Elevating flow cytometric analysis: A comparative study of traditional and image-enhanced methods using the Invitrogen™ Attune™ CytPix™ flow cytometer

Samantha Ellis, Thermo Fisher Scientific, Carlsbad, California

Abstract

There are demonstrative advantages of image-enhanced flow cytometry. These advantages are further strengthened when utilizing machine learning to automate image analysis and evaluate morphological parameters along with antibody based immunophenotypic panels. A series of experiments were conducted using the Invitrogen™ Attune™ CytPix™ flow cytometer to compare traditional flow cytometry with image-enhanced flow cytometry, focusing on aspects such as viability staining, aggregate exclusion, no-wash no-lyse protocols, and cell stimulations. The integration of the imaging capabilities within the Attune CytPix flow cytometer allows both marker expression and cellular morphology to be used to facilitate even higher dimensional flow cytometric analysis. The resulting flow cytometry data identified distinct cell populations within biological samples, which were more precisely characterized using imaging parameters, thereby improving the accuracy of phenotypic analysis. These findings underscore the effectiveness of the Attune CytPix flow cytometer for image-enhanced flow cytometry in multiparametric immune cell phenotyping, providing valuable insights for immunological research.

Introduction

The Attune CytPix flow cytometer possesses advaned features that enable high-throughput workflows, resulting in sensitive and reliable data collection from a diverse range of samples, including human peripheral blood mononuclear cells, mouse splenocytes, bacteria, yeast, and more. With its acoustic focusing technology and built-in high-speed brightfield camera, the Attune CytPix flow cytometer allows for reproducible, real-time photographic capture of flow cytometry samples. These advanced tools offer benefits for various applications. Here, we highlight how image-enhanced flow cytometry can improve the reliability and accuracy of data collection in various experimental scenarios, such as viability staining, aggregation exclusion, erythrocyte differentiation, and morphological changes observed in cell stimulations.

Materials and methods

Sample Preparation and test methods

All samples originated from human blood donated by presumed healthy individuals. Whole blood was used directly for no-wash, no-lyse experiment research. For other research experiments, peripheral blood mononuclear cells (PBMC) were isolated from the blood. The optimal titer of each antibody was aliquoted into flow tubes containing eBioscience™ Flow Cytometry Staining Buffer (00-4222-26, Thermo Fisher Scientific).

Data Analysis

All data was acquired on an Attune CytPix flow cytometer and compensated using the Attune NxT software. All images were captured on the Attune CytPix flow cytometer and processed using the Attune Cytometric software. After collection, FCS data was analyzed using FlowJo™ (BD Biosciences). All data was gated using Forward Scatter Area (FSC-A) vs. Side Scatter Area (SSC-A) parameters and then gated to exclude doublets and non-viable cells or debris, unless analyzed by imaging parameters.

Results

Enhancing viability assessment through image-enhanced flow cytometry

It is common to rely on viability staining alone to differentiate live populations of interest from dead cells. While effective, this method can leave room for doubt in population analysis. Image-enhanced flow cytometry addresses this limitation by providing visual confirmation through images correlated with the viability staining histogram. This dual approach not only enhances the accuracy of distinguishing live versus dead cells but also gives greater confidence in analysis.

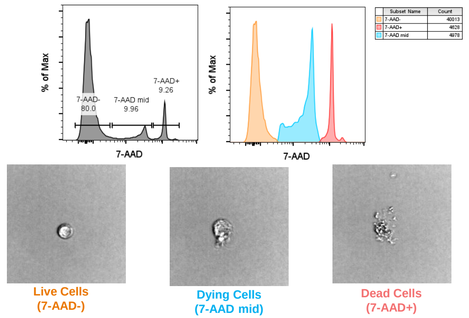

Figure 1. Three distinct populations in heat-killed human PBMC stained with viability dye. Heat-killed human PBMCs stained with 7-AAD exhibited three distinct peaks, an occurrence also observed with other viability dyes. To further differentiate between the 7-AAD-mid and 7-AAD-high populations, imaged cells were backgated to the corresponding peaks. The results indicated that 7-AAD-mid cells may be in the process of dying, while 7-AAD-high or positive cells were extremely dead.

Combining image and size parameters to identify genuine singlets

To exclude aggregated cells and doublets, a combination of size-based parameters, such as Forward Scatter (FSC) and Side Scatter (SSC), is typically used. By incorporating the processed image parameter of Particle Count, identifying true singlets and distinguishing them from doublets, aggregates, and debris becomes easier.

Figure 2A

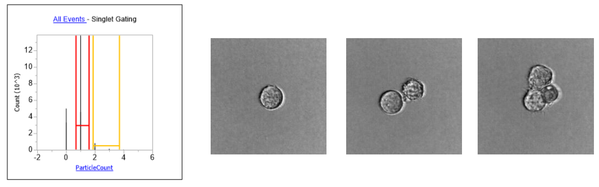

Figure 2. Traditional dot plots with image parameters can produce cleaner data.
2A. A Particle Count value of 1 (red gate) indicates an imaged single cell, while a value of 2 or higher indicates doublets or aggregated cell populations (yellow gate). The singlet image was derived from the red gate, while the doublet and aggregate was derived from the yellow.

Figure 2B

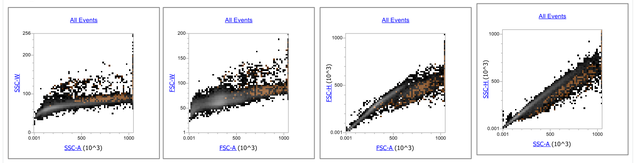

Figure 2. Traditional dot plots with image parameters can produce cleaner data (continued)
2B. Back-gating aggregated image events reveals that the cleanest dot plot for isolating singlets is SSC-A vs. SSC-H.

Efficient Separation of Red Blood Cells from Target Cells in No-Wash, No-Lyse Protocols

No-wash, no-lyse protocols streamline sample preparation, which can minimize cell loss and preserve rare populations within human whole blood. Combined with acoustic focusing technology, the advanced imaging features of the Attune CytPix flow cytometer enable easy separation of erythrocytes from target cells, even at higher flow rates. This combination allows for precise identification and separation of populations within heterogeneous samples.

Figure 3A

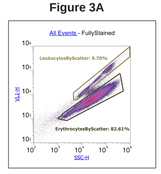

Figure 3B

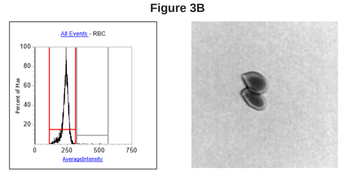

Figure 3C

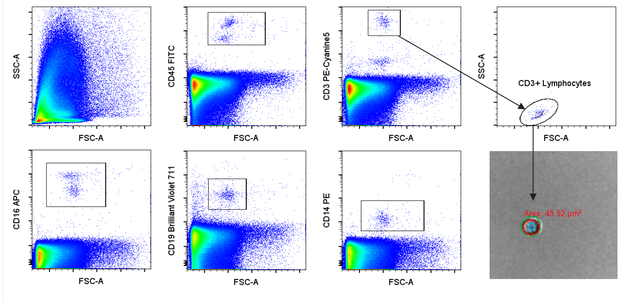

Figure 3. No-wash, no-lyse samples analyzed on the Attune CytPix flow cytometer. 3A. Leukocytes and erythrocytes can be clearly separated and identified by size using the Invitrogen™ Attune™ NxT No-Wash No-Lyse Filter Kit (100022776, Thermo Fisher Scientific). **3B.** Red blood cells (right) can be identified using the imaging parameter of Average Intensity (left), with a value of ~250, while normal cells have a value of ~500. **3C**. A 5-color panel was run on the Attune CytPix flow cytometer. All antibodies were stained within a single sample of 100 uL whole human blood, which was then diluted and run at a high flow rate. Populations of interest, like CD3+ lymphocytes, can be separated from RBCs and confirmed by FSC-A vs. SSC-A, or imaging.

Morphological differences between stimulated and unstimulated cells

Human PBMCs were isolated from whole blood and stimulated with eBioscience™ CD3 Monoclonal Antibody (UCHT1), Functional Grade (16-0038-81, Thermo Fisher Scientific) and eBioscience™ CD28 Monoclonal Antibody (CD28.2), Functional Grade (16-0289-81, Thermo Fisher Scientific) or left unstimulated in culture for three days. Cells were then harvested and stained with eBioscience™ CD25 Monoclonal Antibody (BC96) PE (12-0259-80, Thermo Fisher Scientific). eBioscience™ CD69 Monoclonal Antibody (FN50) APC (17-0699-42, Thermo Fisher Scientific), and eBioscience™ 7-AAD Viability Staining Solution (00-6993-50, Thermo Fisher Scientific).

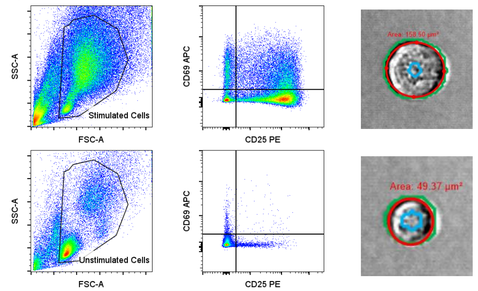

Figure 4. Comparative morphology of stimulated and unstimulated cells. Data was gated using FSC-A vs. SSC-A based on size, SSC-A vs. SSC-H for singlets, FSC-A vs. 7-AAD for live cells, and then CD25 PE vs. CD69 APC to observe effects of stimulations on cell shape. Captured images confirm that stimulated cells can be quite larger (158.50 microns-squared) than unstimulated cells (49.37 microns-squared), as demonstrated in the FSC-A vs. SSC-A dot plots.

Discussion

In several different flow cytometry workflow scenarios, the data collected and analyzed traditionally via dot plots and histograms can be enhanced and improved upon by accompanying image collection and processing. The integration of imaging capabilities allows for more precise identification and characterization of cell populations, providing additional context and verification for flow cytometric data. This approach not only enables distinguishing cell subsets with similar scatter profiles but also helps in identifying morphological changes, verifying cell viability, and excluding aggregates. By combining traditional flow cytometry with real-time imaging, researchers can achieve a higher level of accuracy and reliability in their data, leading to more robust and insightful experimental outcomes.

Acknowledgements

We extend our gratitude to Luise Sternberg for her invaluable contributions to generating application data on the Attune CytPix flow cytometer. We also thank Nicholas Putnam for his expertise and guidance in utilizing the instrument, and Nicolas Gregg for his assistance with sample preparation.

Trademarks/licensing