APPLICATION NOTE

FLOW CYTOMETRY

CyAn™ ADP | 7-Color Immunophenotyping

In research laboratories, there is a constant need to gather more information from the limited sample available. With the CyAn ADP LX 9 Color, what once took many tubes is now accomplished with one tube. We demonstrated a multicolor immunophenotyping protocol which utilizes two scatter parameters as well as seven fluorescent parameters.

Materials and Methods

Blood was collected from a normal, healthy adult in an ACD tube and used within 24 hours. The antibodies shown were selected as a representative group because they are commonly used in a variety of panels. To each of nine tubes, 100 µl of whole blood was added along with the antibody as listed in Table 1.

After antibody addition, the tubes were gently vortexed and incubated for 20 minutes at room temperature in the dark. After incubation, 100 µl Uti-Lyse™ Reagent A was added to tubes 1-8. To tube 9, 200 µl was added. The tubes were gently vortexed and incubated for exactly 10 minutes at room temperature in the dark. After 10 minutes, 1 mL of Uti-Lyse Reagent B was added to tubes 1-8. To tube 9, 2 mL was added. The tubes were gently vortexed and incubated for 10 minutes at room temperature in the dark. After the tubes were centrifuged for 5 minutes at 300 x g, the supernatant was removed and the pellet resuspended in 1 mL HBSS (with 1% BSA, 0.1% NaN3, and 1 mM EDTA). The tubes were then vortexed and centrifuged for 5 minutes at 300 x g. After removing the supernatant, the pellets were resuspended in 500 µl HBSS (with 4% BSA, 0.1% NaN3, and 1 mM EDTA). The tubes were stored at 4 ˚C / 39.2 ˚F until use.

Results

Analysis was done on a CyAn ADP LX 9 Color flow cytometer with the standard three lasers and filter sets. Summit software was used to gate and compensate. Shown in Figure 1 is a representative scatter histogram. Using the FSC/SSC lymphocyte gate to properly set PMT voltage for all parameters, first run the unstained control and adjust PMT voltages as necessary to set the negative population (gated on lymphocytes) in the first log decade of each fluorescent parameter to be used. Once this step is complete, run each control to set the compensation. Place the proper parameter for the single control on the X-axis of the histogram. Rotate through every one of the other fluorescent parameters used on the Y-axis and set compensation if necessary. Note: Compensation will not have to be adjusted between every parameter. However, it is always wise to check just to be sure. This will also ensure that the staining is correct (i.e. if a tandem dye you are using has degraded and is more

Table 1

Tube #	CD45 FITC	CD56 RPE	CD3 RPE-TR	CD14 RPE-Cy5	CD4 RPE-Cy7	CD19 APC	CD8 APC-Cy7
1	—	—	—	—	—	—	—
2	5 µl (CD3 FITC)	—	—	—	—	—	—
3	—	5 µl	—	—	—	—	—
4	—	—	5 µl	—	—	—	—
5	—	—	—	5 µl (CD3 RPE-Cy5)		—	—
6	—	—	—	—	5 µl	—	—
7	—	—	—	—	—	5 µl	—
8	—	—	—	—	—	—	5 µl
9	5 µl	5 µl	5 µl	5 µl	5 µl	5 µl	5 µl

fluorescent in the wrong parameter, it can be found before the experimental tube is run). Do this process for each control tube. It is critical that PMT voltages are not changed once you begin adjusting compensation. If it becomes necessary, all the controls must be run again to ensure compensation is correct. In addition, further tubes are required to properly set the compensation for the CD45/FITC and CD14/RPE-Cy5 parameter. In this case, samples stained with CD3/FITC and CD3/RPE-Cy5 can be used as the compensation controls. Hint: Once you have set the compensation for all parameters, save the protocol you are using in Summit Software. This way, next time you repeat your experiment (using the same antibodies) the compensation will be just about perfect and may only need a little touch up. Once the compensation is complete you are ready to run the experimental sample which is shown in Figures 2-8. Populations were labeled using the annotation feature. Color gating was applied to highlight distinct populations and/or make the data more presentable. The workspace can be exported as graphics as a whole or individual histograms/plots and statistics to Microsoft® Excel.

Discussion

As shown, multicolor immunophenotyping is becoming much easier with the new instrumentation available. By using more markers in each panel, fewer tubes are required using fewer sample and less time. The CyAn ADP LX 9 Color offers an excellent platform for multicolor analysis with the small bench-top footprint, fixed alignment, spatially separated lasers and easily interchangeable filters. The software allows the user to save the PMT settings, compensation, and gating which makes repetition of an experiment easy. Compiling data into a report format is simple with cut and paste tools readily available.

TECHNICAL TIPS

- During cell preparation single controls are used in this protocol to demonstrate instrument set up for a given experiment. Although single controls are always a good idea to troubleshoot staining problems, once the experiment has been set up there is very little variation run to run. The blood used in the cell preparation should not be older than 24 hours. When using the Uti-Lyse, Reagent A is the fixative. Therefore, the 10 minutes incubation time for reagent A must be adhered to so as to avoid fixing red cells as well as the white cells. Reagent B is the lysing agent. As the wash steps occur after the 10 minutes lyse, very few red cells should be in the pellet after centrifugation. Cells can be stored at 4 ˚C / 39.2 ˚F for up to 24 hours before analysis. After 24 hours, the scatter properties will begin to deteriorate.
- Before running samples verify that the instrument has had the appropriate time to warm up and the alignment specifications have been met using a standard bead particle.
- During acquisition single controls should be run to properly set voltages and compensation. Once set, the protocol can be saved which will contain all the settings and can be reused for this same experiment in the future.
- During analysis color gating can be done in Summit Software to highlight populations and show marker overlap.

PRODUCT	CODE
CyAn ADP LX 9 Color	CY201
Monoclonal Mouse Anti-Human CD3/FITC	F0818
Monoclonal Mouse Anti-Human CD3/RPE-Cy5	C7067
Monoclonal Mouse Anti-Human CD45/FITC	F0861
Monoclonal Mouse Anti-Human CD56/RPE	R7251
Monoclonal Mouse Anti-Human CD19/APC	C7224
Uti-Lyse, Erythrocyte Lysing Reagent	S3350
Hank's Buffered Salt Solution	S1754

Graphs

Figures 1 to 8

Gating strategy:

Fig. 1: Create “Lymphocyte gate for compensation”.

Fig. 2: CD3/SSC to create “T Lymphocyte gate”

Fig. 3: CD4/CD8 plot using the combination of “Final Lymphocyte gate” and the “T Lymphocyte gate” to identify CD4+ and CD8+ T Lymphocytes.

Fig. 4: CD45/SSC plot showing initial lymphocytes gate (“CD45 vs. SSC Lymphocyte gate”).

Fig. 5: CD45/CD14 plot using CD45 vs. SSC Lymphocyte gate to create the “Final Lymphocyte gate”, which excludes monocyte contamination.

Fig. 6: CD56/CD8 plot using “Final Lymphocyte gate” to identify the presence of CD8+/CD56+ cells

Fig. 7: CD3/CD56 plot using “Final Lymphocyte gate” to identify NK Lymphocytes (CD56+/CD3-) and NK like Lymphocytes (CD3+/CD56+).

Fig. 8: CD3/CD19 plot using “Final Lymphocyte gate” to identify B lymphocytes.

Figure 1

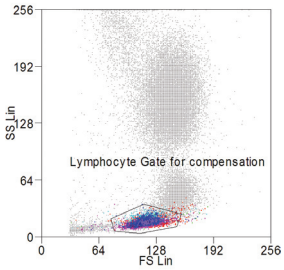


Figure 2

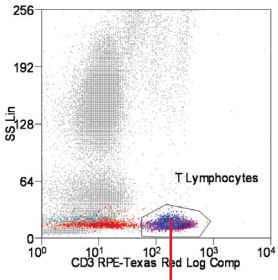


Figure 4

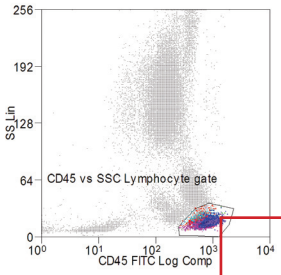


Figure 5

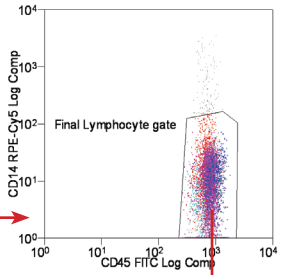


Figure 3

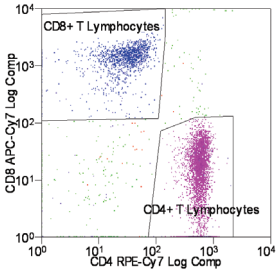


Figure 6

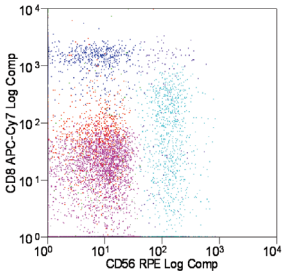


Figure 7

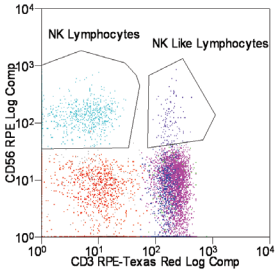
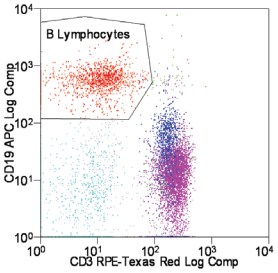


Figure 8



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