27 and CD28 expression. Alongside effector cells, the process also generates long-lived central memory T-cells (CD45RA-CD197 (CCR7)+) and effector

*Only valid for CytoFLEX S (V-B-Y-R) Series Flow Cytometer.

memory T-cells (CD45RA-CD197 (CCR7)-), which are essential for maintaining the capacity for rapid antigen-specific immune responses. The terminal stages of T-cell differentiation are marked by the upregulation of CD57 (effector phenotype) and CD279 (PD-1) (co-inhibitory molecule, exhausted phenotype), indicating the progression toward a differentiated and potentially exhausted T-cell phenotype.

Materials

Instrument and Supplies

1. CytoFLEX LX Flow Cytometer C06779 (Beckman Coulter, Inc.)
2. CytoFLEX mosaic 88 Spectral Detection Module U-V-B-Y-R-I (6 laser UV) (Beckman Coulter, Inc.) **(Table 1)**
3. CytExpert software 2.6 2.6.0.105
4. CytExpert for Spectral software 1.0.0.49
5. Centrifuge (Beckman Coulter, Inc., Avanti J-15R, B99515)
6. Vortex Mixer (Kartell, Italy, TK3S, 1032235)
7. Aspirator (Orange Scientific, ComfoPette+)
8. Screw cap tube 50 mL (Sarstedt, Germany, 62.547.254).
9. Screw cap tube 15 mL (Sarstedt, Germany, 62.554.502)
10. 5 mL flow tubes (Corning, USA, 352054)
11. Transfer pipette
12. Micropipettors
13. Pipette tips (1000 µL, 200 µL, 20 µL) Toledo, USA, 30389212, 30389238, 30389225)
14. Miscellaneous: biological hazard container, aluminum foil, ice

The CytoFLEX mosaic Spectral Detection Module represents the next advancement in the CytoFLEX platform, enabling both CytoFLEX S* and LX instruments to operate in spectral mode in addition to their conventional mode. When paired with the CytoFLEX LX Flow Cytometer, the CytoFLEX mosaic 88 Spectral Detection Module allows operation in either spectral or conventional modes. Users can either choose to upgrade their existing CytoFLEX LX or S* instruments to include spectral functionality or opt for a bundled system that includes both the CytoFLEX Flow Cytometer and the CytoFLEX mosaic Spectral Detection Module.

The CytoFLEX mosaic Spectral Detection Module is available in two configurations: the CytoFLEX mosaic 88, compatible with the CytoFLEX LX Flow Cytometer (offering 88 detection channels), and the CytoFLEX mosaic 63 Spectral Detection Module, compatible with CytoFLEX S BRVY series (offering 63 detection channels). For this application note, we used CytoFLEX mosaic 88 Spectral Detection Module, and specifications are mentioned in Table 1.

CytoFLEX Flow Cytometer	CytoFLEX mosaic Spectral Detection Module	Channel (FSC/ SSC/FL)	FSC	355 nm (SSC/FL)	405 nm (SSC/FL)	488 nm (SSC/FL)	561 nm (SSC/FL)	638 nm (SSC/FL)	808 nm (SSC/FL)
CytoFLEX LX Instrument (UV355)	Spectral Detection Module, CytoFLEX mosaic 88	1/6/81	1	1/20	1/20	1/16	1/12	1/10	1/3

Table 1: Instrument Configuration

*Only valid for CytoFLEX S (V-B-Y-R) Series Flow Cytometer.

Reagents and Antibodies

1. DURAClone IM T-cell subsets tube, 25 tests, B53328, RUO contains the following: 25 tests of the DURAClone IM T-cell subsets tube (i.e., a single tube is a single test), 3 compensation kits, each kit containing ten tubes, each of a single color (CD3-APC-A750, CD4-APC, CD8-A700, CD27-PC7, CD28-ECD, CD45-Krome Orange, CD45RA-FITC, CD57-Pacific Blue*, CD197 (CCR7)-PE, CD279 (PD-1)-PC5.5) and LIVE/DEAD Near IR 876 **(Table 2)**
2. BD CompBeads (cat. 552843)
3. ArC amine reactive Compensation Beads (Thermo Fisher Scientific, Inc., cat. A10346)
4. Blood collection tubes with anticoagulant (K2EDTA)
5. Heat Inactivated Fetal Bovine Serum (FBS) supplemented with 10% dimethyl sulfoxide (DMSO)
6. Phosphate Buffered Saline (PBS) Buffer
7. FACS buffer (PBS supplemented with 2% FBS)
8. CytoFLEX Daily QC Fluorospheres (Beckman Coulter, Inc., C65719)
9. CytoFLEX Daily IR QC Fluorospheres (Beckman Coulter, Inc., C06147)
10. CytoFLEX sheath fluid (Beckman Coulter, Inc., B51503)
11. Flow cleaning agent (Beckman Coulter, Inc., A64669)

Marker	Fluorochrome	Clone	Catalog	Company
CD3	APC-A750	UCHT-1	B53328	Beckman Coulter, Inc.
CD4	APC	13B8.2		
CD8	A700	B9.11		
CD27	PC7	1A4.CD27		
CD28	ECD	CD28.2		
CD45	Krome Orange	J33		
CD45RA	FITC	2H4		
CD57	Pacific Blue	NC1		
CD197 (CCR7)	PE	G043H7		
CD279 (PD-1)	PC5.5	PD1.3.5		

Table 2: Antibody Panel Details

Methods

Specimen Collection

1. Up to 30 mL of blood specimen was collected in vacuettes containing ethylenediamine-tetraacetic acid (EDTA) as an anticoagulant. Blood was processed immediately.
2. Isolation of peripheral blood mononuclear cells (PBMCs) was performed using Ficoll-Hypaque technique according to standard procedures.
3. PBMCs were stored in liquid nitrogen in fetal bovine serum (FBS) supplemented with 10% dimethyl sulfoxide (DMSO).

Sample Preparation and Staining

1. Isolated PBMCs were thawed and rested for 1 hour at 37°C in a 5% CO_2 atmosphere in complete RPMI culture medium. For further details on PBMC thawing, please refer to the protocol used in point 2 below.
2. Two million (2×10^6) of PBMCs were stained with LIVE/DEAD® Fixable Near-IR 876 (Invitrogen, cat. L34980) for 20 minutes at room temperature (RT) in PBS.

3. Cells were washed with 2 mL of PBS for 5 minutes at 500 x g.
4. The cells were initially resuspended in 100 µL of freshly prepared FACS buffer (PBS + 2% FBS) and subsequently transferred into a DURAClone IMT tube (Beckman Coulter, Inc. Brea, CA) containing CD45-Krome Orange, CD3-APC-A750, CD4-APC, CD8-A700, CD27-PC7, CD57-Pacific Blue, CD279 (PD-1)-PC5.5, CD28-ECD, CCR7-PE, and CD45RA-FITC for staining at room temperature for 20 minutes, protected from light.
5. Cells were washed with 2 mL of FACS buffer for 5 minutes at 500 x g and resuspended in 0.5 mL of FACS buffer for acquisition.

Color Compensation Settings

A. Compensation Setup

1. Compensation beads:

1. Vortex the vial BD CompBeads (Cat. 552843) and add one drop of positive BD CompBeads to all the DURAClone IMT cell panel tubes.
2. Incubate as per the manufacturer's instructions.
3. Wash beads with FACS or similar buffer.
4. Prepare an unstained beads tube by adding one drop of negative BD CompBeads.
5. Both the unstained beads (1 tube) and the single-stained control beads (10 tubes) were resuspended with 300 µL of FACS buffer each.

a. For CytoFLEX LX Flow Cytometer

Run the tubes on the CytoFLEX LX instrument to create compensation matrices. For setting compensation on CytExpert 2.6 software, use single-stain control and apply the compensation to the data.

b. For CytoFLEX mosaic 88 Spectral Detection Module

Run the tubes on the CytoFLEX mosaic 88 Spectral Detection Module to create unmixing matrices. For setting compensation on CytExpert for Spectral software, use single-stain control for unmixing and unmix the data.

2. Amine Reactive Compensation Beads (ArC beads-viability)

1. Vortex the vial and prepare tube with one drop of positive ArC beads as per the manufacturer's instructions.
2. Stain the ArC beads with the viability dye. Follow the staining protocol.
3. Wash and run the tubes to adjust compensation for the viability LIVE/DEAD® Fixable Near-IR 876 (Invitrogen, cat. L34980) dye.

Data Acquisition

Flow cytometry allows for the identification of cellular populations of interest based on the detection of forward and side scattering light as well as light emission from fluorescently labeled cellular antigens or compartments.

1. Resuspend cells at the recommended concentration (20×10^6 cells per mL). Note: The thawed cells should be stained with the viability dye.
2. Add 100 µL of the cell suspension to the DURAClone tube.

3. Incubate as per DURAClone IFU instructions.

   Note: Although BD CompBeads and amine-reactive compensation beads vary in size, their autofluorescence exhibits a comparable spectral signature. Therefore, the same unstained beads tube (one drop of negative BD CompBeads) was used for both BD CompBeads and amine-reactive compensation beads during the compensation setup.

1. Data Acquisition on CytoFLEX LX Flow Cytometer

1. Ensure the flow cytometer is properly aligned according to the manufacturer's recommendations—refer to CytoFLEX mosaic Spectral Detection Module IFU (D172052).
2. Run CytoFLEX daily QC (C65719, Beckman Coulter, Inc.) and daily IR QC Fluorospheres (C06147, Beckman Coulter, Inc.) to verify instrument alignment before the sample acquisition.
3. Note: For instrument setup and daily QC, refer to the CytoFLEX LX Flow Cytometer IFU (B49006).
4. Samples are acquired by applying the Cytosettings from the Quality Control.
5. 10,000 events as the main population in the scatterplot were collected for compensation beads.
6. At least 400,000 lymphocytes in the scatter plot were collected for cell analyses.

2. Data Acquisition on CytoFLEX mosaic 88 Spectral Detection Module

1. Ensure the flow cytometer is properly aligned according to the manufacturer's recommendations (refer to CytoFLEX mosaic Spectral Detection Module IFU) (D17052). Run CytoFLEX daily QC (C65719, Beckman Coulter, Inc.) and daily IR QC Fluorospheres (C06147, Beckman Coulter, Inc.) to verify instrument alignment before the sample acquisition.
2. Note: For instrument setup and daily QC, refer to the CytoFLEX mosaic Spectral Detection Module IFU (D172052).
3. Samples are acquired by applying the Cytosettings from the Quality Control.
4. Ten thousand events as the main population in the scatterplot were collected for compensation beads.
5. At least 400,000 lymphocytes in the scatterplot were collected for cell analyses.

Data Analysis

1. Data Analysis on CytExpert Software

1. Open a new workspace on CytExpert software and add sample .fcs files and unmixing .fcs files in separate folders.
2. Apply calculated compensation to the sample .fcs files.
3. Define the gated populations as shown in Figure 1 and Table 3.

2. Data Analysis on CytExpert for Spectral Software

4. Open a new workspace on CytExpert for Spectral software and add sample .fcs files and unmixing .fcs files in separate folders.
5. Apply calculated unmixing to the sample .fcs files.
6. Define the gated populations as shown in Figure 1 and Table 3.

Data Analysis

Gating Strategy

1. Create an appropriate analysis protocol to define the population gates and the series of dual parameter plots for analysis of the reagent specificities.
2. Set the discriminator on the Forward Scatter (FS) parameter to a low enough value to ensure lymphocytes are included in the acquisition.
3. Create a time vs. viability dye (LIVE/DEAD Near IR 876) plot to select singlet cells, and exclude dead cells, electronic noise and events with acquisition inconsistencies.
4. Use the Forward Scatter High (FSC-H) and Forward Scatter Width (FSC-W) to exclude doublets.
5. Use Forward Scatter Area (FSC-A) vs. Side Scatter Area (SSC-A) to include lymphocytes.
6. Use CD45-KrO (Krome Orange) vs. CD3-APC-A750 to gate the CD3+CD45+ T-cells.
7. Use a CD4-APC vs. CD8-A700 plot to create separate regions to further split T-cells into CD4+ or CD8+ T-cells.
8. For CD4+ and CD8+ cells, draw a quadrant to delineate the following phenotyping T-cell subsets by CD45RA-FITC vs. CCR7-PE Plot. These populations were characterized based on their CCR7 and CD45RA expression into:
   i. Naïve T-cells (N): CCR7+ CD45RA+
   ii. Central memory (CM): CCR7+ CD45RA−
   iii. Effector memory (EM): CCR7− CD45RA−
   iv. Effector memory RA (EMRA): CCR7− CD45RA+
8. Finally, CD4+ and CD8+ T-cell subpopulations (N, CM, EM and EMRA) were assessed for their CD27 and CD28 expression (markers of differentiation and longevity) or for their PD-1 and CD57 expression (markers of senescence/exhaustion).

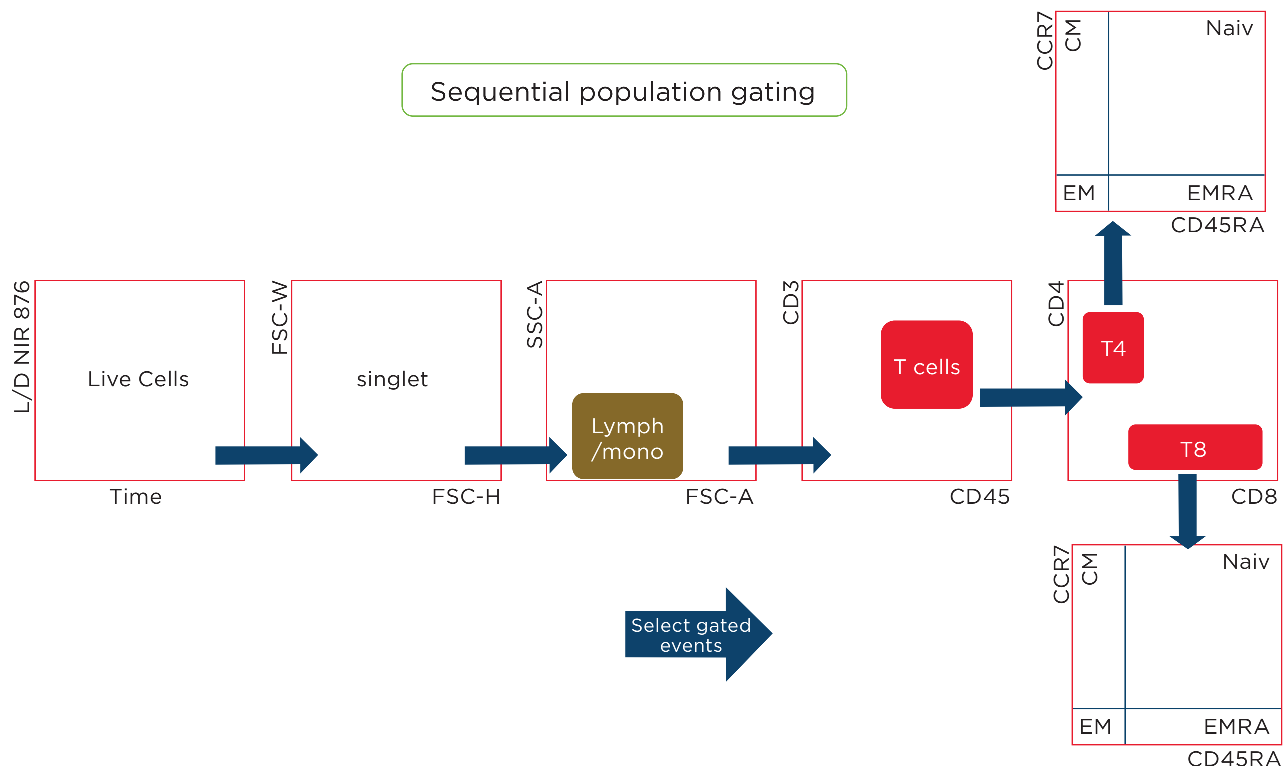

Figure 1: Sequential gating strategy for T-cell populations, starting with singlet and viability gating, followed by lymphocyte selection, CD3+ T cell identification, and subsequent gating of CD4+ and CD8+ subsets, with further subsetting based on differentiation and activation markers.

Results and Discussion:

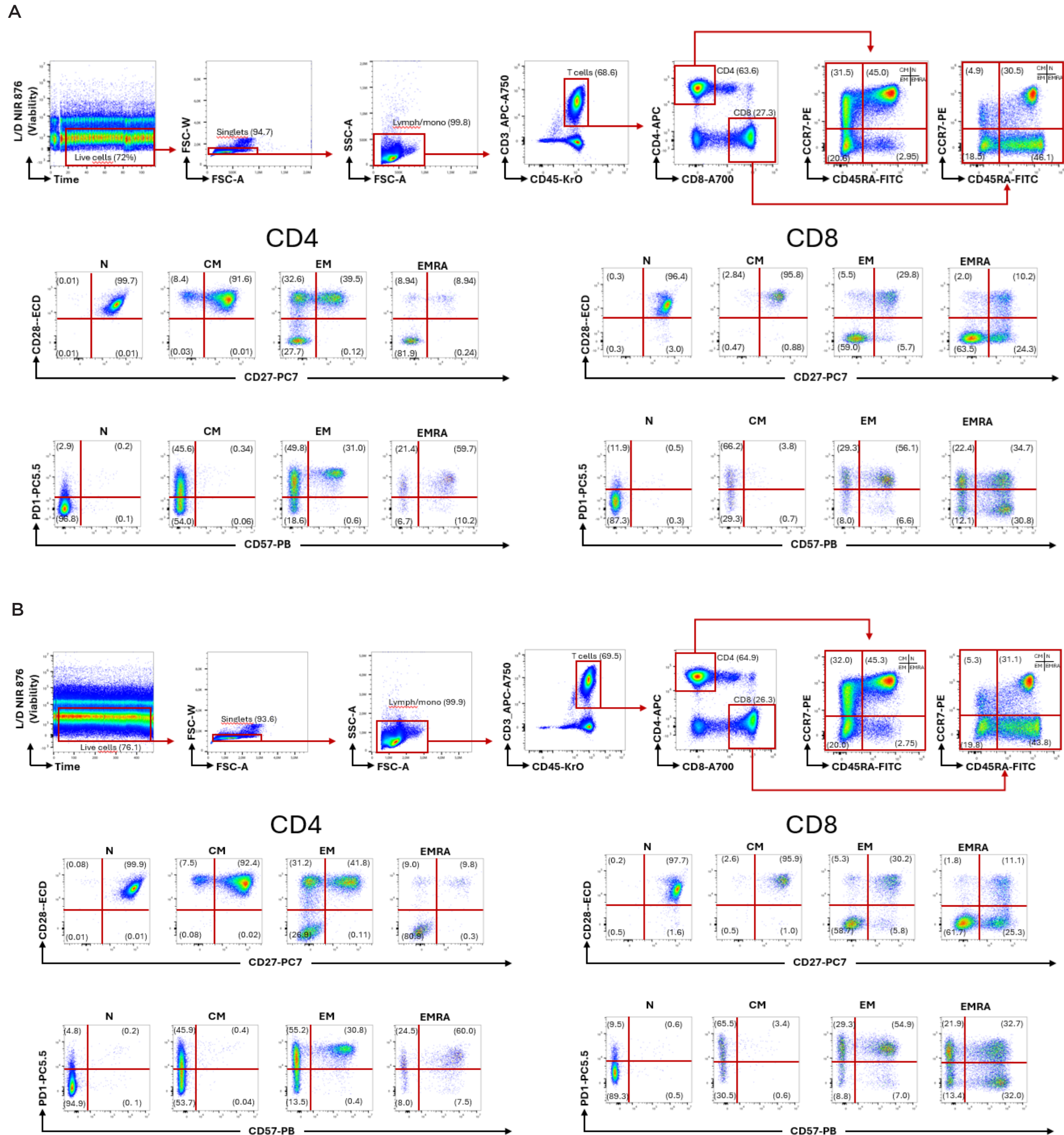

Figure 2: (A) Dot plots showing the sequential gating strategy for T-cell analysis using conventional flow cytometry (CytoFLEX LX Flow Cytometer), with percentages of positive populations indicated for each step, including singlet, viability, lymphocyte, CD3+CD45+, CD4+, CD8+, and T-cell subsets defined by CD45RA vs. CCR7. Further analysis of CD27/CD28 and PD-1/CD57 expression is also shown. **(B)** Equivalent dot plots were obtained using the CytoFLEX mosaic 88 Spectral Detection Module paired with the CytoFLEX LX Flow Cytometer. These plots display a similar gating strategy with percentages of positive populations indicated.

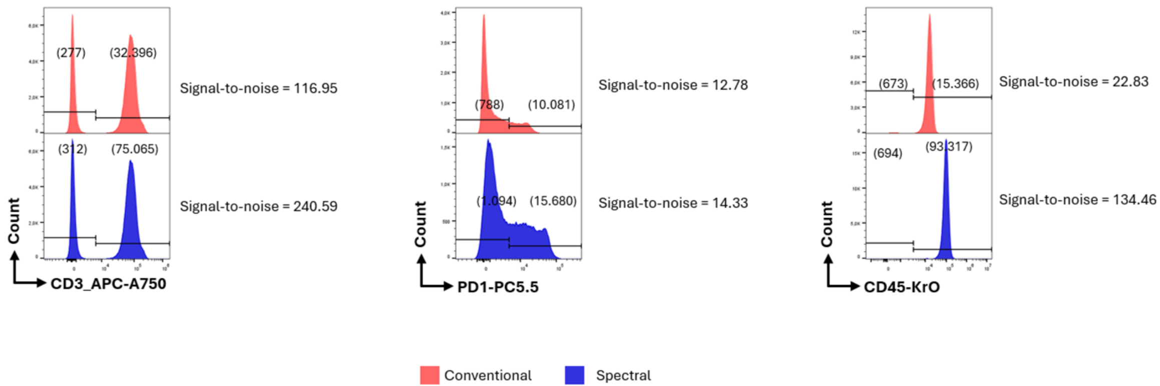

Figure 3: Comparison of conventional (CytoFLEX LX Flow Cytometer) and spectral mode for signal-to-noise ratio across markers: CD3 APC-A750, PD-1–PC5.5, and CD45-KrO. Signal-to-noise ratios are highlighted for each marker, showing enhanced sensitivity and resolution in spectral mode, particularly evident in CD3 APC-A750 (240.59 vs. 116.95) and CD45-KrO (134.46 vs. 22.83).

The results show that the population subsets identified by conventional and spectral flow cytometry are similar, with both methods providing consistent percentages across major cell populations **(Figure 2).** For example, both techniques identified viable cells and subsets with nearly identical efficiency, as shown by the high percentages for lymphocyte gating (spectral mode: 99.9%, conventional mode: 99.8%) **(Table 3)**. This consistency suggests that spectral flow cytometry mode and conventional flow mode generated similar results and both modes are equally efficient in T-cell subset analysis.

Spectral flow cytometry mode, however, demonstrated clear improvements in signal-to-noise ratio, particularly for markers like CD3-APC-A750 (240.59 vs. 116.95) and CD45-KrO (134.46 vs. 22.83) **(Figure 3)**. The CytoFLEX mosaic Spectral Detection Module enhances sensitivity, improving resolution of overlapping fluorophores and reducing spillover and background noise. This implies that spectral flow cytometry mode can be particularly useful for identifying dim markers and low-abundance populations, which can be difficult to detect with conventional methods.

While conventional flow cytometry remains effective for routine immunophenotyping, spectral flow cytometry offers significant benefits for more complex analyses. Its ability to resolve overlapping spectra and detect rare or dim populations makes it a useful complement to traditional methods. The modular design of the CytoFLEX platform allows for seamless integration of both conventional and spectral modes, offering flexibility for a wide range of experimental needs.

Cell Subset	Spectral Flow (%)	Conventional Flow (%)	Observations
Viable Cells	76.1	72	Both methods are equally efficient.
Singlets (FSC-A vs. FSC-W)	93.6	94.7	
Lymphocytes (SSC-A vs. FSC-A)	99.9	99.8	
T-Cells (CD3+ CD45+)	69.5	68.6	
CD4+ T- Cells	64.9	63.6	
Naïve T-cells (N): CCR7+ CD45RA+	45.3	45.0	
Central memory (CM): CCR7+ CD45RA−	32.0	31.5	
Effector memory (EM): CCR7− CD45RA−	20.0	20.6	
Effector memory RA (EMRA): CCR7− CD45RA+	2.75	2.95	
CD8+ T- Cells	26.3	27.3	
Naïve T- cells (N): CCR7+ CD45RA+	31.1	30.5	
Central memory (CM): CCR7+ CD45RA−	5.3	4.9	
Effector memory (EM): CCR7− CD45RA−	19.8	18.5	
Effector memory RA (EMRA): CCR7− CD45RA+	43.8	46.1	

Table 3: Performance Comparison of Spectral vs. Conventional Flow Cytometry in T-Cell Subset Analysis

References

1. B53328, DURAClone IM T-cell Subsets Tube, 25 Tests, RUO

2. De Biasi, S., Lo Tartaro, D., Neroni, A. et al. Immunosenescence and vaccine efficacy revealed by immunometabolic analysis of SARS-CoV-2-specific cells in multiple sclerosis patients. Nat Commun 15, 2752 (2024)

Acknowledgment

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Abbreviations

A700	Alexa Fluor* 700
APC	Allophycocyanin
APC-A750	Allophycocyanin Alexa Fluor750
BD	Becton Dickinson
CD	Cluster of Differentiation
CD45-RA	CD45 isoform RA
ECD	R Phycoerythrin-Texas Red-X
FITC	Fluorescein isothiocyanate
FSC	Forward Scatter
IR	Infrared
KrO	Krome Orange
mL	Milliliter
µL	Microliter
PBS	Phosphate Buffered Saline
PC5.5	Phycoerythrin-Cyanine 5.5
PC7	Phycoerythrin-Cyanine 7
PE	Phycoerythrin
SSC	Side Scatter
T cells	T lymphocytes
UV	Ultraviolet
U-V-B-G-Y-I	UV-Violet-blue-Yellow Green-Red-IR

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*Pacific Blue ("PB") is a trademark or registered trademark of Thermo Fisher Scientific Inc. (formerly Molecular Probes Inc.)
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