Cell Classification in Human Peripheral Blood using the Amnis ImageStream® Flow Imaging System

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Abstract

Amnis Corporation's ImageStream® 100 (IS100) combines the quantitative power of flow cytometry with high-resolution brightfield, darkfield, and fluorescent cellular imagery. The system simultaneously generates six images of each cell in flow and can acquire data sets consisting of ten thousand cells in minutes, while offering fluorescence sensitivity equal to or better than existing flow cytometers. The image data are analyzed using Amnis' IDEAS software, which automatically calculates over 200 morphometric and photometric features. Associated statistics for each cell allow for the identification of unique cell groups based on their morphological characteristics as well as fluorescence intensity. The software offers the ability to view the imagery associated with any cell in a fluorescence intensity scatter plot, perform "virtual cell sorts" of user-specified sub-populations, and generate custom features of biological significance (e.g. N/C ratio). In this study, human peripheral blood mononuclear cells were stained with a fluorescent DNA binding dye to reveal nuclear morphology, as well as fluorescently labeled anti-CD45 and anti-CD14. Five images of each cell were acquired: brightfield, darkfield (laser side scatter), and three fluorescent colors including nuclear imagery. The object was to identify morphometric parameters in the brightfield, darkfield, and nuclear imagery that would prove useful in cell classification. Parameters with discriminating power included cellular size and texture, darkfield intensity and granularity, and nuclear fluorescence intensity, texture, and shape. Cell types that could be automatically discriminated using these parameters in lieu of immunofluorescent markers included lymphocytes, monocytes, neutrophils, eosinophils and basophils.

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Introduction

Differential counting and assessment of peripheral blood leukocyte morphology can be an important source of data in a wide range of clinical and experimental situations and provides a means to follow subjects longitudinally through a study by repeated evaluations. Up to this point, peripheral blood differential analysis has been performed via the 2 dimensional Coulter approach, which does not provide morphological information, or by manually assessing the morphological characteristics of a blood smear via microscopy, which is a tedious and subjective task and generally involves a relatively small sample size. The ImageStream 100 flow imaging system (IS100) is a recently developed technology by Amnis Corporation that is able to acquire cellular imagery in flow at rates up to 100 cells per second. The system produces up to six simultaneous images of each cell, including brightfield, darkfield and four colors of fluorescence.
This technology was applied to the analysis of normal human peripheral blood with the goal of obtaining a differential leukocyte analysis. In this study, human peripheral blood leukocytes were evaluated on the IS100 after staining with fluorochrome conjugated anti-CD45 and CD14. It was found that a bivariate plot of CD45 intensity with darkfield intensity produced 5 distinct populations of cells. The actual identity of the cells could be confirmed by the associated imagery. In this manner, lymphocytes, neutrophils, monocytes, basophils and eosinophils could be identified.

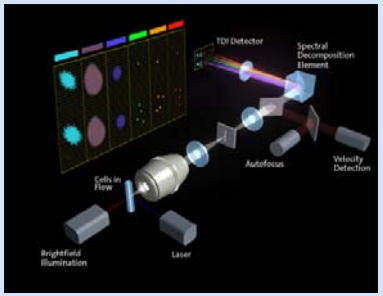


Figure 1: The ImageStream Architecture

ImageStream is a novel technology designed to image rapidly moving objects in flow with high sensitivity, high image fidelity, and in multiple simultaneous imaging modes. As shown in the Figure above, cells are hydrodynamically focused within a flow cuvette and are illuminated from the side and from behind with lasers or other light sources. Fluorescence, side scatter, and transmitted light from cells is imaged by an objective lens and relayed to a spectral decomposition element, which divides the imagery into spectral bands located side-by-side across the detector. Different spectral bands are used for different imaging modes or different colors of fluorescence imagery. For example, laser side scatter produces a darkfield image in the laser's 488nm spectral band while transmitted red light produces a brightfield image in the red spectral band. Because all the channels are in spatial register, image analysis is greatly facilitated and the imagery can be readily reconstructed for visual interpretation after quantitative analysis. High sensitivity is achieved by operating the CCD in Time Delay Integration (TDI) mode. TDI imaging is a method of electronically panning the detector to track object motion. TDI operation results in signal collection times that can exceed ten milliseconds, orders of magnitude longer than conventional flow cytometry, while preserving image fidelity and throughput.

Materials and Methods

Human peripheral blood was obtained from AllCells (Berkeley, CA). Whole blood was stained for 15 minutes on ice with FITC conjugated anti-CD45 mAb and PE conjugated anti-CD14 mAb (CalTag Laboratories, Burlingame, CA). RBCs were then lysed by incubation with FacsLyse (BD Biosciences, San Diego, CA) for 10 minutes at room temperature. After 2 washes in PBS, DRAQ5 (Biostatus Ltd., Leicestershire, UK) was added as a nuclear stain. The cells were then run directly on the IS100 and a file of 20,000 events collected. After acquisition, the data were compensated for inter-channel crosstalk using Amnis' IDEAS data analysis software package, which automatically calculated a crosstalk compensation matrix based on data from control cell samples stained with either FITC or PE but not both. Of the 20,000 total events imaged, 15,000 were identified as single cells and further analyzed as described below.

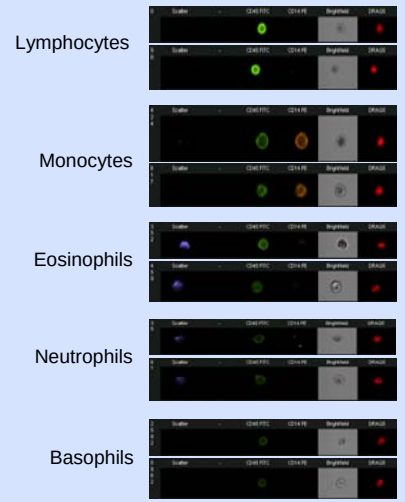
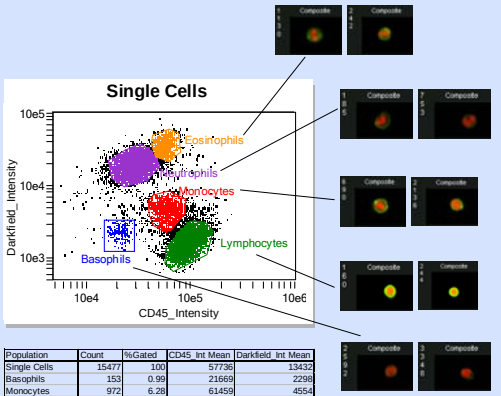


Figure 2: Selected Image Galleries

Image Galleries from the IDEAS analysis program are shown above. Each row represents the collected imagery from an individual cell, from channel 1 on the left to channel 6 on the right. In this experiment, darkfield scatter is in channel 1, CD45-FITC in channel 3, CD14-PE in channel 4, brightfield imagery in channel 5 and DRAQ5 nuclear stain in channel 6.

Cells were classified by their composite imagery (see Figure 3) and also by the use of phenotypic markers such as CD14, as is shown in this experiment. In such instances, back-gating on the unique phenotypic marker allowed confirmation of the classification (data not shown).



Population	Count	%Gated	CD45_Int Mean	Darkfield_Int Mean
Single Cells	15477	100	57736	13432
Basophils	153	0.99	21669	2298
Monocytes	972	6.28	61459	4554
Lymphocytes	4917	31.8	95666	1668
Neutrophils	7093	45.8	30740	20339
Eosinophils	1012	6.54	55594	35828

Figure 3: Bivariate Plot of Darkfield Scatter Intensity vs. CD45-FITC Intensity Reveals Five Cell Populations

A bivariate plot of CD45 staining intensity vs. darkfield scatter intensity of human peripheral blood leukocytes is shown. Gating validity was assessed by examining the morphology of the cell populations, with representative composite images shown for each cell population. Composite imagery shown here was produced by combining nuclear imagery (channel 6) with the fluorescent imagery (channel 3). In additional data sets, phenotypic markers were included (CD3, CD14, etc.) and positive cells were back-gated onto the CD45 vs. darkfield scatter plot to validate the gating.

Conclusion

The utility of the ImageStream100 for the differential analysis of human peripheral blood leukocytes is demonstrated here using darkfield scatter intensity and a single phenotypic marker, CD45. The novel advantage the ImageStream provides over the standard flow cytometric differential is the ability to visually confirm the population designations defined by the phenotypic markers. The acquired imagery can then be used to assure that the phenotypically defined populations indeed match classically accepted morphological cellular definitions obtained by standard image analysis from Romanowsky stained blood films.

Attempts to achieve this differential based solely on physical morphometric measures of cytoplasm and nuclear features is currently ongoing at Amnis. This would allow fluorescence image channels to be dedicated to fluorescent markers that would add insight into changes in subpopulations of cells, cell function or the presence of abnormal cell populations.

Additionally, the ability to acquire imagery of large numbers of cells (in the tens of thousands) adds statistical power to the analysis of intra- or inter-sample differences. Currently, the IS100 acquires imagery at rates up to 100 cells per second. Thus, the acquisition of a 10,000 event file requires approximately 2 minutes.

Digital imagery also has the advantage of allowing longitudinal sample comparisons without the need for physical searching for slides and associated data. Again, in this type of situation, the large number of events in typical data files would make analysis more robust compared to other morphology based analytical methods.

In conclusion, image acquisition and differential analysis of human peripheral blood cells was performed using the ImageStream 100 platform and analyzed with IDEAS software package. The data presented here demonstrate the utility of the this technology for advancing the capabilities to automatically classify peripheral blood cell subsets based on imagery.

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