Detection and quantitation of eosinophils with other leukocyte subsets in human peripheral blood using the NovoCyte™ flow cytometer

During hematopoiesis, eosinophils differentiate from myeloid cells in the bone marrow prior to migration into the blood. They are responsible for combating parasitic infections and also play an essential role in inflammatory responses and injury occurring during an allergic response. The traditional method to identify and quantify eosinophils in inflammatory tissues and exudates relies on morphologic criteria and manual counting (i.e. prominent eosinophilic granules will appear as orange-red intracellular structures after H&E staining). However, limitations to this approach result from variability in sample preparation and subjective interpretation of inclusion criteria for inflammatory cells based on morphology alone. In this application note, Dr Paul Hutchinson and his team at National University of Singapore collaborated with ACEA Biosciences and utilized a flow cytometry-based assay to identify eosinophils in human blood. This method applies a more rigorous technique for detection and allows for the exact quantitation of the cell populations of interest.

Human whole blood (treated with EDTA anti-coagulant) was taken from a normal donor and stained with antibodies against CD4, CD8, and CCR3. Red blood cells were then lysed in BD FACSlyse solution and the sample was washed and fixed in 2% paraformaldehyde. Human peripheral blood leukocytes samples were separately acquired using the ACEA NovoCyte™ flow cytometer as well as a competitor’s instrument. Data analysis was performed with ACEA’s NovoExpress™ software. We found that the wide dynamic range of detection (7.2 logarithmic decades) provided by the NovoCyte™ flow cytometer made it easy to identify eosinophils and lymphocytes in the same plot (Fig 1A). In contrast, the limited dynamic range of the competitor’s instrument restricted our ability to identify these two cell types in the same plot. In fact, adjusting PMT voltages to accurately identify the lymphocyte population resulted in poor resolution of the eosinophils and vice versa. The NovoCyte™ flow cytometer also allowed us to clearly distinguish monocytes from granulocytes (Fig 1A&B). Absolute cell counts from each of these populations are easily obtained using the NovoExpress™ software coupled with the highly accurate syringe pump within the NovoCyte™ system.

We then wanted to further verify that we had accurately identified the eosinophils based on their FSC/SSC profile. Since eosinophils are intrinsically autofluorescent, we were able to gate on the autofluorescent cells in an unstained sample and backgate these to a FSC/SSC dot plot (Fig 2A&B). CCR3 is a chemokine receptor found to be primarily on eosinophils. When comparing autofluorescent cells to CD4 and CD8 lymphocytes, the autofluorescent cells had much higher levels of CCR3 than both the CD4 and CD8 positive populations (Fig 2C&D). Backgating these populations to a FSC/SSC plot localized the CD4 and CD8 positive cells to where lymphocytes normally reside and the autofluorescent/CCR3 positive cells to where we would expect the eosinophils to reside (Fig 2E).

Figure 1. Analysis of eosinophils in whole blood simultaneously with other leukocytes. Human peripheral blood leukocytes were used for acquisition on ACEA’s NovoCyte™ flow cytometer and a competitor’s flow cytometer. Data were analyzed using the NovoExpress™ software. The population highlighted in brown represents the eosinophils present in each sample (A&B). The full range scale was displayed for the data acquired by the competitor’s instrument (5-decade dynamic range). Two different leukocyte populations representing monocytes and granulocytes were also clearly distinguished for the NovoCyte™ data (A) but not the competitor’s flow cytometer (B). The direct absolute count, “Abs. Count”, column in the statistical table (C) indicates the No. of cells/µl in blood (total and sub-populations).
Table 1: This table summarizes the antibody combination used in the experiment excited by the 488nm and the 640nm lasers.

<table>
<thead>
<tr>
<th>Antibody Specificity</th>
<th>Fluorochrome</th>
<th>Laser (Excitation)</th>
<th>FL Channel on NovoCyte</th>
<th>Filter (Emission)</th>
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<tr>
<td>CD4</td>
<td>FITC</td>
<td>488nm</td>
<td>BL1</td>
<td>530/30</td>
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<tr>
<td>CD8</td>
<td>PE</td>
<td>488nm</td>
<td>BL2</td>
<td>585/40</td>
</tr>
<tr>
<td>CCR3</td>
<td>APC</td>
<td>640nm</td>
<td>RL1</td>
<td>675/30</td>
</tr>
</tbody>
</table>

Figure 2. Identification of eosinophils using autofluorescence and CCR3. Human blood was stained with CD4, CD8, and CCR3 antibodies and acquired using ACEA’s NovoCyte™ flow cytometer followed by analysis using NovoExpress™ software. Using the backgating technique, the autofluorescent cells detected using FITC/PE (A) backgate to the where eosinophils are expected in the FSC/SSC dot plot (B). Eosinophils are not only confirmed based on their strong intrinsic auto-fluorescence in the FITC and PE channels (C) but also through their CCR3 positive staining (D). The CD4+ and CD8+ lymphocytes are both negative for CCR3 and all cells are shown backgated to a FSC/SSC plot (E).

This flow cytometer-based assay offers rapid, accurate and unbiased identification and quantitation of eosinophils in human peripheral blood as compared to conventional methods of staining and manual counting. The identification of eosinophils is based on both its FSC/SSC profile and strong intrinsic auto-fluorescence in the FITC and PE channels. An additional eosinophil marker, CCR3, was used to further confirm the identity of the auto-fluorescent population. Since eosinophils are captured together with lymphocytes, monocytes, and granulocytes, the wide dynamic range offered by the NovoCyte™ flow cytometer greatly facilitates the resolution of all of these cell types in the same FSC/SSC plot. We were even able to obtain superb separation of the monocyte and granulocyte populations based solely on their scatter profiles. Highly accurate absolute cell counts for each of these cell types was also possible, enabling the generation of additional analytic data.

References