Construction of multicolor antibody panels for the flow cytometric analysis of murine thymic stromal cells

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Application Note

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Abstract

Murine thymic stromal cells support the regulated development of thymocytes and are critical for their positive and negative selection. Thymic stromal cells are diverse and heterogeneous, and contain multiple hematopoietic and non-hematopoietic subsets. Consequently, multiple markers are needed to identify and characterize individual subsets.

This application note describes the process of how a customer built upon a basic three-color antibody staining panel to develop five, seven, nine, and eleven-color antibody staining panels, using an iterative process, for the analysis of thymic stromal cells. The development of these staining panels allows for detailed analyses of stroma from primary murine thymus preparations.

Introduction

Biological overview

The thymus is central to T cell development, and consists both of developing, immature thymocytes, and stromal cells. Stromal cells support thymocyte growth along a regulated series of steps, and shape the T cell repertoire, performing important functions such as positive and negative selection.[1,2] The thymus is involved in a variety of normal and pathological processes including age-related involution, radiation-induced T cell lymphopenia, and thymic graft-versus-host-disease (GVHD).[3,4] Many processes affect thymocytes, which are of hematopoietic origin. However, they can also affect the biology and composition of the hematopoietic and non-hematopoietic stromal cells, resulting in complex and indirect effects on thymocyte development and function.

Although thymocytes can be readily described with a basic two-color staining panel (CD4 and CD8), comprehensive characterization of the stromal cell components of the thymus is best achieved using multicolor flow cytometry. In this application note, we describe the construction of a panel that allows for the identification of thymic hematopoietic and non-hematopoietic stromal cell components as shown in Figure 1.

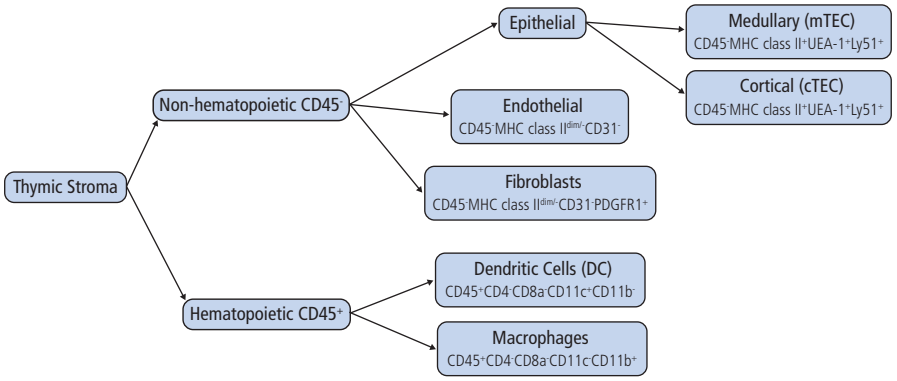

Figure 1: Different cell types found in the thymic stroma.

Abbreviations: MHC - major histocompatibility complex; UEA - *Ulex europaeus* agglutinin; PDGFR - Platelet-derived growth factor receptor: mTEC – medullary thymic epithelial cells; cTEC – cortical thymic epithelial cells.

The role of thymic stroma in disease

The study of different cell types in murine thymic stroma is an important tool in understanding the role of the thymus in disease. GVHD and prolonged immunodeficiency are common side effects of allogeneic bone marrow transplantation (allo-BMT). A typical transplant involves the transfer of donor bone marrow into a recipient who has undergone chemotherapy and/or radiation.

Donor alloreactive T cells present in the transplanted bone marrow mediate both GVHD, in which they attack host tissues, as well as desirable graft-versus-tumor (GVT) activity. Although GVHD is a systemic inflammatory process, it damages specific organs such as the intestines and thymus. The organ-specificity of GVHD has been a continued subject of study. However, differential use by donor alloreactive T cells of a variety of trafficking, co-stimulatory/co-inhibitory, and cytolytic molecules is believed to mediate organ-specific GVHD and GVT. Thymic GVHD may be important for delaying donor T cell reconstitution, but the molecular pathways by which alloreactive donor T cells damage the thymus are unclear. Furthermore, the target cell subsets in the thymus that are involved in thymic GVHD, and their interactions with alloreactive T cells, are not well defined.

Previous studies have suggested that donor alloreactive T cells do not directly damage donor bone marrow derived thymocytes but, instead, the thymic stromal cells that support their development. Thymic stromal cells contain significant non-hematopoietic components, and therefore remain of host origin even after allo-BMT. It is theorized that damaged thymic stromal cell components are then unable to support the prompt and correct development of thymocytes, leading to prolonged T cell immunodeficiency after allo-BMT.

Flow cytometry based research studies of the many cell types present in the thymus are one way in which the different cell subsets can be distinguished from each other. Information on the relative expression of surface and intracellular markers present in particular hematopoietic and non-hematopoietic subpopulations will contribute to the understanding of the role of the many cell types present in this complex organ, the mechanisms involved in thymic GVHD, and prolonged post allo-BMT immunodeficiency.

Objective

The objective of this study was to demonstrate how a customer constructed a flexible multicolor staining panel that can be used in flow cytometry based research studies to probe the biology and cell subsets of thymic stromal cells. This application note will outline how an 11-color panel was constructed to meet this objective.

Methods

Reagents and Materials

Product Number	Product Description	Vendor
550994	CD45 PerCP-Cy™5.5	BD Biosciences
551487	Streptavidin- PE-Texas Red® (SAv-PE-TR)	BD Biosciences
553036	CD8a PerCP	BD Biosciences
553049	CD4 PE	BD Biosciences
553051	CD4 APC	BD Biosciences
553141	CD16/CD32 (Unlabeled)	BD Biosciences
553373	CD31 PE	BD Biosciences
553735	Ly51 PE	BD Biosciences
553972	Hamster IgG κ PE	BD Biosciences
554258	CD95 (Fas/APO-1) PE	BD Biosciences
557657	CD11b APC-Cy™7	BD Biosciences
558107	CD4 Pacific Blue™	BD Biosciences
Custom Conjugate	MHC class II Alexa Fluor® 700	BD Biosciences
Custom Conjugate	CD11c BD Horizon™ V450	BD Biosciences
Custom Conjugate	PDGFR1 Biotin	BD Biosciences
Custom Conjugate	CD31 PE-Cy7	BD Biosciences
n/a	RPMI 1640 medium	Core facility preparation
12-5883-83	DR5 (CD262, Trail-R2) PE	eBioscience
17-5321	MHC class II APC	eBioscience
50-720-3279	DNAse I (Roche 10104159001)	Fisher Scientific
50-720-3639	Collagenase D (Roche 11088874103)	Fisher Scientific
D1306	DAPI	Invitrogen
URCP-50-2K	SPHERO™ Ultra Rainbow Calibration Particles (6 peaks), 5–5.9 µm	Spherotech
n/a	UEA-1 FITC	Vector Laboratories

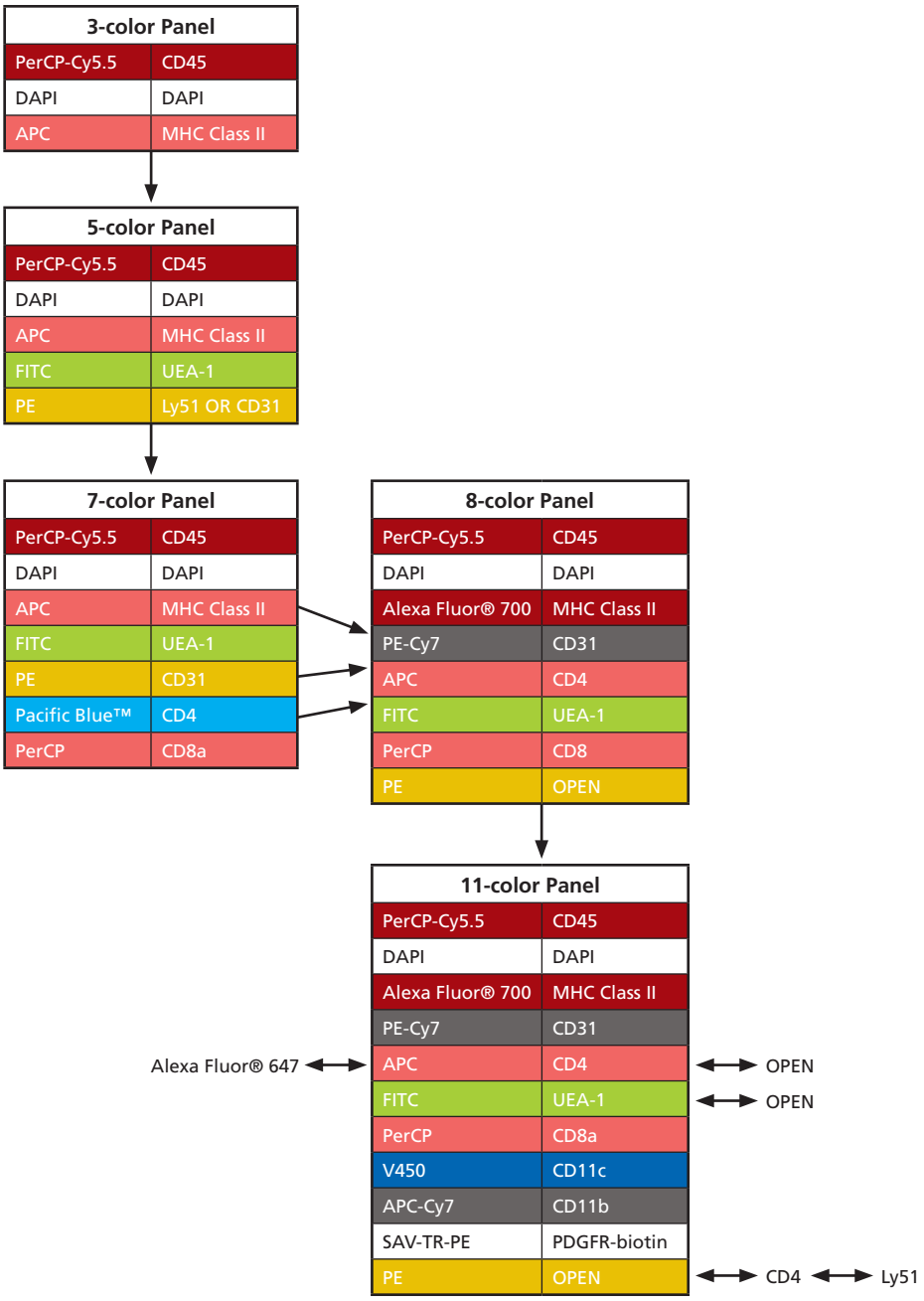

Figure 2: A flow chart summarizing the building of the 11-color panel.

Instrument

Flow cytometry was performed using a Special Order BD™ LSR II flow cytometer with the configuration shown in Table 1.

Table 1. Cytometer Configuration

Laser	Power (mW)	Emission Wavelength (nm)	Detector	PMT	Dichroic Mirror	Bandpass filter
Ultraviolet (UV)	20	355	Trigon – 2 Photomultiplier Tubes (PMTs)	A	530/30	450
				B	450/50	BLANK
Violet	25	405	Octagon – 7 PMTs	A	825/50	755
				B	705/70	690
				C	660/40	635
				D	605/40	600
				E	585/42	570
				F	560/40	505
				G	450/50	BLANK
Blue	20	488	Octagon – 7 PMTs	A	780/60	735
				B	695/40	675
				C	670/14	635
				D	610/20	600
				E	575/26	550
				F	530/30	505
				G	488/10	BLANK
HeNe (Red)	17	633	Trigon – 3 PMTs	A	780/60	755
				B	710/50	690
				C	660/20	–

Excellent instrument stability was noted with respect to voltages and PMT sensitivity. The Special Order BD LSR II cytometer performance was checked using SPHERO Rainbow Calibration Particles. Although BD Biosciences recommends daily QC, this Special Order BD LSR II cytometer was found to be stable and QC was performed less frequently. The instrument was checked with consistent sources of primary thymic stromal cells obtained from young (6–8 week old) female C57BL/6 (B6) and BALB/c mice, and revealed no significant "drift" in spillover percentages. No significant differences in biological stromal cell composition were noticed over a nine-month period.

Mice

Female B6 and BALB/c mice were obtained from the Jackson Laboratory (Bar Harbor, ME). All mouse experiments were approved by the Memorial Sloan-Kettering Cancer Center (MSKCC) Institutional Animal Care and Use Committee. Mice were housed in the MSKCC pathogen-free facility in sterilized micro-isolator cages and were given normal chow and autoclaved hyper-chlorinated drinking water (pH 3.0).

Tissue digestion

Thymic stromal cells are significantly larger in size than thymocytes, and not readily obtained in sufficient quantities via standard syringe mashing of a thymus through a 70-µm filter or via dissociation with frosted slides. We used a well described enzymatic digestion procedure to obtain thymic stromal cells.[5,6]

Briefly, the thymus was first removed from the thoracic cavity of a euthanized young (6–8 week old) female B6 or BALB/c mouse. Fatty and connective tissue were removed, and the thymus gently incised in both lobes with a scalpel or razor blade to release thymocytes. The incised thymus was then placed into digestion medium (5–7 mL per thymus), composed of 0.15% (w/v) collagenase D, and 0.01% (w/v) DNAse I in RPMI 1640 medium + 5% Fetal Calf Serum (FCS). The thymus was then digested at 37°C. Every 10–15 minutes, the digesting thymus was agitated up and down using a pipette to release additional thymocytes and stromal cells, and macroscopic fragments were allowed to settle under gravity. The cell-containing supernatant was removed and saved, and additional digestion medium added. The procedure was repeated until no macroscopic thymic fragments remained (typically three to four exchanges of digestion medium). All supernatant fractions were combined and centrifuged at 225*g* for 5 minutes at 4°C to obtain a pellet, which contained both thymocytes and stromal cells.

Cell staining

Fc receptors (CD16/CD32) are present on certain thymic stromal cell populations and were blocked to reduce nonspecific antibody binding. Isolated thymocytes and stromal cells were incubated in 20 µg/mL anti-CD16/CD32 antibody for 5 minutes at 4°C. Cells were then stained without washing, with fluorochrome conjugated antibodies using standard techniques (15 minutes, 4°C). Cells were centrifuged at 300*g* (5 minutes, 4°C) and resuspended in DAPI for flow cytometric analysis.

Data analysis

Data analysis was performed using BD FACSDiva™ v6.1 or FCS Express software. Isotype and/or fluorescence minus one (FMO) controls were used to facilitate gating as appropriate.

Panel building

3-color panel

Non-hematopoietic thymic stromal cells can be most simply defined as the $CD45^-$ cell fraction present in the digest supernatant. Hematopoietic populations ($CD45^+$) that are present consist of thymic dendritic cells and macrophages. The study was started by assessing the digestion protocol and confirming that the digest supernatant was composed of an acceptable cell yield and composition (especially of $CD45^-$ MHC class II^+ epithelial cells). Since the thymic stromal cell enzymatic digestion process can result in limited toxicity and cell death, DAPI was used to assess cell viability. The 3-color staining panel used for this initial experiment is shown in Table 2, and the flow cytometry data thus generated are shown in Figure 3.

Table 2. 3-color staining panel

Fluorochrome	Marker
PerCP-Cy5.5	CD45
DAPI	Viability
APC	MHC class II

$DAPI^{lo}$ events (viable cells) were displayed in a FSC vs. SSC plot and an atypical gate was used to exclude lymphocytes and enrich for "large" cells, including the stromal cells of interest. The CD45 status of these large cells was determined using a CD45 vs SSC plot and a $CD45^-$ gate. The final plot shows the MHC class II^+ (epithelial) status of these $DAPI^-CD45^-$ stromal cells.

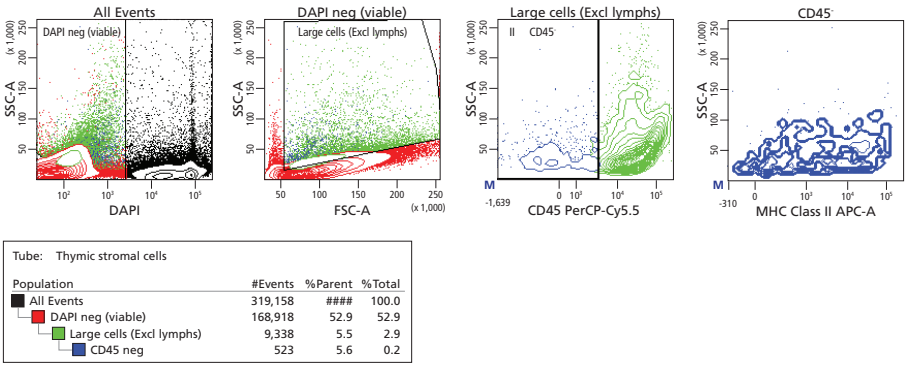

Figure 3: Analysis of thymic digest supernatant for the presence of CD45-MHC Class II$^+$ thymic stromal cells.

A standard digest of thymus from a young (6–8 week old) unmanipulated female B6 or BALB/c mouse typically results in >5% CD45$^-$ cells using this gating scheme.[6] This percentage can vary significantly. It depends upon the skill of the experimenter in performing the stromal cell digestion, animal age and sex, the cell populations included in the forward and side-scatter gate, and other experimental interventions and treatments such as total body irradiation, chemotherapy, or experimental GVHD.

Using these markers (CD45, MHC Class II, and DAPI), it was confirmed that approximately 5% of viable cells were CD45$^-$ cells and 15–30% of these cells were also of epithelial origin (MHC class II$^+$, Figure 3).

5-color panel

Building upon this basic 3-color panel, new antibodies were titrated and then evaluated for potential addition to the panel. The markers UEA-1 FITC, Ly51 PE, and CD31 PE can be used to further subset thymic epithelial cells (UEA-1 and Ly51), and to identify thymic endothelium (CD31). UEA-1 and Ly51 have opposite staining patterns, with UEA-1 staining the medullary thymic epithelium (mTEC) and Ly51 the cortical thymic epithelium (cTEC), but either marker is sufficient for a basic analysis of mTEC vs. cTEC.

Table 3. 5-color staining panel

Fluorochrome	Marker
FITC	UEA-1
PE	CD31
PerCP-Cy5.5	CD45
DAPI	Viability
APC	MHC class II

UEA-1 FITC, Ly51 PE, and CD31 PE were titrated over a dilution range from 1:50 to 1:6400. Optimal dilutions were determined visually as the first dilution at which populations were visible and not significantly clustered on the top axis. Data are presented in Figure 4 (red boxes). The addition of these markers allowed for the elucidation of endothelial cells (CD31$^+$) and subsetting of epithelial cells within the thymic stroma of medullary (UEA-1$^+$ and Ly51$^-$) and cortical (UEA-1$^-$ and Ly51$^+$) origin.

These data show that a 1:3200 (UEA-1), 1:800 (Ly51), and 1:400 (CD31) dilution of the antibody conjugates were optimal for use with thymic stromal cells in a 5-color panel.

Based upon these data, and the biological redundancy between UEA-1 FITC and Ly51 PE, each of which is sufficient by itself to distinguish between medullary and cortical epithelial cells, we elected to add UEA-1 FITC and CD31 PE to our staining panel as shown in Table 3, and temporarily set aside Ly51 PE.

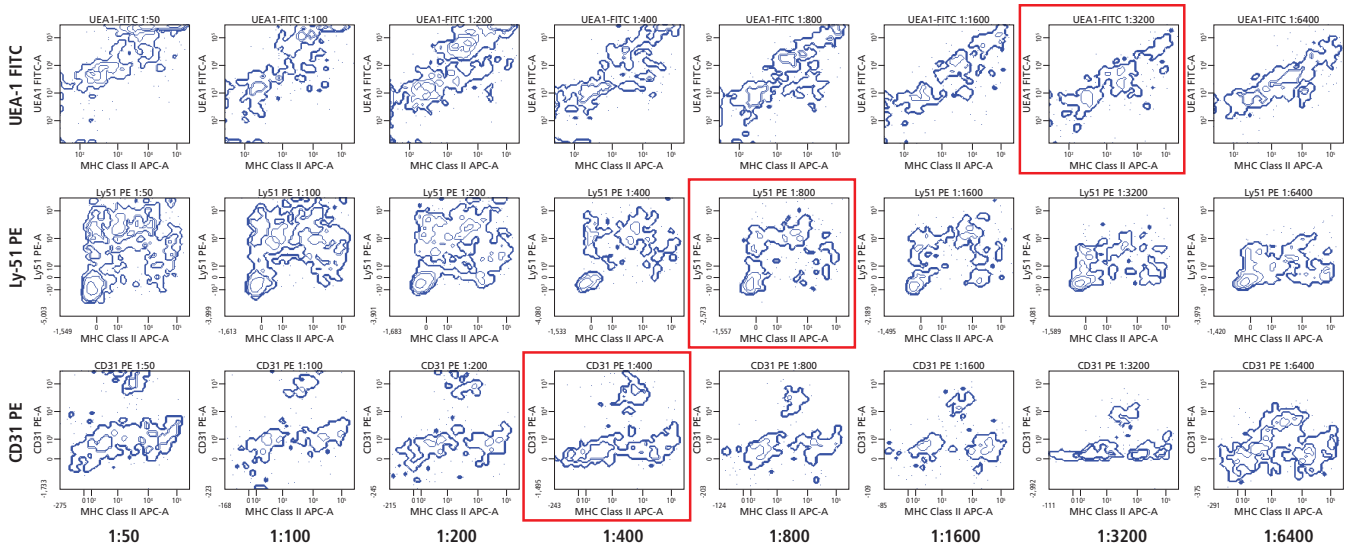

Figure 4: Titration of UEA-1 FITC, Ly51 PE, and CD31 PE in the context of the 3-color panel. Optimal concentrations are indicated by red boxes.

Table 4. 7-color panel

Fluorochrome	Marker
FITC	UEA-1
PE	CD31
PerCP	CD8a
PerCP-Cy5.5	CD45
DAPI	Viability
Pacific Blue™	CD4
APC	MHC class II

Table 5. 8-color panel

Fluorochrome	Marker
FITC	UEA-1
PE	*open*
PerCP	CD8a
PerCP-Cy5.5	CD45
PE-Cy7	CD31
DAPI	Viability
APC	CD4
Alexa Fluor® 700	MHC class II

7-color panel

The next cell subsets that were added to the panel were thymic dendritic cells ($CD45^+CD4^-CD8a^-CD11c^+$) and thymic macrophages ($CD45^+CD4^-CD8a^-CD11b^+$), which are hematopoietic ($CD45^+$) stromal cell components of the thymus. To detect and discriminate these cell types, CD4 and CD8a were added to the panel. These are classic thymocyte markers.

CD4 Pacific Blue™ and CD8a PerCP were selected and used at a 1:100 dilution, since they were routinely used at this concentration in the laboratory (data not shown). The new 7-color panel is shown in Table 4.

8-color panel

Since the goal was to obtain a panel that could also be used to stain and characterize thymic epithelium, endothelium, dendritic cells, macrophages, and fibroblasts, the commercial availability of fluorochrome conjugates for CD11b (macrophages) and CD11c (dendritic cells) was assessed. CD11c V450 and CD11b APC-Cy7 were chosen.

To facilitate these changes and to increase panel flexibility, the original 7-color panel shown in Table 4 required reconfiguration. MHC class II was moved from APC to Alexa Fluor® 700, and CD31 was moved from PE to PE-Cy7 thus opening up the PE channel for additional PE-conjugated antibodies. These two reagents were prepared by BD as custom conjugates. Finally, CD4 Pacific Blue™ was moved to APC.

Alexa Fluor® 700 has a lower stain index than APC and, based on previous experience and data previously obtained in the laboratory, it was decided that a dilution of 1:800 for MHC class II Alexa Fluor® 700 and CD4 APC and a 1:400 dilution of CD31 PE-Cy7 were appropriate (data not shown). The new 8-color panel is shown in Table 5, and an example plot showing the expression of CD31 (endothelium) vs. MHC class II (epithelium) gated on $DAPI^-CD45^-$ stromal cells is shown in Figure 5.

Cells were gated on $DAPI^-$ and $CD45^-$ events, as previously shown in Figure 3.

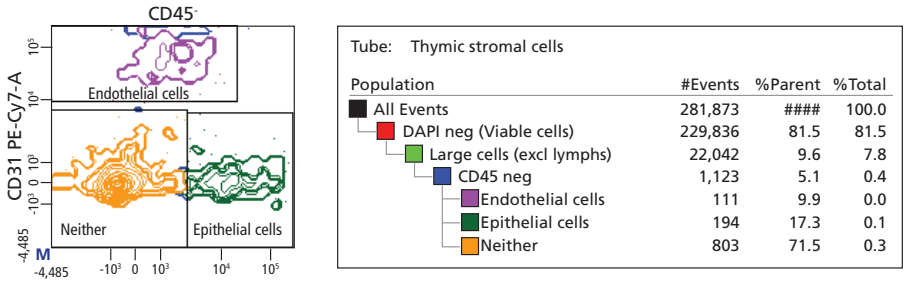

Figure 5. Discrimination of endothelial and epithelial thymic stromal cell subsets (CD45$^-$ DAPI$^-$) using MHC class II Alexa Fluor® 700 and CD31 PE-Cy7.

Table 6. 9-color panel

Fluorochrome	Marker
FITC	UEA-1
PE	*open*
PerCP	CD8a
PerCP-Cy5.5	CD45
PE-Cy7	CD31
DAPI	Viability
V450	CD11c
APC	CD4
Alexa Fluor® 700	MHC class II
APC-Cy7	CD11b

9-color panel

Working with the 8-color panel, CD11c V450 (1:100) and CD11b APC-Cy7 (1:400) were added to provide the ability to discriminate dendritic cells and macrophages, respectively, as shown in Table 6. Dilutions were based on previously obtained data (data not shown). Representative data using this panel to identify these cell types in the thymic digest are shown in Figure 6.

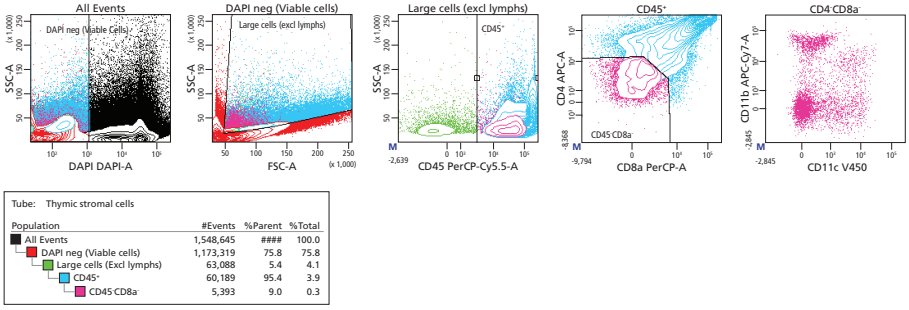

Figure 6. Identification of dendritic cells and macrophages in the hematopoietic fraction of the thymic digest. Cells were gated for DAPI$^-$CD45$^+$CD4$^-$CD8a$^-$ populations, and then a bivariate plot of CD11c (dendritic cells) vs. CD11b (macrophages) was displayed.

Table 7. 11-color staining panel

Fluorochrome	Marker
FITC	UEA-1
PE	*open*
PE-TR	PDGFR1 (biotin SAv)
PerCP	CD8a
PerCP-Cy5.5	CD45
PE-Cy7	CD31
V450	CD11c
DAPI	Viability
APC	CD4
Alexa Fluor® 700	MHC class II
APC-Cy7	CD11b

11-color panel

Finally, the 11-color panel was completed with the addition of PDGFR1-biotin (1:100) and SAv-PE-TR (1:800) for the identification of fibroblasts, as shown in Table 7. Both reagents were routinely used in the laboratory and titrated in the same way as the example shown in Figure 4. Representative data showing how this panel was used are shown in Figure 7.

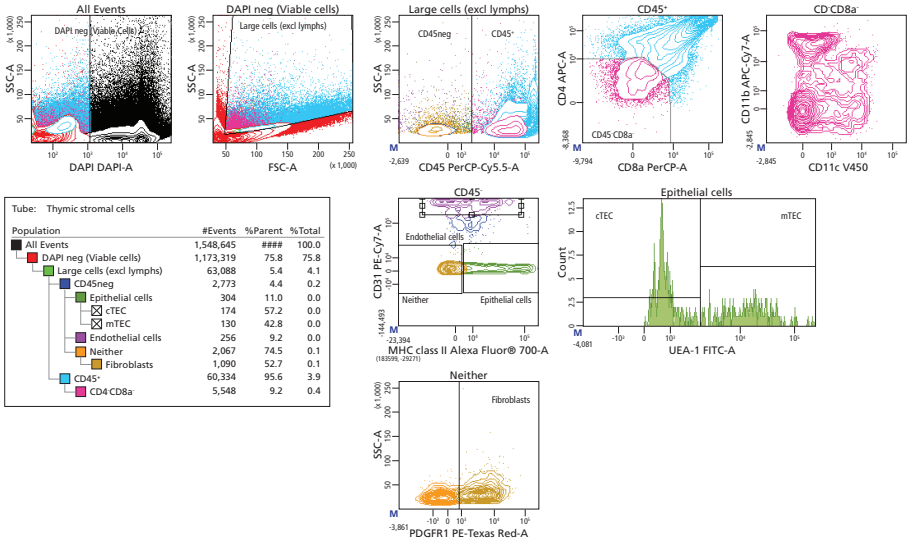

Figure 7. Subsetting of thymic stromal cells with the final 11-color staining panel showing all thymic stromal cell subset populations.

Panel flexibility

Because of instrument availability and capabilities, most vendors, including BD Biosciences, initially conjugate antibodies to FITC, PE, and APC, since these fluorochromes can be detected on the widest range of instruments. This 11- color panel was constructed as a "starting point," and has the advantage of defining complex cell subsets and evaluating their relative composition and percentages. It also has the flexibility of "slotting in" various other reagents to further characterize the biology of the cell subsets that the basic panel defines.

Of the three fluorochromes initially chosen, it was decided to leave the PE channel "open" because it has a higher stain index than FITC and has a somewhat better reagent availability than APC. However, the panel was designed with the possibility of also opening up the FITC and APC channels if required. CD4 APC can be readily switched to CD4 PE without any other adjustments. This allows the use of APC and Alexa Fluor® 647 conjugated antibodies, or secondary detection reagents that can be detected in the APC channel.

To open up the FITC channel, anti-UEA-1, which is a polyclonal rabbit conjugated antibody, was replaced with Ly51. Anti-UEA-1 is not available conjugated to PE or APC, compared to Ly51, which is available conjugated to PE. As previously stated, UEA-1 and Ly51 have opposite staining patterns, with UEA-1 staining the medullary thymic epithelium (mTEC) and Ly51 the cortical thymic epithelium (cTEC). Either marker is sufficient for a basic analysis of mTEC vs. cTEC.

Use of the 11-color panel

The 11-color staining panel was used to identify the death receptors Fas (receptor for Fas ligand) and DR5 (receptor for TRAIL) on thymic stromal cells. Fas and DR5 are both low-density markers, so Fas PE and DR5 PE were used because this fluorochrome has a high stain index. Representative data using the staining panel to stain for the death receptor DR5 are shown in Figure 8.

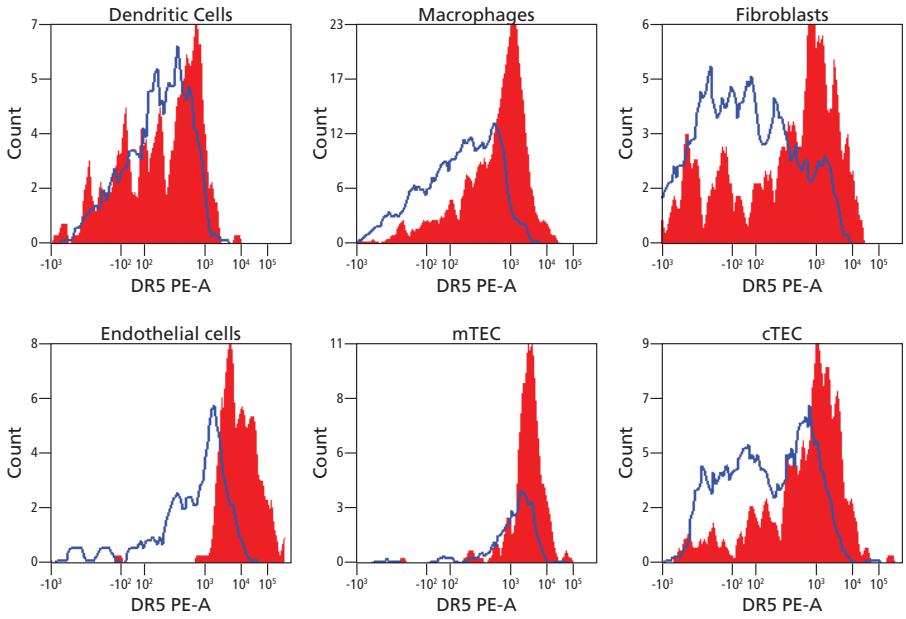

Figure 8. Expression of the death receptor DR5 by dendritic cells, macrophages, fibroblasts, endothelium, mTEC, and cTEC present in thymic stromal cells. Blue indicates hamster isotype control, and red indicates specific staining.

Thymic stromal cells were stained with the 11-color panel shown in Table 7. The PE channel was used for DR5 or an appropriately matched hamster isotype control. Dendritic cells, macrophages, fibroblasts, endothelium, mTEC, and cTEC were gated as described previously. The expression of the death receptor DR5 was then determined on these cell subsets.

Discussion

This application note demonstrates how a customer incrementally developed a complex staining panel by first building upon a small panel containing the most essential markers for a given objective, and then adding markers. Reagents labeled with common fluorochrome conjugates were used first and evaluated. It was then determined that markers labeled with these fluorochromes could then be moved to less common fluorochromes, when available. This process can be usefully iterated to arrive at flexible antibody staining panels for a wide variety of cell types.

Tips for developing a multicolor panel

- Successful development of multicolor flow panels can be time consuming and can require careful application of complex design principles. Allow sufficient time for testing and design rework.
- Information, with suggested guidelines on choosing reagents and reducing compensation problems, is available in *Selecting Reagents for Multicolor Flow Cytometry*.[7] As a general rule:
  - Use fluorochromes with a high stain index for low density markers and fluorochromes with a lower stain index for higher density markers.
  - Use stain indices to aid your decision in selecting appropriate antibody dilutions during titrations.
  - As new fluorochromes are added, check that the original markers continue to perform as expected and are not compromised by spectral overlap. Watch for the appearance of false positives.[8]
- Consider the use and objective of each marker when choosing fluorochromes. Markers used to gate or define high percentage populations might not need to be as stringent as those markers used to probe the biology of the cells or detect rare events.

  For example: In the final 11-color panel (Table 7) all the markers except for the open PE channel are used to delineate cell subsets. The bright (PE) channel was reserved to define the biology of individual cell subsets, such as the expression of the death receptors (Fas and DR5).
- Guidance on experimental design and appropriate controls is provided in *Flow cytometry controls, instrument setup, and determination of positivity*.[9]
- When deciding on an open channel, investigate which candidate markers will most likely be utilized in these channels and which fluorochrome conjugates are readily available. Consider custom conjugates or indirect staining.
- Modern digital instruments such as the Special Order BD LSR II flow cytometer have excellent sensitivity and can detect a wide variety of fluorochromes. This significantly enhances the flexibility of constructing staining panels.
- We have found anecdotally, that for biological markers of intermediate density, increasing the concentration (titration) of antibody staining may be sufficient to allow its detection on comparatively dimmer channels such as PerCP and Alexa Fluor® 700.
- Many BD antibodies are titrated on a cell type expressing a particular marker as part of our quality control process. Information is often provided in the technical data sheet on the recommended volume of antibody to stain 10^6 cells. Additional titration (optimization) might be required for detection of markers on different cell types.
- For consistent results, maintain your flow cytometer and perform QC on a regular basis. BD Cytometer Setup and Tracking Beads are designed to automate the performance tracking of your cytometer.

BD Horizon™ V450 reagents are engineered for use with BD FACS™ brand flow cytometers equipped with violet lasers. With a maximum absorption of 404 nm and an emission peak of 448 nm, these reagents have improved spectral characteristics compared to Pacific Blue™ reagents when used on these instruments. For additional information, visit **bdbiosciences.com/colors/horizonv450**.

BD Biosciences is constantly expanding its portfolio of conjugates for flow cytometry. If you cannot find a conjugate in our catalog, please try our Custom Conjugation Service, which offers more choices for multicolor experiment design. Contact your BD sales representative for more information about the Custom Conjugation Program.

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