APPLICATION NOTE

FLOW CYTOMETRY

CyAn™ ADP | A 4-Color Combination with Minimal Spectral Overlap for B Cell Subsetting

In today's busy laboratory the need to quickly process and extract as much accurate and important information as possible from a sample is becoming more and more important. Multicolor flow cytometry is an ideal approach to this problem. However, the more colors used, the greater the issues with spectral overlap between fluorochromes. Potential consequences include problems with compensation and increasing possibility of reporting erroneous results. Careful choice of fluorochromes can reduce the amount of compensation required to a minimal level.

Materials and Methods

In this example, we used a MultiMix™ reagent that consists of CD20 FITC, CD5 RPE and CD19 APC. This combination is used to identify the total T and B lymphocyte populations and the CD5+ sub set of B lymphocytes. Since the MultiMix reagents are pre-titrated, the user simply adds the required volume of the MultiMix reagent to an aliquot of patient's cells. The fluorochrome combinations, FITC, RPE and APC are chosen specifically to reduce problems with spectral overlap. By adding CD45 Pacific Blue as a drop-in reagent we can easily expand from a 3- to a 4-color system. The advantage of using CD45 Pacific Blue is that virtually no compensation is required between the Pacific Blue and the FITC, RPE or APC fluorochromes. Thus, for this four-color combination the only compensation required is between RPE and FITC and FITC and RPE. The FITC and RPE are excited by the blue (488nm) laser, the APC by the red (635nm) laser and the Pacific Blue by the violet (405nm) laser.

The data shown was collected using a CyAn ADP LX 9 Color flow cytometer equipped with the standard laser and filter sets. The red blood cells were lysed using UtiLyse™ lysing reagent.

Peripheral blood from a normal healthy adult donor was collected by venepuncture into a tube containing EDTA anticoagulant. Testing was performed within 24 hours.

Instrument Set Up

Prepare Tubes 1–4 as show in Table 1. Add 10 µL of single CD3 reagent to Tubes 2–3. To Tube 4 add 20 µL of the MultiMix reagent and 5 µL of CD45 Pacific Blue. To all tubes add 100 µL of well-mixed blood; mix and incubate at room temperature in the dark for 15 minutes. A lysing reagent is added to remove the red blood cells. A lysing reagent containing fixative UtiLyse was used in this application, following manufacturer's instructions. When lysing is complete, centrifuge at 300 x g for five minutes and aspirate the supernatent. Add 2 mL of PBS containing 2% BSA and centrifuge at 300 x g for five minutes and aspirate the supernatent, then resuspend the cells in approximately 300 µL of PBS.

Acquire Tube 1 (unstained cells). Data is acquired using a FSC trigger. Adjust the FSC and SSC gains and PMT to position the lymphocytes, monocytes and granulocytes so they can be distinguished clearly. Adjust the threshold setting to eliminate the majority of the debris, being careful not to clip the lymphocyte population (see Figure 1). Create a series of histograms for the FITC, RPE, Pacific Blue and APC parameters and apply

Table 1

Tube #	Name	FITC	RPE	Pacific Blue	APC
1	Unstained	–	–	–	–
2	CD3 FITC	CD3	–	–	–
3	CD3 RPE	–	CD3	–	–
4	TC 663	CD20	CD5	CD45	CD19

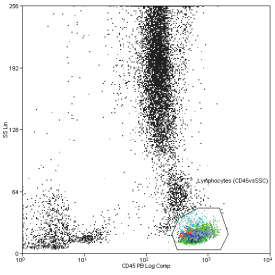
the lymphocyte gate to them. Adjust the PMT voltages so the negative population gives a median channel number of around 3.5. Then, without adjusting the PMT voltages acquire Tubes 2 and 3, and acquire 5,000 gated lymphocytes.

Set the compensation using the “Auto Compensation” tool in Summit software v4.3. Analyze Tubes 2 and 3 using the lymphocyte gate, following the on screen instructions (Figure 2). The compensation settings can be verified using the “VisiComp” function in Summit software v4.3.

Once PMT voltages and compensation has been set, the MultiMix TC663 + CD45 Pacific Blue tube can be acquired.

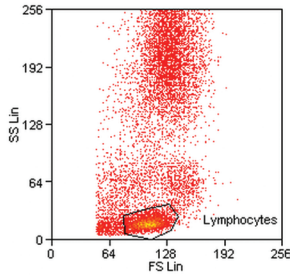
To aid in the visual identification of the various cell types, gates were created defining the population of interest and color gating was applied. Once the various plots have been created and positioned, and the PMT voltages and compensation settings derived, the protocol can be saved. When opened, the protocol will automatically recall these settings, so only minor changes may be required to optimize the settings. For optimal instrument performance, it is advisable to regularly clean the instrument and implement a daily quality control procedure to monitor the instrument performance that will alert the user to potential problems.

Figure 3



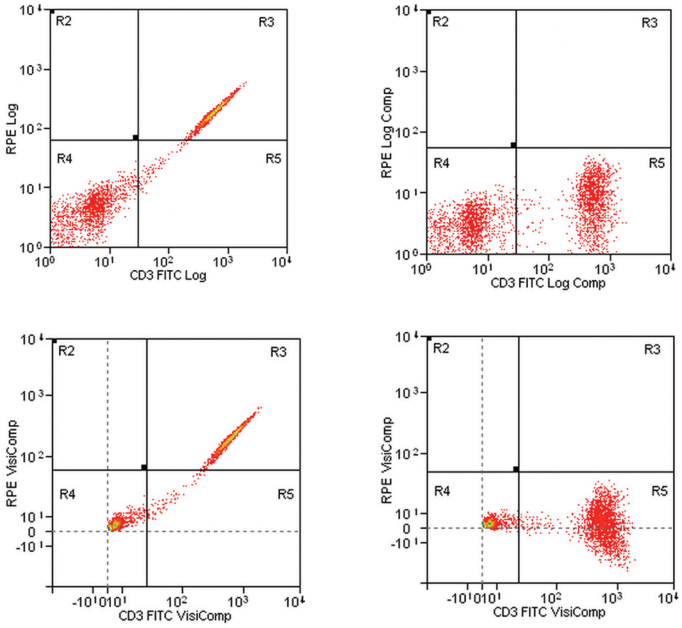
Setting of the CD45 Pacific Blue vs. Side Scatter lymphocyte gate region.

Figure 1



Forward Scatter (FSC) vs. Side Scatter (SSC) plot showing the positioning of the various white blood cell populations and the gate used to define the lymphocyte population.

Figure 2



Plots showing pre- and post-Auto Compensation. The upper plots show the normal Log mode and the lower show VisiComp mode.

TECHNICAL TIPS

> When using UtiLyse reagent A, do not exceed the 10-minute incubation time to prevent fixation of the red blood cells. Once lysing and washing is complete, the sample can be stored for 24 hours at 4 °C (39 °F) before scatter parameters begin to deteriorate.

> Before running samples, ensure the instrument has had the appropriate time to warm up.

Results

There was minimal compensation required between the four fluorochromes used in the experiment (see Table 2).

Figures 4, 5 and 6 show the CD20/CD5/CD19 staining patterns obtained from the CD 45 vs. SSC defined lymphocyte gate.

Table 2

Compensation settings derived from the Auto Compensation tool using the single stained CD3 tubes.

Fluorochrome	CD20 FITC	CD5 RPE	CD45 Pacific Blue	CD19 APC
CD20 FITC	100	0.6543	0	0
CD5 RPE	31.018	100	0	0
CD45 Pacific Blue	0	0	100	0
CD19 APC	0	0	0	100

Figure 4

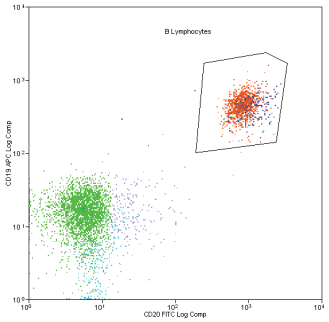


Figure 5

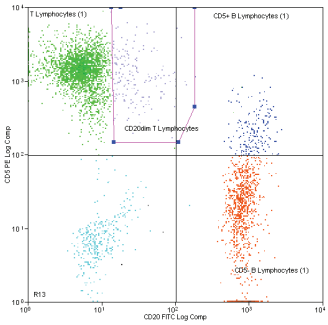
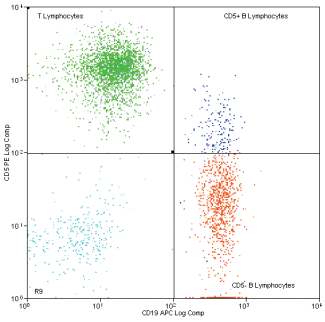


Figure 6



Discussion

Using the CyAn ADP LX 9-Color allows the use of a four-color mix of fluorochromes that have little spectral overlap, resulting in minimal compensation requirements.

This example shows how CD45 Pacific Blue can be used as a "drop in" to the MultiMix reagent to create a four-color mix in which the only compensation required is between RPE and FITC and RPE and FITC. Thus, a simple, easy-to-use combination is made to estimate T, B, CD5+ B-cell and CD20^{dim} T-cell sub sets.

References

1. Hultin L.E, Hauser M.A, Hultin P, Giorgi J.V. CD20 (pan B cell) antigen is expressed at a low level on a sub population of normal T Lymphocytes. *Cytometry 14, 196 – 204.*

PRODUCT	CODE
CyAn ADP LX 9 Color	CY201
MultiMix Triple Color CD20 FITC/CD5 RPE/CD19 APC	TC663
CD45 Pacific Blue	PB986
CD3 FITC	F0188
CD3 RPE	R0810
UtiLyse	S3350

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