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18–Color Human Blood Phenotyping Made Easy with Flow Cytometry

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Introduction

As flow cytometry continues to develop increasing capabilities, the addition of lasers, more detectors and better signal processing, high parameter applications have begun to move out of specialty labs and into common practice. Users often find the process of establishing these high parameter applications intimidating. High-quality data requires multiple iterations of antibody-fluorochrome combinations that make up a panel and exhaustive testing to ensure sound results. Innovations in flow cytometry signal detection ease the process of panel design and data generation.

Objectives

- How to create a high parameter panel starting from a DURAClone backbone
- Where to apply Fluorescence Minus One (FMO) controls to ensure gating confidence

The implementation of avalanche photodiodes (APDs) for signal detection in the CytoFLEX offers two key benefits which enable easier panel design. First, APDs are more sensitive than photomultiplier tubes (PMTs) over a wider range of the spectrum. Second, this higher photo-sensitivity results in less measurement error, which minimizes spillover due to spreading.[1] Minimization of spillover spreading in high parametric experiments, allows better discrimination between dim and negative populations resulting in less critical channel selection for dim markers. Taking it one step further, ease of design can be enhanced by the use of dried, unitized reagent panels such as DURAClone. The use of DURAClone IM panels as a “backbone” allows the researcher to drop in additional stains as needed, while keeping many parameters stable and pre-optimized.

Combining the innovative technologies in CytoFLEX and DURAClone allows the creation of high parametric experiments with less effort for design and set-up. In this note, we will demonstrate the ease of panel design and generation of sound data, using the CytoFLEX LX and DURAClone IM T Cell Subset panel. Moreover, with the high number of detection channels on the CytoFLEX LX, a single DURAClone IM backbone can be modified to fit multiple experimental designs and needs, allowing for quick response to new questions in the lab. This paper demonstrates how these technologies combine for quick design and testing of an 18-color panel.

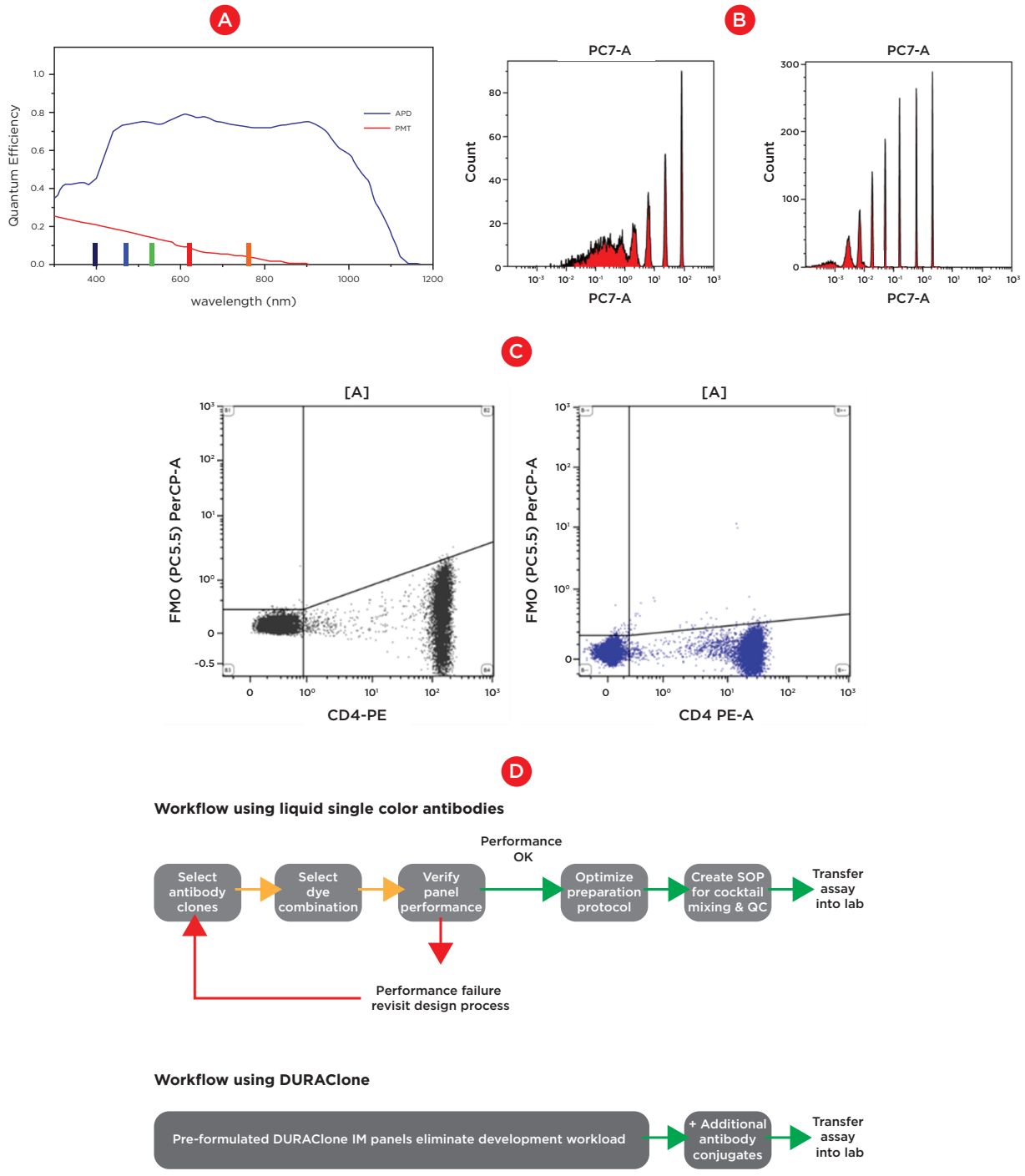

Figure 1. Comparison between PMT based and APD based systems.

Panel A: Graph representing Quantum Efficiency (QE), i.e., photon-electron conversion yield, of APDs and PMTs over the spectral range. This higher photon-electron conversion yield reduces measurement error thus facilitating higher sensitivity and resolution. Graph adapted from "A Comparison of Avalanche Photodiode and Photomultiplier Tube Detectors for Flow Cytometry" by Paul Wallace et al, 2008, Proceedings of SPI, Vol 6859.[2]

Panel B: Comparison of Spherotech 8 peak beads on PMT (left) and APD (right) shows better resolution of the dimmest beads due to increased QE, especially for emission wavelengths greater than 650 nm.

Panel C: Higher QE also reduces data spreading into adjacent detectors in APD based systems (right) compared to PMT based systems (left).

Panel D: Comparison of antibody panel design workflow with and without using DURAClone. DURAClone panels make building large panels less labor intensive by providing pre-optimized stable reagents for the backbone of the panel. Use DURAClone alone, or test and add more colors.

Materials

Product	Manufacturer	Part Number
CytoFLEX Daily QC Fluorospheres	Beckman Coulter Life Sciences	B53230
CytoFLEX Sheath Fluid	Beckman Coulter Life Sciences	B51503
CytoFLEX Daily IR QC Fluorospheres	Beckman Coulter Life Sciences	C06147
VersaComp Antibody Capture Beads	Beckman Coulter Life Sciences	B22804
VersaLyse Lysing Solution	Beckman Coulter Life Sciences	A09777
Dulbecco's Phosphate Buffered Saline	CORNING cellgro	21-031-CV
Brilliant Stain Buffer	BD Biosciences	566349
DURAClone IM T Cell Subsets	Beckman Coulter Life Sciences	B53328
CD20 BUV395	BD Biosciences	563781
HLA-DR BUV661	BD Biosciences	565074
CD19 BUV737	BD Biosciences	564304
CCR4 BV605	BioLegend	359417
CD95 BV650	BioLegend	305641
CD25 BV785	BioLegend	302637
CD33 PC5	Beckman Coulter Life Sciences	IM2647U
iFluor860 (IR fixable viability dye)	AAT Bioquest	1408
Whole EDTA blood (24-HR post venipuncture)	N/A	N/A
740/35 Band Pass Filter	Beckman Coulter Life Sciences	B78217
CytoFLEX LX UV	Beckman Coulter Life Sciences	C11186

Tips for Success

- When using multiple Brilliant Violet or Brilliant Ultraviolet dyes, Brilliant Stain Buffer must be added to the DURAClone tube (or any multicolor tube containing more than one of these dyes), before adding these dyes to prevent dye interactions that may result in artifacts.
- Vortex DURAClone tube immediately following addition of specimen and additional antibody conjugates to ensure proper mixing of reagents.

Protocol

1. Stain DURAClone compensation controls (included with DURAClone kit)
    a. Place one of each compensation control tube into a rack
    b. Add one drop each of positive and negative VersaComp beads to each tube
    c. Incubate for 20 minutes at room temperature, protected from light
    d. Add 1 mL PBS+1% BSA to each tube and centrifuge at 300x *g* for 6 minutes
    e. Decant supernatant
    f. Vortex
    g. Re-suspend in 400 µL of buffer PBS+1% BSA
2. Create drop-in reagent compensation controls (for additional single colors)
    a. Add one drop each of positive and negative VersaComp beads to tube
    b. Incubate for 20 minutes at room temperature, protected from light
    c. Add 1 mL buffer PBS+1% BSA to each tube and centrifuge at 300 x *g* for 6 minutes
    d. Decant supernatant
    e. Vortex
    f. Re-suspend in 400 µL of buffer PBS+1% BSA

3. Stain Whole Blood

    a. Add 50 µL of Brilliant Staining Buffer to each DURAClone tube

    b. Add titrated test amounts of each drop-in antibody and iFluor 860 to each DURAClone tube

    c. Label an additional 12 x 75 mm tube as unstained whole blood

    d. Add 100 µL of fresh whole blood to each mixed antibody reagent tube and unstained whole blood tube

    e. Vortex at high speed for 6-8 seconds

    f. Incubate tubes for 15 minutes, protected from light

    g. Add 2 mL of VersaLyse

    h. Vortex each tube at high speed for 1-3 seconds

    i. Incubate each tube at room temperature, protected from light

    j. Centrifuge each tube at 200 x *g* for 5 minutes

    k. Aspirate the supernatant and discard

    l. Gently tap the cell pellet to suspend into residual supernatant

    m. Add 5 µL of the IR Fixable dye to each mixed antibody reagent tube

    n. Incubate for 20 minutes at room temperature, protected from light

    o. Perform a wash step by re-suspending the cell pellet in 3 mL 1X PBS+1% BSA

    p. Aspirate the supernatant and discard

    q. Gently tap the cell pellet and resuspend the cell pellet in 500 µL of 1X PBS+1% BSA

Acquisition

4. Daily Startup

    a. Run the CytoFLEX System Startup Program

    b. Verify the detector configuration

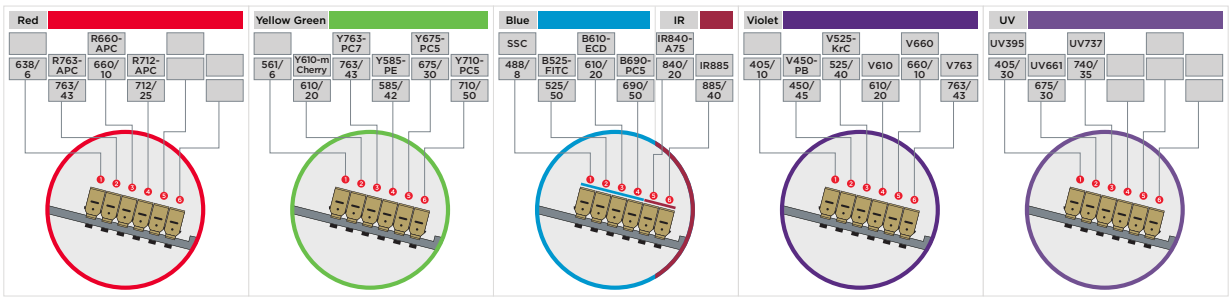

Figure 2. Detector Configuration of a CytoFLEX LX equipped with the UV laser.

    c. Run the Quality Control procedure according to the user manual: CytoFLEX Series Instructions for Use (IFU), document number B49006

5. Create Compensation Experiment

    a. For your DURAClone controls and drop-ins, include the lot # to allow for updating the Compensation controls when lots change

    b. Select Bead in the Sample Type column to reflect your single color controls

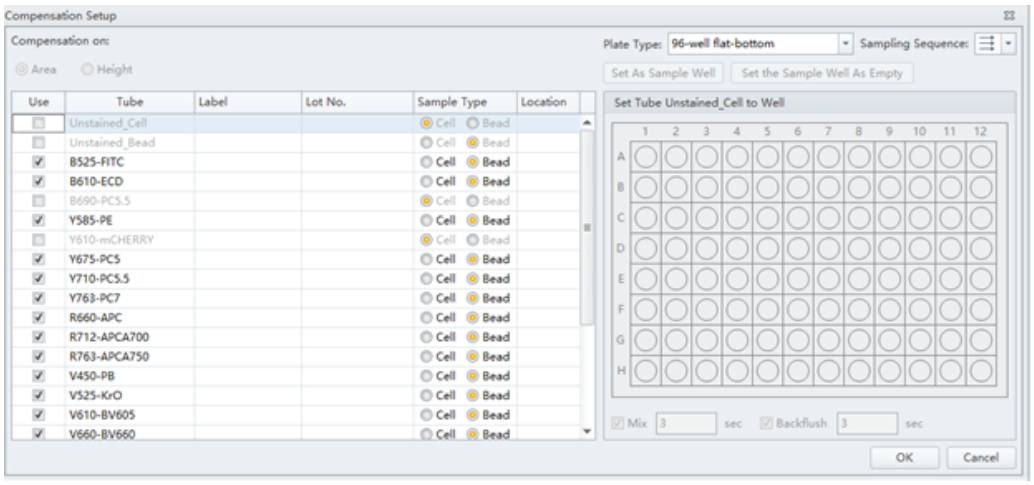

Figure 3. Compensation setup window, where the operator may select channels and sample type.

c. Uncheck using a universal negative, as VersaComp beads have a negative peak in each tube

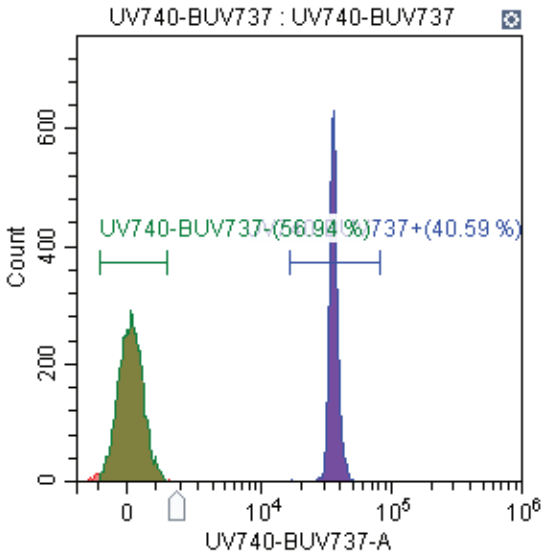

Figure 4. Example of single color run and gating.

d. Record each compensation sample

   i. Move the scatter gate to contain singlet beads

   ii. Use the Log-Linear slider to tighten the negative population and move the gate to capture the negative population

   iii. Move the gate to capture the positive population

e. Calculate the compensation matrix and save values to the Compensation Library

6. Create Experiment in CytExpert

   a. Import the created compensation library and convert the matrix based on current gains

   b. Create the plots

   c. Record

Results & Discussion

375 nm			405 nm					488 nm			561 nm					638 nm			808 nm	
405/305	675/30	740/35	450/45	525/40	610/20	660/10	763/43	525/40	610/20	690/50	585/42	610/20	675/30	710/50	763/43	660/10	712/25	763/43	840/20	885/40
BUV395	BUV661	BUV737	PAC BLUE	KROME ORANGE	BV605	BV650	BV786	FITC	ECD	B690	PE	Y610	PC5	PC5.5	PC7	APC	APC-A700	APC-A750	IR840	IR885/40
CD20	HLA-DR	CD19	CD57	CD45	CCR4	CD95	CD25	CD45RA	CD28		CCR7		CD33	CD279 (PD-1)	CD27	CD4	CD8	CD3		Viability

Figure 5. 17–Marker, 18–Color Panel Design. The above panel shows the marker-fluorochrome combinations used in this study. The DURAClone IM T cell Subset backbone, which consists of 10 colors, is outlined in red. The channels that were not used are shaded in gray.

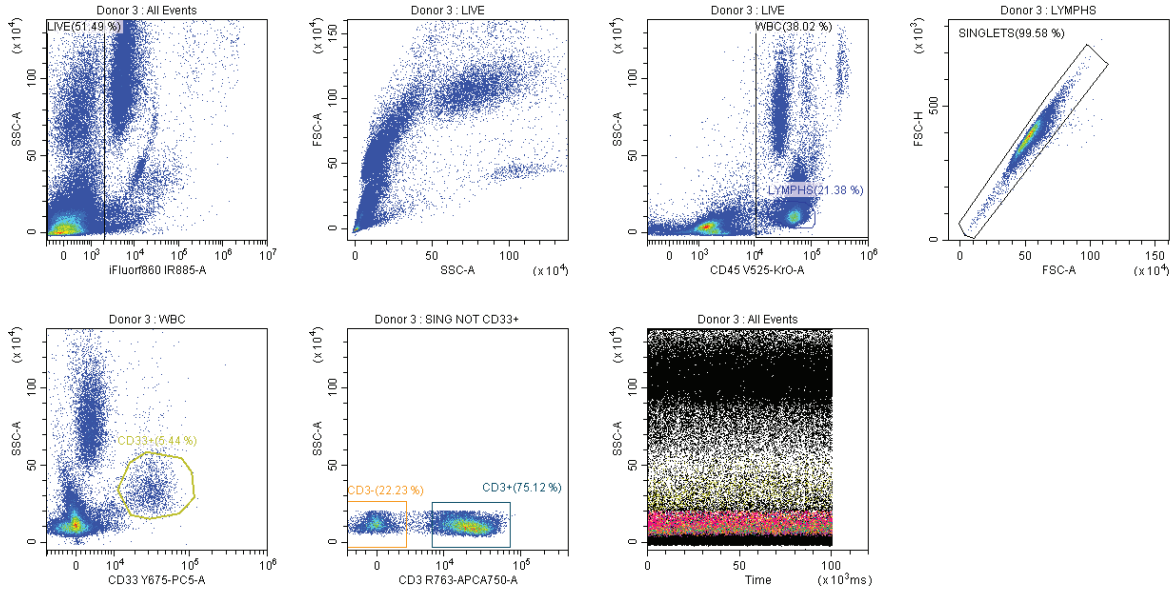

Figure 6a. High purity gating strategy

CD4+ T Cells

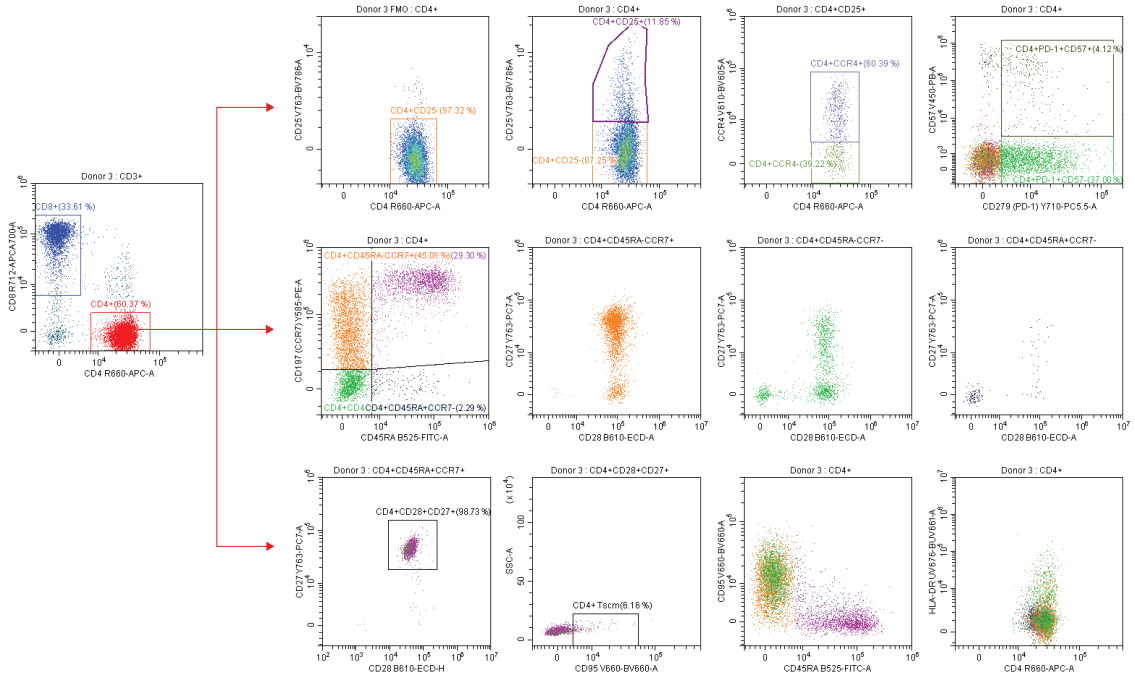

Figure 6b. CD4 T Cell Subset Analysis

CD8+ T Cells

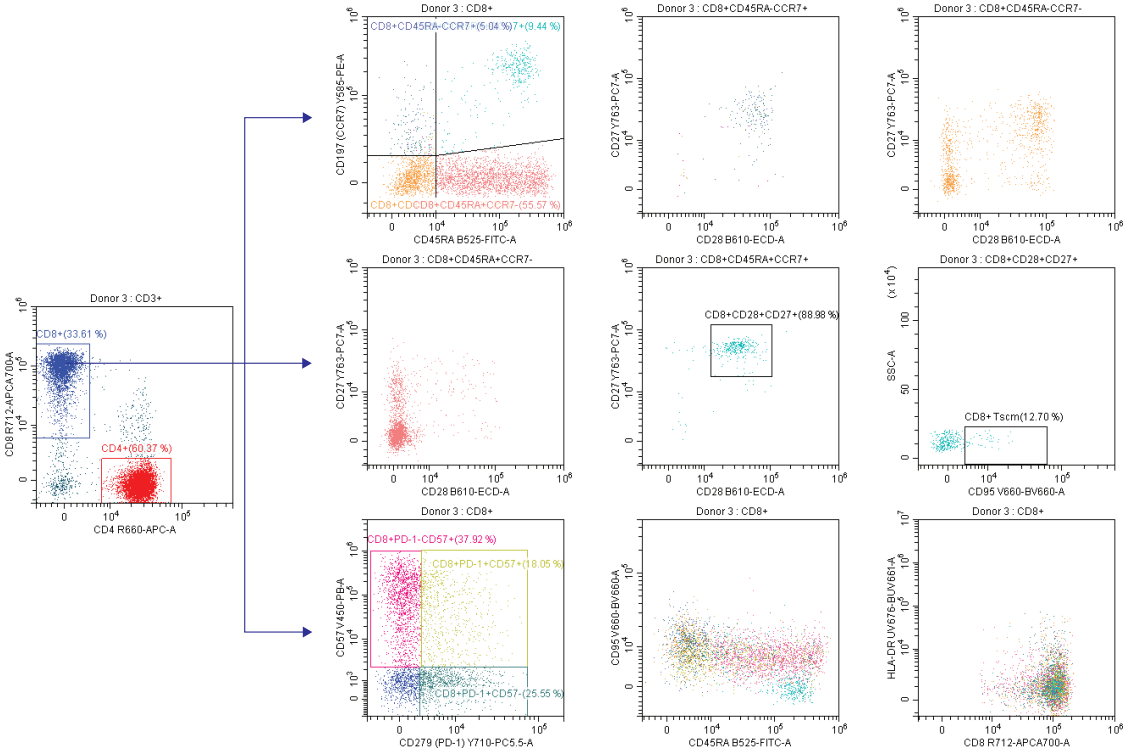

Figure 6c. CD8 T Cell Subset Analysis

B-Cells

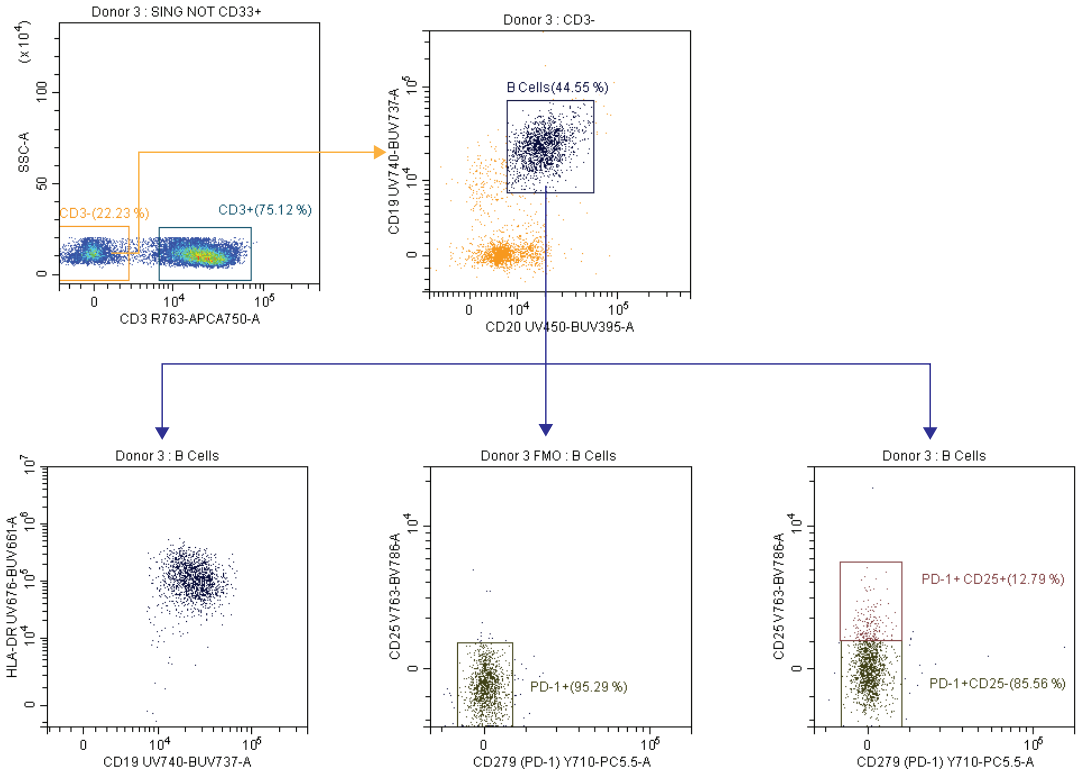

Figure 6d. B Cell Subset Analysis

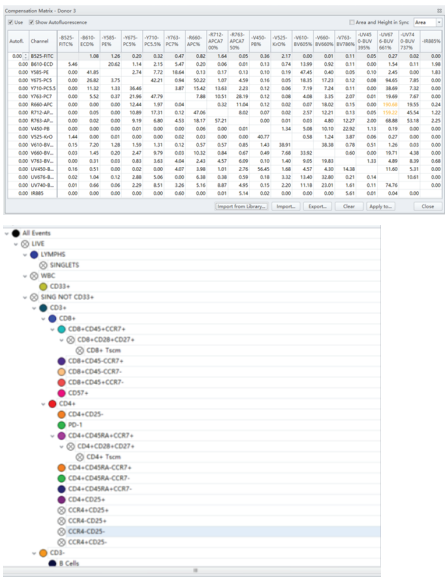

Figure 7. Compensation Matrix and Hierarchal Gating

A total of three healthy donors, 24-hours post venipuncture, were tested during this experiment. Beginning with the DURAClone IM T Cell subset as our backbone, seven markers and a viability dye were added to complete the panel. Marker-fluorochrome combinations adhere to the standard principles of multicolor design and also consider the availability of conjugates for the desired markers.

Prior to delving into T cell analysis, a preliminary gating strategy is applied. Using the IR fixable viability dye, dead cells are excluded. Live cells are then gated into a SSC vs CD45 plot, which is used to identify the White Blood Cell (WBC) and a gate is drawn around the WBCs to exclude debris. The lymphocyte gate is drawn around the population that has low SSC, high CD45 fluorescence profile. Lymphocytes are further differentiated by gating out doublets and removing monocytes ($CD33^+$) from the analysis. Lastly, we add the time plot to monitor and verify the system's fluidics and stability during acquisition (Figure 6a).

A total of 25 populations are assessed here in the T cell subsets (Figures 6b and 6c). To begin this analysis, we draw a region on $CD3^+$ cells that fall in the lymphocytes region. This allows the separation of $CD4^+$ and $CD8^+$ T cells from other cell types that could express these markers, such as NK cells (CD8) and monocytes (CD4).

Taking a deeper look at the $CD4^+$ T Cell subset, a population of $CD4^+CD25^+$ T cells comprises the Regulatory T cells. It is difficult to gate this population with confidence, especially in multicolor panels. CD25 also has a large potential for spreading, so a Florescence Minus One (FMO) stain was performed to increase gating confidence. Gating on $CD4^+$ cells, we can also assess CCR4 expression as a function of Tregs: while the CCR4 marker plays a critical role in the homing of skin cells, a subset of $CCR4^+$ said to control suppression of effector Regulatory T cells.[3]

Combining CD45RA with CCR7, we can begin to look at the various stages or pathways T cell subsets undergo during activation; i.e., naïve ($CD45RA^+CCR7^+$), central ($CD45RA^-CCR7^+$), memory, effector ($CD45RA^+CCR7^-$) and effector memory cells ($CD45RA^-CCR7^-$). Each phenotype can be further assessed by looking at the various expressions of the CD27 and CD28 co-stimulatory molecules.

Program Death Cell-1 (PD-1) is a member of the CD28 superfamily and is responsible for enhancing regulatory T cells, while impeding effector T cell function. PD-1 versus CD57 is assessed to identify exhaustive ($PD\text{-}1^+CD57^+$) and activated ($PD\text{-}1^+CD57^-$) T cell phenotypes. Lastly, CD95 is responsible for cell-mediated apoptosis. CD95 is primarily expressed on memory T cells but a small portion of T cells with naïve phenotype also expresses CD95 and are considered so-called stem cell like T cells, memory type (Figure 6b).[4]

Taking a look at the CD8 T cell subsets, a similar approach that is used to look at CD45RA, CCR7, CD28 and CD27 marker subsets as we did with CD4. The $CD8^+CD57^+$ subset denotes terminally differentiated T cells with high cytotoxic but low proliferative capacity. CD95 expression as a function on CD45RA is once again assessed in this T cell subset (Figure 6c).

Although not as richly stained in this panel as the T cells, B cells can also be seen ($CD19^+$, $CD20^+$, HLA^-DR^+) gating on the $CD3^-$ population. Although there is no consensus on how to gate regulatory B Cells (Bregs), PD-1 and CD25 are looked at in attempt to identify this population, with the understanding that the $PD\text{-}1^+CD25^+$ subset will contain other B cell subsets.[5, 6] If more detail is desired in this population or other cell types, the open channels in the backbone panel should allow for ease of changes (Figure 6d).

Conclusions

In this Application Note we illustrate the ease of using DURAClone dry unitized reagent assays as a backbone for deeper immunophenotyping panels. Beginning with a backbone and having open channels on a cytometer allows for fast results and the security of knowing that several of the parameters in the panel are pre-optimized. This method also allows for increased flexibility in the lab, as drop-in reagents can be set up to ask specific research questions. Further, the use of DURAClone IM tubes cuts down on staff time spent dispensing reagents, and the possibility of error when pipetting multiple reagents into multiple samples tubes.

Finally, the sensitivity of APD detectors in the CytoFLEX allows for flexible high parameter panel design. Dimly expressed markers no longer require placing that marker exclusively on the high performing channels as is the case with placing CD25 BV786 on the V763 channel. Although an FMO was performed, separation between $CD4^+CD25^-$ and $CD4^+CD25^+$ was clearly visible, highlighting the system's ability to resolve dim vs negative populations.

References

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