Studying Mouse Thymocyte Development using Multiparametric Flow Cytometry: An Efficient Method to Improve an 8-Color Panel on the BD FACSVerse™ System

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Application Note

Contents

Abstract

The maturation of T cells in the thymus is a highly orchestrated process. Any deviation in this process can lead to immunodeficiency, autoimmunity, or cancer. During the development of T cells, the expression of many surface molecules varies on the thymocytes, which can be monitored using multiparametric flow cytometry. In this application note, we describe an 8-color immunophenotyping antibody panel, against surface protein markers, for studying mouse thymocytes using the BD FACSVerse™ system. Using the BD FACS™ Universal Loader (Loader) and optimized mixing conditions, fluorescent antibodies for the panel were titrated to determine their optimal concentrations for this study. This panel could be used as an initial screening tool to analyze normal or abnormal immunophenotypic changes during T-cell developmental processes in mouse-model systems.

Introduction

T lymphocytes (T cells) play important roles in humoral and cell-mediated immune responses. Their maturation in the thymus is an intricate process. T-cell progenitors originate in the bone marrow and eventually migrate into the thymus, becoming thymocytes, where they differentiate and mature into naïve T cells. The structure of the thymus includes the cortex and the medulla, which play different roles during T-cell development. Progenitor cells enter the thymus and accumulate in the cortex. As they differentiate and mature, these thymocytes gradually move into the medulla and eventually migrate outside the thymus into the peripheral circulation as naïve T cells. Any deviation from this normal process can lead to immunodeficiency, autoimmunity, or cancer.[1-3] A brief summary of the current understanding of T-cell development in the thymus is shown in Figure 1 and in the sidebar.

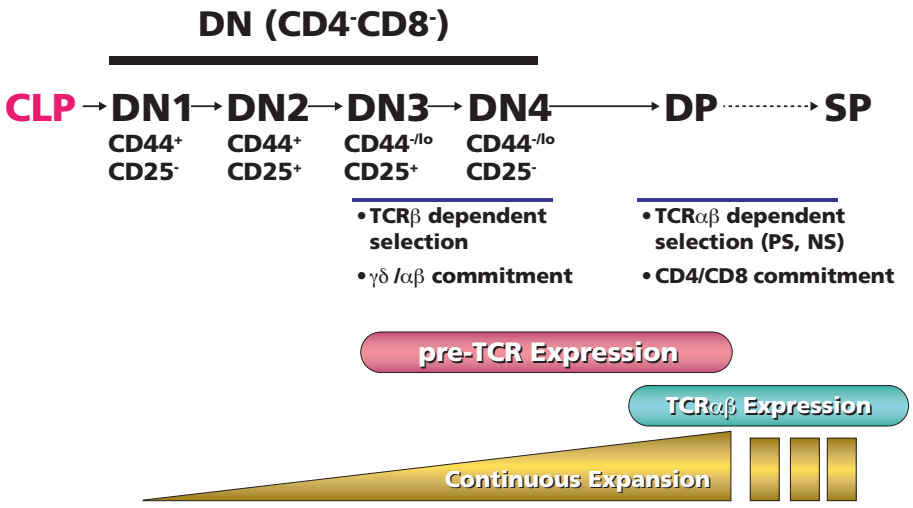


Figure 1. Schematic view of T-cell development in the mouse thymus.

CLP, common lymphoid progenitor; DN, double negative; DP, double positive; SP, single positive; TCR, T-cell receptor; PS, positive selection; NS, negative selection.

Multiparametric flow cytometry and transcriptome profiling are among the popular technologies used in studying thymocyte development.[2,3,6,7] Multiparametric flow cytometry is an efficient method of monitoring the expression of various molecules related to the developmental stages of T cells at a single-cell level. In the mouse model, various panels of fluorescent antibodies directed against different combinations of cell surface molecules have been reported to be useful for analyzing the cellular and molecular mechanisms of thymic T-cell maturation.[2,3,7,8] This application note is based on an in vivo study[7] and demonstrates the utility of a panel that includes CD4, CD8α, CD44, CD25, TCR β, CD5, CD69, and CD45R/B220 markers. Further, using various tools from BD Biosciences such as the BD FACSVerse system with the optional BD FACS Universal Loader and antibodies labeled with new fluorochromes (eg, BD Horizon™ PE-CF594), the existing panel design by Wang et al (2009) was improved.

The BD FACSVerse system is a high-performance flow cytometer that can incorporate the BD™ Flow Sensor option for volumetric measurement, automated procedures for setting up the instrument and assays, and configurable user interfaces designed for maximum usability for researchers. These functions are integrated to provide simplified routine applications while simultaneously providing powerful acquisition and analysis tools for more complex applications. The Loader enables use of either multiple tubes or multiwell plates for sample

Overview of Thymocyte Development

With the alternative expression of paired surface T-cell receptors (TCRs), T cells can be divided into two general groups, αβ T cells or γδ T cells. The αβ T cells are one of the most studied T-cell populations and play important roles in immune responses.

The developmental process of naïve T cells starts in the bone marrow where CLPs develop. The CLPs then migrate to the thymus and eventually commit to specific T-cell lineages such as αβ and γδ T cells. Based on the coexpression of CD4 and CD8, three major stages of thymic αβ T-cell development have been defined: the DN stage ($CD4^{-/low}CD8^{-}$); the DP stage ($CD4^{+}CD8^{+}$); and the SP stage ($CD4^{+}CD8^{-}$ or $CD4^{-}CD8^{+}$). The thymocytes that are destined to become γδ T lineage cells also go through the DN stage, but the following stages of their CD4 and CD8 expression are less well understood.

Based on the intrathymic reconstitution potential of the cells, cell cycle status, and TCR gene configuration, the DN stage is further divided in the following sequence: DN1 → DN2 → DN3 → DN4. The coexpressed levels of CD25 (IL-2 receptor alpha subunit) and the adhesion molecule CD44 are commonly used surface markers to identify the four substages. Some key events that determine the fate of the thymocytes during DN stages include TCR β chain rearrangement, pre-TCR expression, and γδ or αβ T-cell commitment. The surface expression of TCR β chains serves as one of the hallmarks for αβ T-cell lineage commitment.

After a successful combination of a TCRα chain and a TCR β chain, the αβ TCR is tested for its ability to recognize a ligand, ie, MHC-peptide complex. Thus, the DP thymocytes are required to bind the ligands for their survival and maturation (Positive Selection). Meanwhile, the DP thymocytes might undergo apoptosis if the ligands they bind activate naïve T cells that are bearing the same receptors (Negative Selection). Changes in the surface expression of CD5 and CD69 are commonly used as indicators of this process. CD5 has been reported as a negative regulator of thymocytes and indicates the intensity of interactions between TCRs and self MHC-peptides.[4] In addition, transient expression of CD69 indicates thymocytes that are undergoing or have just finished the process of TCR-mediated positive selection.[5]

During the process of positive and negative selection, αβ thymocytes also commit to either the $CD4^{+}$ (normally MHC class II restricted) or $CD8^{+}$ (normally MHC class I restricted) T cells.

acquisition. When used with the Worklist in BD FACSuite software, the Loader automates the acquisition of multiple samples.

The BD FACSVerse system also provides optional filters for various fluorochromes (eg, GFP, YFP, PE-Texas Red®, and Alexa Fluor® 700) to improve signal resolution and allow separation of emission signals that might overlap in the standard optical configuration. This option provides an additional level of flexibility for design of multiparametric panels. For example, with the optional 613/18 bandpass filter, the new fluorochrome conjugate BD Horizon PE-CF594 can be used. This fluorochrome can be excited by a 488-nm laser and emits at similar wavelengths to PE-Texas Red® with maximum emission at 612 nm. It is a very bright dye and has significantly less spillover into PerCP and PE-Cy™7 detectors. It also exhibits consistent spillover values and eliminates the need for lot specific compensation.

Objective

The objective of this application note is to demonstrate how to optimize and improve an existing multicolor fluorescent antibody panel for studying mouse thymic T-cell development using the following tools from BD:

- The BD FACSVerse system with the Loader option, using the Worklist function of BD FACSuite™ software to acquire and analyze multiple samples quickly and efficiently
- Optimization of the Loader mixing settings and titration of the reagents in 96-well plates
- Use of the new dye, BD Horizon PE-CF594, in conjunction with the optional 613/18 bandpass filter of the BD FACSVerse flow cytometer

Methods

Antibodies

Antibody Specificity	Clone	Antibody Stock Conc. (mg/mL)	Fluorochrome	Vendor	Product Number
Rat anti-Mouse CD16/CD32 (Mouse BD Fc Block™)	2.4G2	0.5	Purified	BD Biosciences	553142
Rat anti-Mouse CD4	RM4-5	0.2	PE-CF594	BD Biosciences	562314
Rat anti-Mouse CD8a	53-6.7	0.2	APC-H7	BD Biosciences	560182
Rat anti-Mouse CD45R/B220	RA3-6B2	0.2	BD Horizon™ V500	BD Biosciences	561226
Rat anti-Mouse CD25	7D4	0.5	FITC	BD Biosciences	553072
Rat anti-Mouse CD44	IM7	0.2	PE-Cy™7	BD Biosciences	560569
Hamster anti-Mouse TCR β	H57-597	0.2	APC	BD Biosciences	553174
Rat anti-Mouse CD5	53-7.3	0.2	BD Horizon™ V450	BD Biosciences	561244
Hamster anti-Mouse CD69	H1.2F3	0.2	PE	BD Biosciences	553237

Reagents and materials

Product Description	Vendor	Product Number
Mirror Filter ASSY PE-Texas Red®	BD Biosciences	653447
Stain Buffer (BSA)	BD Biosciences	554657
BD FACSuite™ CS&T research beads	BD Biosciences	650621
BD Falcon™ 96-well library storage plate, round-bottom, 340 µL	BD Biosciences	351190
BD Falcon™ 15-mL conical tubes	BD Biosciences	352097
Hanks' Balanced Salt Solution (HBSS) 1X	Life Technologies	14025-092
Trypan Blue 0.5% Solution	VWR®	95037-580
Fetal Bovine Serum (FBS)	ATCC®	30-2020

BD FACSVerse Instrument Configuration

Wavelength (nm)	Detector	Dichroic Mirror (nm)	Bandpass Filter (nm)	Fluorochrome
488	A	752LP	783/56	PE-Cy7
	B	605LP	613/18*	PE-CF594
	D	560LP	586/42	PE
	E	507LP	527/32	FITC
640	A	752LP	783/56	APC-H7
	B	660/10	660/10	APC
405	A	500LP	528/45	BD Horizon V500
	B	448/45	448/45	BD Horizon V450

*PE-Texas Red® optional filter.

Specimens

Sample Type	Source Species	Gender	Age	Vendor
Cryopreserved Thymocytes	BALB/c Mice	Female	3.5 weeks	Antibody Solutions

Thymocyte Preparation and Staining in a 96-well Plate

1. The cryopreserved thymocytes were thawed briefly at 37°C and diluted 1:1 with 1X HBSS in a 15-mL conical tube. Two milliliters of FBS was then laid under the diluted cell suspension.
2. Cells were pelleted by centrifugation (300*g*, 4°C, 7 min) and the supernatant was removed.
3. The cell pellet was washed once by resuspending in 2 mL of ice cold Stain Buffer followed by centrifugation (300*g*, 4°C, for 7 min). The supernatant was removed and cells were resuspended in ice cold Stain Buffer. The concentration of viable cells was then determined using the Trypan Blue exclusion method.
4. Cells were transferred into wells of a 96-well plate (5 x 10^5 viable cells in 200 µL of Stain Buffer per well).
5. Before staining with antibodies, the cells in the plate were pelleted by centrifugation (930*g*, 4°C, 2 min). The supernatant was carefully discarded and the pellets were loosened by vortexing briefly.
6. The cells in each well were incubated on ice for 30 minutes with 0.5 µg of Mouse BD FC Block™, in a total volume of 80 µL of Stain Buffer containing single or multiple antibodies, depending on the experiment layout (Table 3).
7. After incubation, cells were again centrifuged (930*g*, 4°C, 2 min) and washed once with 200 µL of Stain Buffer.
8. The cells were finally resuspended in 200 µL of Stain Buffer, and the plate was protected from light and kept on ice until the samples were acquired.

Study Overview

The overall study was accomplished in three consecutive steps. The mixing conditions were first optimized using unstained thymocytes. Using the optimized mixing conditions for the Loader, the working concentrations of each individual anti-mouse antibody in the panel were then determined by titration. Finally, the thymocytes were phenotyped by multiparametric flow cytometry using the panel.

Instrument Setup

The workflow for BD FACSVerse instrument setup is shown in Figure 2. The default configuration of the BD FACSVerse cytometer was modified by replacing the 700/54 filter with the optional 613/18 filter. BD FACSuite software

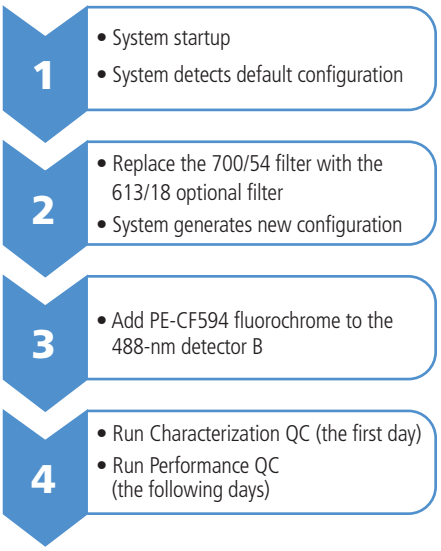


Figure 2. Overview of the instrument setup process.

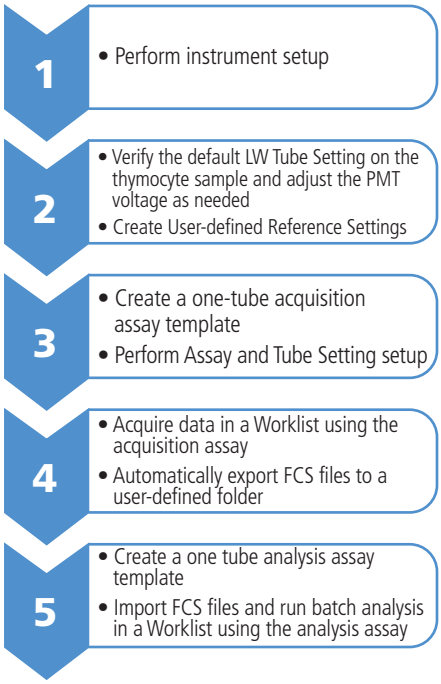
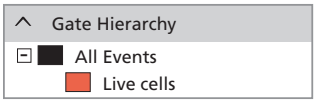


Figure 3. Overview of the data acquisition and analysis process.

A



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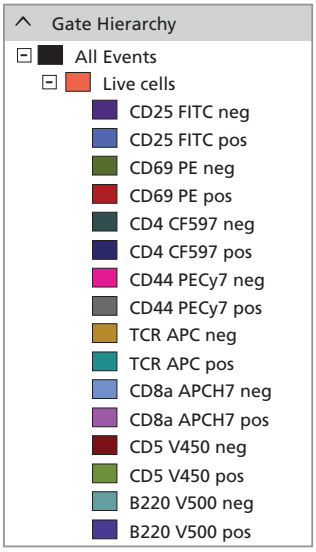


Figure 4. Gating hierarchies.
A. For data acquisition.
B. For data analysis.

automatically recognized the new optical filter and generated an optical configuration based on the new combination of mirrors and filters. The PE-CF594 fluorochrome was then added to the Blue B detector channel, and assigned to the new 613/18 filter in BD FACSuite software as outlined in the *BD FACSVerse™ System User's Guide*.[9] Characterization Quality Control (CQC) was initially performed to generate a baseline performance for the new configuration. Through the course of this study, Performance Quality Control (PQC) was performed on a daily basis to track the instrument performance. Each automated quality control procedure was performed with BD FACSuite CS&T research beads as outlined in the *BD FACSVerse™ System User's Guide*.[9]

Data Acquisition and Analysis

The workflow for data acquisition and analysis using the BD FACSVerse system is shown in Figure 3. The data was acquired and analyzed in a Worklist, using user-defined (UD) assays in BD FACSuite software.

After the instrument was set up, the default lyse/wash (LW) Tube Settings were first verified using the single-stained thymocytes. The PMT voltages were adjusted to place the population of interest on scale as needed. Further, UD Reference Settings for compensation were created following the instructions from the *BD FACSVerse™ System User's Guide*.[9] Next, a UD assay was created for a single tube using the UD Reference Settings, with a gating hierarchy shown in Figure 4, panel A. The UD assay for acquisition was set up initially by performing Assay and Tube Setting setup using CS&T research beads, and then was updated as necessary. The UD assay was used to acquire multiple samples in a Worklist. The FCS files were automatically exported to a user-defined folder.

After acquisition, another single tube UD assay for analyzing data was created with the same UD Reference Settings used for acquisition. The assay included a gating hierarchy (Figure 4, Panel B) and a report containing analysis plots. The FCS files generated during acquisition were imported into the new Worklist, and batch analysis was performed using the newly created UD assay.

Optimization of Loader Mixing Conditions

Unstained thymocytes were prepared as described and dispensed into 96-well plates at a concentration of 5 x 10^5 cells per well in a total volume of 200 µL. Multiple plates were prepared and were kept on ice for at least 30 minutes to ensure that the cells settled down to the bottom of each well. The plates were then run on the Loader under different mixing conditions as shown in Table 1.

Table 1. Loader mixing conditions tested in the study.

Mixing conditions	1	2	3	4	5	6 (default)
Initial Mixing Duration (Seconds)	30	30	30	20	20	10
Interim Mixing Duration* (Seconds)	30	30	30	20	20	5
Mixing Intensity (rpm)	700	1,000	1,400	1,000	1,400	1,400

*Interim mixing set to a 5-minute interval.

During acquisition, dead cells and debris were excluded by scatter gating and live cells were gated using gating strategy shown in Figure 4, panel A. See the **Tips and Tricks** section for details on gating live cells during acquisition. The stopping criterion for the acquisition was set at 6 minutes or 100,000 events in the "Live cells" gate, whichever was met first during the acquisition.

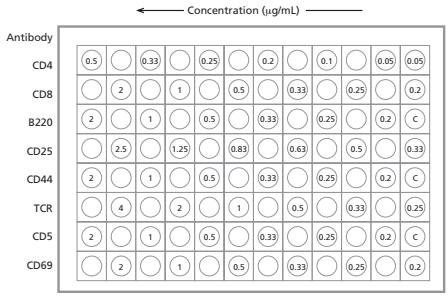


Figure 5. Custom layout for titration of the antibodies in a 96-well plate.

Each row contains a series of dilutions of an individual antibody. Unstained thymocytes were used as controls (C).

Titration of Antibodies

The thymocytes seeded in the 96-well plate were stained with a series of dilutions of individual antibodies as described in Table 2. The samples were acquired on a BD FACSVerse system, in a Worklist using the Loader and optimized mixing conditions. Unstained thymocytes were used as negative controls. A custom 96-well plate layout was used to acquire samples as shown in Figure 5.

Two types of gating hierarchies were used for data acquisition and analysis, respectively (Figure 4). The stopping criterion for acquisition was set at 6 minutes or 40,000 events in the "Live cells" gate, whichever was met first during the acquisition.

Table 2. Titration of the antibodies.

Antibody specificity	Concentration (µg/mL)					
CD4 PE-CF594	0.5	0.33	0.25	0.2	0.1	0.05
CD8a APC-H7	2	1	0.5	0.33	0.25	0.2
CD45R/B220 BD Horizon V500	2	1	0.5	0.33	0.25	0.2
CD25 FITC	2.5	1.25	0.83	0.63	0.5	0.33
CD44 PE-Cy7	2	1	0.5	0.33	0.25	0.2
TCR β APC	4	2	1	0.5	0.33	0.25
CD5 BD Horizon V450	2	1	0.5	0.33	0.25	0.2
CD69 PE	2	1	0.5	0.33	0.25	0.2

Immunophenotyping Mouse Thymocytes using 8-Color Multiparametric Flow Cytometry

Thymocytes seeded in 96-well plate were stained with the 8-color panel along with FMO controls as shown in Table 3.

Table 3. Antibody combinations of the 8-color panel and FMO controls.

Sample Type		CD25 FITC	CD69 PE	CD4 PE-CF594	CD44 PE-Cy7	TCR Cβ APC	CD8 APC-H7	CD5 BD Horizon V450	CD45R/B220 BD Horizon V500
8-color Panel		+	+	+	+	+	+	+	+
FMO Controls	CD25 FITC		+	+	+	+	+	+	+
	CD69 PE	+		+	+	+	+	+	+
	CD4 PE-CF594	+	+		+	+	+	+	+
	CD44 PE-Cy7	+	+	+		+	+	+	+
	TCR Cβ APC	+	+	+	+		+	+	+
	CD8 APC-H7	+	+	+	+	+		+	+
	CD5 BD Horizon V450	+	+	+	+	+	+		+
	CD45R/B220 BD Horizon V500	+	+	+	+	+	+	+	

The gating hierarchy for data acquisition is shown in Figure 6, panel D. The stopping criterion for data acquisition was set at 6 minutes or 100,000 events in the "Live cells" gate for the 8-color panel and the FMO controls.

The gating hierarchy for initial data analysis is shown in Figure 6, panel E. To exclude the aggregates, two sequential gates of scatter width (W) vs height (H) signals were applied as shown in Figure 6, panels A and B. The singlet population was then gated by forward scatter (FSC-A) vs side scatter (SSC-A) to exclude the dead cells and debris, as shown in Figure 6, panel C.

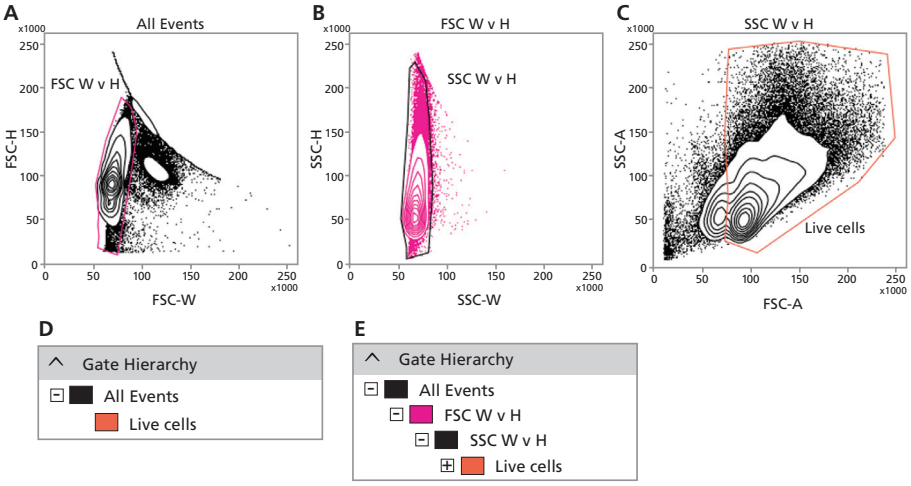


Figure 6. Gating strategy for live cell events for immunophenotyping.

A. Contour plot for FSC-W vs FSC-H; B. Contour plot for SSC-W vs SSC-H; C. Contour plot for FSC-A vs SSC-A; D. Gate hierarchy for data acquisition; E. Gate hierarchy for data analysis.

Data Analysis

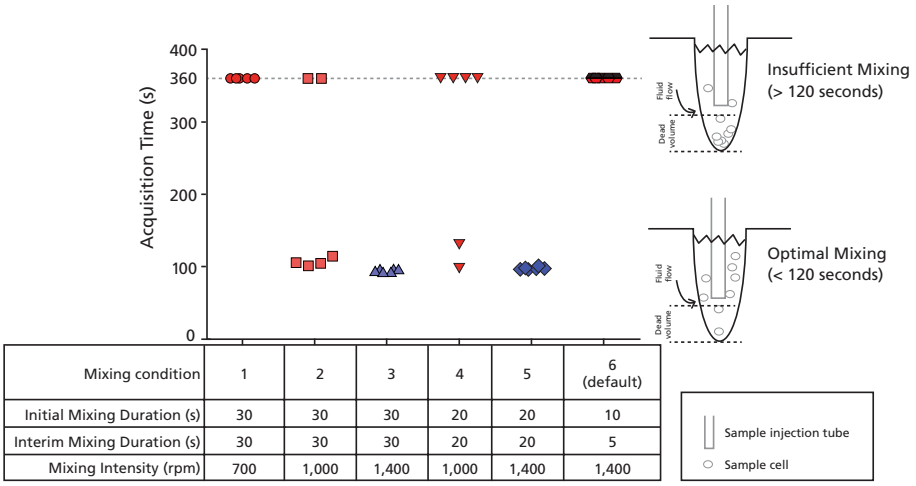
The data was acquired, analyzed, and presented using BD FACSuite software version 1.0.2. The graphs for antibody titration were plotted using Graphpad Prism® v5.04 (Graphpad Software, Inc).

Results

Optimization of Loader Mixing Conditions for the Thymocytes

Optimization of Loader mixing conditions during acquisition was the first step in this study. The duration and intensity of initial and interim mixes were investigated and six conditions were tested as described in Table 1. A sample concentration was predetermined to ensure that 100,000 events from the "Live cells" gate would be acquired within 120 seconds from the 200-µL sample volume when fully resuspended in Stain Buffer. The hypothesis was that if the mixing conditions were optimal, the samples in each well of the plate would be acquired in less than 120 seconds. Insufficient mixing would result in the cells not being fully suspended in the solution, and the acquisition would take longer. To prevent acquisition of insufficiently suspended sample over a long period of time and to avoid introducing air into the system, a stopping criterion of 6 minutes was also applied to each acquisition.

Six samples placed at the different locations in a 96-well plate were acquired after the cells were allowed to settle in the plate for at least 30 minutes. As shown in Figure 7, in mixing conditions 3 and 5, the desired numbers of events were achieved for all 6 samples within the expected 120-second limit. When mixing conditions 1, 2, 4, and 6 were used, it took more than 120 seconds to acquire 100,000 live cell events, with some variability among the 6 samples under each condition. The data suggests that the default mixing setting (condition 6) was not optimal for mouse thymocytes, and a prolonged duration time coupled with the strongest mixing intensity gave optimal results. Based upon these results, we decided to use mixing condition 5, which yielded the best combination of time use and sample suspension, in the remainder of the study.



Mixing condition	1	2	3	4	5	6 (default)
Initial Mixing Duration (s)	30	30	30	20	20	10
Interim Mixing Duration (s)	30	30	30	20	20	5
Mixing Intensity (rpm)	700	1,000	1,400	1,000	1,400	1,400

Figure 7. Optimizing mixing conditions of the Loader for studying mouse thymocytes.

Determination of Antibody Titers

Although most of BD anti-human antibodies are ready to use, we recommend titration of the BD anti-mouse antibodies based on a given application to obtain optimal results. Thus, optimizing the concentration of each antibody used in the panel was the second step in this study.

A dilution series of each antibody was used for staining thymocytes (Table 1), and the data was analyzed for median fluorescence intensity (MFI) and stain index, defined as D/W where D is the difference between positive and negative populations and W is equal to 2 standard deviations (SD) of the negative population. Figure 8 shows the data from a representative sample of CD4 PE-CF594 antibody (stock concentration at 0.2 mg/mL) titration along with unstained control. Panel A shows the separation of negative and positive populations as overlaid histograms when the antibody concentration was optimal. The statistics calculated from the interval gates of histograms were used to calculate the MFI and stain index, and are plotted against antibody concentration in panel B. From the titration curve, the optimal concentration of the antibody was chosen at the highest dilution where the stain index and MFI started to plateau. For the CD4 PE-CF594 antibody, a concentration of 0.2 µg/mL was chosen for the panel. Similarly, the concentrations of other antibodies in the panel were titrated, and the optimal concentrations are listed in Table 4.

Table 4. Optimized concentrations of antibodies in the panel.

Antibody	Optimized Conc. (µg/mL)
CD4 PE-CF594	0.2
CD8a APC-H7	1.3
CD45R/B220 BD Horizon V500	1.3
CD25 FITC	2.5
CD44 PE-Cy7	0.5
TCR β APC	1.0
CD5 BD Horizon V450	1.3
CD69 PE	1.0

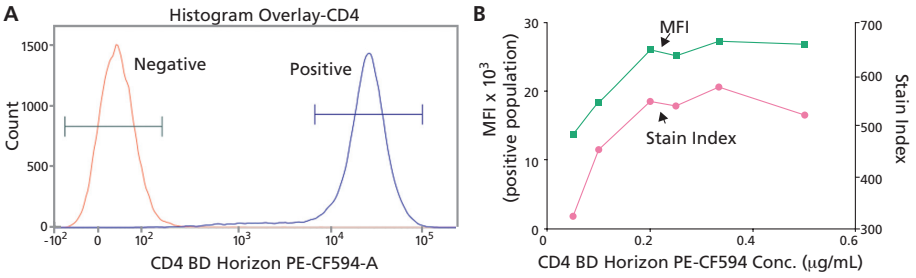


Figure 8. Titration of CD4-PE-CF594.

A. Histogram overlays for unstained and stained populations. B. Titration curve based on the MFI and stain index.

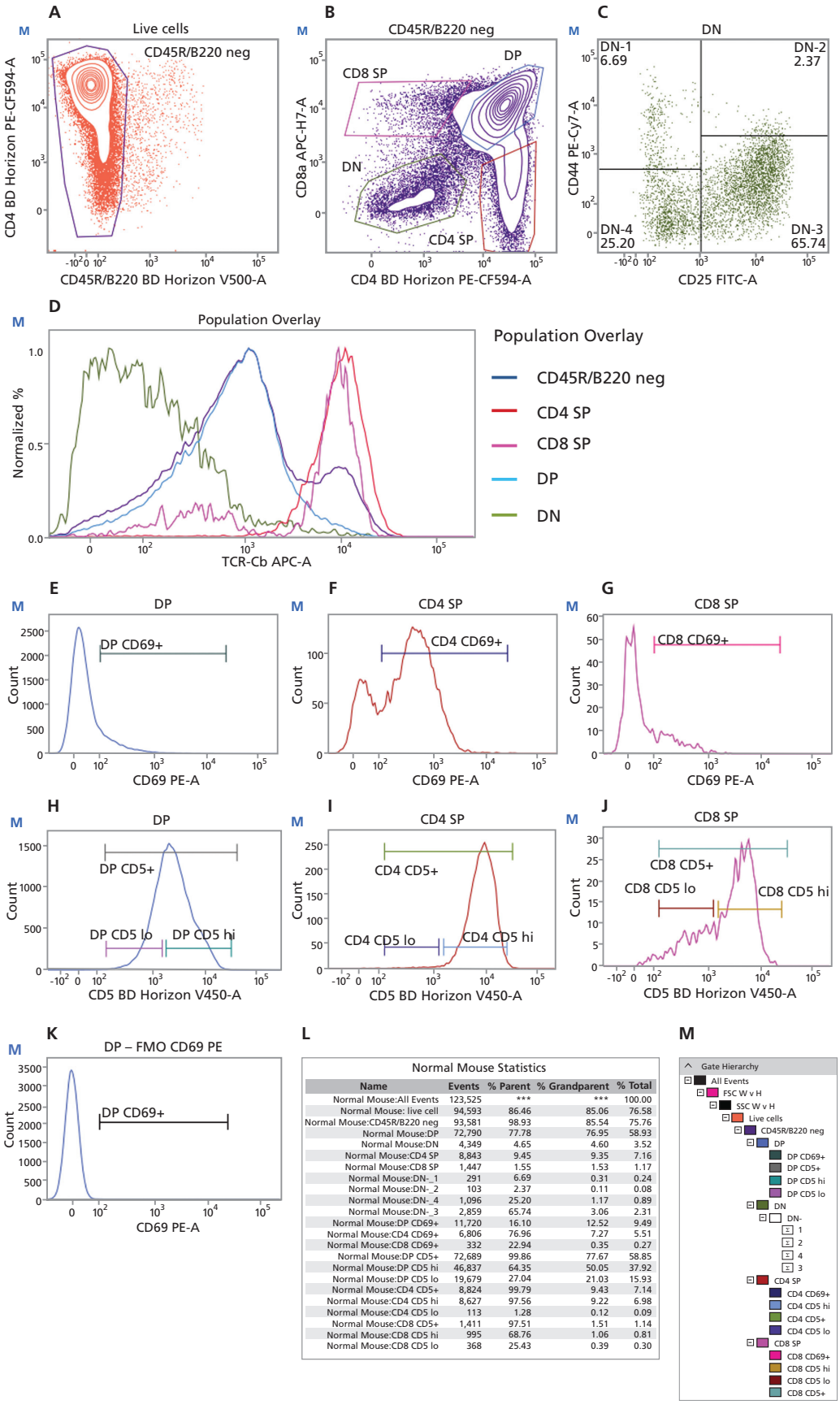
Immunophenotyping Mouse Thymocytes by 8-Color Multiparametric Flow Cytometry

An 8-color antibody panel against the surface markers listed in Table 4 was optimized to phenotype the thymocytes from normal 3.5-week-old female BALB/c mice. The results from the panel have been organized in two sets of plots. The first set of plots shows the population distribution in the major stages as defined by CD4, CD8, CD44, and CD25 expression (Figure 9, panels A to C). As shown in panel A, CD45R/B220 negative events were gated as a first step, since this was the cell population of interest in this study. These events were then used in a CD4 vs CD8 contour plot to identify the DN, DP, and SP cell populations (Figure 9, panel B). Data for the DN cell population was further analyzed using a CD44 vs CD25 plot to identify the four substages (DN1 to DN 4) as shown in panel C.

As shown in the statistics table (panel L), the thymi from the BALB/c mice used in this study had close to 5%, 78%, 9%, and 2% of CD45R/B220$^-$ thymocytes that were DN, DP, CD4 SP, and CD8 SP, respectively. The percentages of the DN cells in the DN1, DN2, DN3, and DN4 stages were 7%, 2%, 66%, and 25%, respectively. These results are within the normal range for a normal mouse.

After the first set of plots identified the major stages in αβ T-cell lineage development, the second set of plots was organized to show the expression of the markers related to the signaling process in those stages (panels D to J). Since the maturation of thymocytes is heavily dependent on receptor signaling, including pre-TCR and TCR complexes, the first plot is an overlay of the histograms for the surface level of the TCR β chain among the DN, DP, and SP populations (panel D). The pattern of the surface TCR β expression on the whole thymocyte population was a three-peak distribution, which was a combination of a low level of surface pre-TCR on the DN cells, an intermediate level of surface TCR complexes on the DP cells, and a high level of surface TCR complexes on the single-positive cells. This was further illustrated by the overlays on the same histogram from the individual populations.

Panels E to J show the levels of the CD69 or CD5 expression on the cell surfaces of DP and SP cells. CD69 and CD5 were included in the panel because they are indicators for the signaling process in the various stages.[10,11] After thymocytes enter the DP stage during development, the cells undergo positive and negative selection processes to refine the T-cell repertoire. The successfully selected cells that migrate into the peripheral circulation are MHC restricted and self-tolerant. This process incorporates a series of TCR-dependent signaling activities. Among them, upregulation of two surface markers, CD69 and CD5, has been widely used as an indicator to study the process. Transiently expressed surface CD69 indicates that the thymocytes that are undergoing or have just finished the process of TCR-mediated positive selection. In addition, the level of CD5 expressed on thymocytes at the DP stage is thought to indicate the intensity of interactions between TCRs and self MHC-peptides. Therefore, in this study, both CD69 and CD5 were evaluated on the DP and SP events. For CD69 expression, the positive marker was set based on a comparison with the FMO control (panel K). For CD5 expression, in addition to the positive marker, which was set based on the FMO control, dim (CD5^{lo}) and bright (CD5^{hi}) populations were marked based on the heterogeneous staining pattern of CD5. The heterogeneous expression of CD5 has also been reported by others,[10] and any difference in the CD5-marked population can be used to study developmental mutants.



Normal Mouse Statistics

Name	Events	% Parent	% Grandparent	% Total
Normal Mouse:All Events	123,525	***	***	100.00
Normal Mouse: live cell	94,593	86.46	85.06	76.58
Normal Mouse:CD45R/B220 neg	93,581	98.93	85.54	75.76
Normal Mouse:DP	72,790	77.78	76.95	58.93
Normal Mouse:DN	4,349	4.65	4.60	3.52
Normal Mouse:CD4 SP	8,843	9.45	9.35	7.16
Normal Mouse:CD8 SP	1,447	1.55	1.53	1.17
Normal Mouse:DN-_1	291	6.69	0.31	0.24
Normal Mouse:DN-_2	103	2.37	0.11	0.08
Normal Mouse:DN-_4	1,096	25.20	1.17	0.89
Normal Mouse:DN-_3	2,859	65.74	3.06	2.31
Normal Mouse:DP CD69+	11,720	16.10	12.52	9.49
Normal Mouse:CD4 CD69+	6,806	76.96	7.27	5.51
Normal Mouse:CD8 CD69+	332	22.94	0.35	0.27
Normal Mouse:DP CD5+	72,689	99.86	77.67	58.85
Normal Mouse:DP CD5 hi	46,837	64.35	50.05	37.92
Normal Mouse:DP CD5 lo	19,679	27.04	21.03	15.93
Normal Mouse:CD4 CD5+	8,824	99.79	9.43	7.14
Normal Mouse:CD4 CD5 hi	8,627	97.56	9.22	6.98
Normal Mouse:CD4 CD5 lo	113	1.28	0.12	0.09
Normal Mouse:CD8 CD5+	1,411	97.51	1.51	1.14
Normal Mouse:CD8 CD5 hi	995	68.76	1.06	0.81
Normal Mouse:CD8 CD5 lo	368	25.43	0.39	0.30

Figure 9. Immunophenotyping panel for studying T-cell development in mouse thymocytes.

Representative data from pooled thymocytes of three normal female BALB/c mice.

A. Contour plot of CD45R/B220 vs CD4 to gate on thymocytes;

B. Contour plot of CD4 vs CD8 to identify DP, DN, and SP events;

C. Dot plot of CD25 vs CD44 with quad gate to identify events in the substages of the DN population;

D. Histogram of TCRβ chain staining with overlays of CD45R/B220 neg, DP, DN, CD4 SP, and CD8 SP populations;

E–G. Histograms of CD69 staining of the DP, CD4 SP, and CD8 SP populations, respectively;

H–J. Histograms of CD5 staining of the DP, CD4 SP, and CD8 SP populations, respectively;

K. Histogram of negative CD69 signals of DP events from the FMO CD69 control for setting the gate position of CD69⁺;

L. Statistics table for the gated populations;

M. Gating hierarchy for data analysis.

Discussion

T cells play an important role in humoral and cell-mediated immune responses. The normal development and maturation of T cells in the thymus are critical, and abnormalities during this process can lead to immunodeficiency, autoimmunity, or cancer. Understanding T-cell development in the thymus using mouse-model systems can help in understanding abnormalities in the developmental process that might be responsible for immune dysfunction. This proof of principle study provides a multiparametric flow cytometry panel to monitor the major developmental stages of thymocytes into naive T cells. The application note highlights the improved 8-color fluorescent antibody staining panel for the cell surface molecules and a workflow to efficiently use the BD FACSVerse system to optimize the panel.

During optimization of the flow cytometry panels, especially for titration of individual antibodies, multiple tubes need to be acquired and analyzed. The Worklist function in BD FACSuite software, when combined with the Loader option, enables running of multiple samples in tubes or multiwell plates in a high-throughput manner. When using a Loader to acquire the data in 96-well plates, sample sedimentation needs to be considered during acquisition. Because of "dead volume" (ie, the volume of the solution that remains in the carrier that the instrument cannot acquire), faster acquisition of events is preferred before the dead volume is reached, to avoid air bubbles in the system. To accomplish this, the concentration of the sample as well as the mixing conditions should be optimized to ensure the optimal number of events and the homogenous distribution of the cells in the solution during acquisition. For mouse thymocytes, we found that the default setting was not optimal, and longer durations of the initial and interim mixing were needed.

Previous publications from BD have discussed the challenges involved in panel design and highlighted the importance of selecting reagents for multiparametric flow cytometry.[11-13] When choosing fluorochromes for the antibodies, it is good practice to assign as many bright fluorochromes as possible with minimal spectral overlap. For example, PE-CF594 was assigned to CD4 in this panel instead of APC-Cy7, which was used in the previous study,[7] because of the brightness and consistent spillover of PE-CF594.

The stain index is a useful metric to normalize signals over background and to measure reagent brightness. It measures the effective brightness of a reagent on a given instrument based on the difference between the positive and the negative population and the spread of the negative population. In this study we used this metric to optimize the concentration of each antibody. We found that six or seven titers starting with 2–4 µg/mL of antibodies, with a dilution factor of 2 to 10, provided enough dynamic range for selection of optimal concentration. For some very bright fluorochromes such as CD4 PE-CF594, a second round of titration was needed to find the optimal concentration. The antibody titration experiment was efficiently performed in a 96-well plate, and the data was automatically acquired by the Loader into a Worklist. Similarly, using the Worklist, the data was also analyzed in BD FACSuite software to automatically calculate the MFI values and the stain index.

The selection of antibody specificities against the particular target in the panel (ie, the clone of the antibody) is also important based on the biology and experimental question being addressed.[14] For example, the anti-TCR β antibody used in the panel was from a clone, H57-597, that has been widely used to identify TCR β chain.[15] The advantage of using H57-597 over other anti-TCR antibody clones in this study was its stable interaction with the constant region of the TCR β chain, which reveals both pre-TCR and TCR complexes. Further,

the APC fluorochrome was assigned to the antibody, which was selected based on the density and distribution of the antigens (pre-TCR and TCR) on the surface of the thymocytes. It is always important to match the antigen with heterogeneous expression with one of the brightest fluorochromes available.

To have an objective gating strategy to identify the different populations, it is good practice to include appropriate controls such as FMO controls along with the panel. The gating strategy used in this study was based on the existing study[7] with slight modification. However, depending upon the data, other gating strategies can also be used. In addition, the gating strategy can be adjusted depending on whether the thymocytes have been frozen, to exclude aggregates of varying scatter height and width.

Overall, the panel provided in this study could help to quickly identify the potential abnormal stage(s) during mouse T-cell development in the thymus. For example, it can be used for screening thymocytes from genetically modified mice compared to normal mice. The panel can also provide valuable initial information about the abnormal stage during the development, which can be further investigated by techniques such as gene expression and/or a post-translational modification profile of the transcription factors, to discern the possible cause of the abnormality.

Conclusions

A multiparametric flow cytometry panel for studying development of mouse thymocytes has been described in this application note. Workflows for BD FACSVerse instrument setup, acquisition, and analysis to optimize the panel have been presented. The Loader option combined with the Worklist provides a powerful tool for handling multiple samples during titration of antibodies. For efficient acquisition of mouse thymocytes on the Loader, higher intensity and longer duration of mixing conditions were required. BD Horizon PE-CF594, a brighter alternative to PE-Texas Red®, can be used in the panel with the optional 613/18 bandpass filter on the BD FACSVerse flow cytometer. This 8-color panel can be used as an initial screening tool to identify abnormalities at various stages of mouse T-cell development in the thymus.

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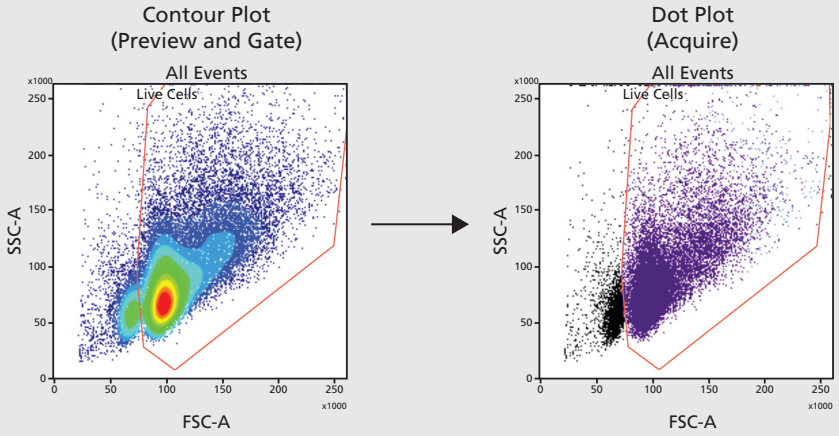
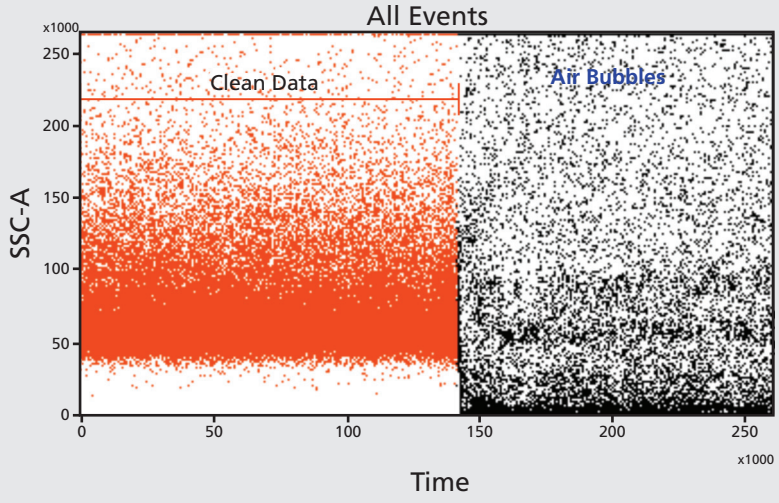
Tips and Tricks

- When running a multiwell plate on the Loader, the plate layout can be customized and multiple samples can be acquired as a group after being individually selected in BD FACSuite software. To prevent accidental misplacing of sample entries in the Worklist while using the Loader for a custom layout, the position of the wells can be locked using BD FACSuite software.
- To accurately define the "Live cell" gate during acquisition, cells can be previewed and gated using a contour plot. After defining the gate boundary, a dot plot can be used instead of the contour plot for easy monitoring of acquisition (see the following example).



- To avoid the influence of spillover value (SOV) on the position of the single-stained population, compensation should be turned off when antibody titration data is analyzed.
- A gate on time vs scatter parameters (shown below) can be used to exclude the data events for the samples that have air bubbles introduced during acquisition.



- To minimize the effect of fluorescence spillover on accurate identification of the different populations during multiparametric flow cytometry, the compensation matrix should be enabled. The BD FACSVerse system provides a default compensation matrix for most of the common fluorochromes and also provides the ability to generate a user defined compensation matrix (Reference Settings).
- During the process of creating a compensation matrix, the unstained cells can be mixed with the single-stained sample as an internal control to help identify the negative population needed for calculating compensation. This spiking technique is especially helpful for the antigens that are positive in the majority of the cell population.
- The selection of antibody specificities against the particular target in the panel (ie, the clone of the antibody) should be based on the biology and experimental question being addressed.
- To have an objective gating strategy to identify the different populations, it might be necessary to include single-stained as well as FMO controls.
- For a complete list of the relative stain indexes of fluorochromes offered by BD Biosciences, visit **bdbiosciences.com/documents/Multicolor_Fluorochrome_Guide.pdf**.
- Visit **bdbiosciences.com/colors** for more details about multicolor flow cytometry reagents.
- For details about BD Horizon™ PE-CF594 Reagents, visit **bdbiosciences.com/documents/BD_PE_CF594_Reagents_Datasheet.pdf**

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