

## Analysis of Complement 3b deposition on Rituximab opsonized cells using ImageStream® Multispectral Imaging Cytometry

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### BACKGROUND AND TECHNOLOGY

The Amnis ImageStream system offers a powerful new approach for elucidating the mechanisms of action of therapeutic monoclonal antibodies directed toward cellular targets. The instrument is operationally similar to a flow cytometer, measuring fluorescent intensity from each cell after intersection with a laser light source. However, in addition, the ImageStream system simultaneously captures high resolution multispectral images of each cell at a rate of 300 events per second or more. As many as six independent images may be collected, including a brightfield image, a darkfield image and up to four independent fluorescent images. The system

provides detailed morphological information about the cellular localization of the therapeutic antibody as well as statistically significant assessments of the population of treated cells. The combination of these two capabilities allows a new level of integrated quantitative analysis – analysis both of the individual cell and of the cell population – thereby providing information on the possible mechanism of action as well as the potential identification of variant (e.g., non-responding) subpopulations.

### EXPERIMENTAL DESIGN AND RESULTS

One excellent example of this capability is presented here in a study of the mechanism of action of Rituximab (RTX). RTX is a therapeutic monoclonal antibody directed against CD20 that was approved in 1997 for the treatment of Non-Hodgkin's lymphoma and is also being examined as a treatment for chronic lymphocytic leukemia (CLL). The complete mechanism of anti-tumor activity of RTX in vivo has not yet been determined, although preclinical studies and clinical investigations have supported models of apoptosis, Fc receptor-mediated antibody-dependent cellular cytotoxicity (ADCC), and complement-dependent cytotoxicity (CDC).

Results of previous studies have shown that binding of RTX to CD20+ cells activates the complement cascade and analysis by fluorescence microscopy demonstrated that on some cells, the C3b fragment of complement was co-

located with the antibody. This study was designed to evaluate the cellular localization of the therapeutic antibody as well as statistically significant assessments of the population of treated cells.

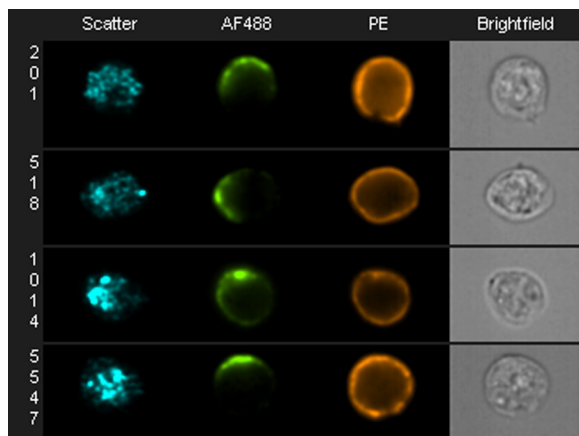


Figure 1. ImageStream Image Gallery Analysis. Channel images displayed include laser side scatter; RTX-AF488, mAb 7C12 (PE) and Brightfield.

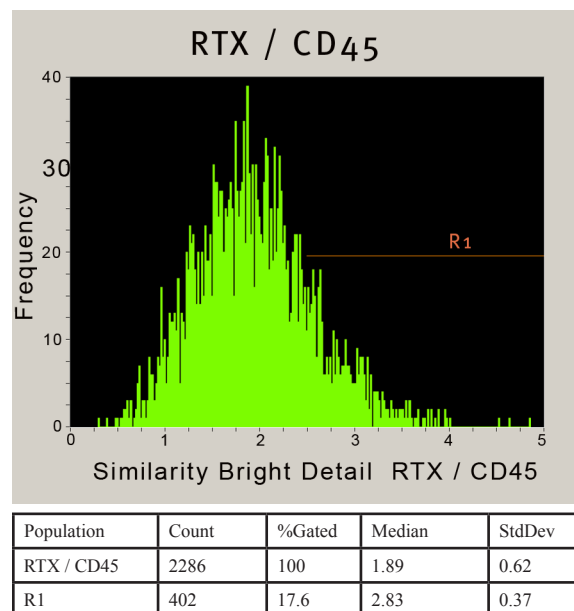


Figure 2. Population analysis of the similarity in cell-surface distribution between RTX-AF488 and anti-CD-45 (PE).

localized with bound RTX. For the set of experiments in this report, B cells were opsonized with RTX (labeled with AlexaFluor® 488 (AF488)) and analyzed for C3b deposition after incubation in normal human serum as a source of complement. If, as the model predicts, RTX bound to CD20 on the surface of cells triggers covalent deposition of C3b and its fragments, then this can be determined by staining with a C3b-specific antibody such as mAb 7C12. As a negative control, cells were stained with anti-CD45 after RTX binding.

## IMAGESTREAM MULTISPECTRAL ANALYSIS OF RTX/C3B CO-LOCALIZATION

The Raji B lymphoma cell line or B cells isolated from a patient with Chronic Lymphocytic Leukemia (CLL) were incubated with RTX (AF488) in normal human serum and then stained with either anti-CD45 (PE) or anti-C3b mAb 7C12 (PE). Samples were then analyzed on the ImageStream System. Representative multispectral images with RTX and anti-CD45 mAb are shown in Figure 1. The RTX (AF488) stained images of the Raji cells are shown in Channel 3 and the anti-CD45 (PE) stained images are shown in Channel 4 (laser light scattering is shown in Channel 2 and brightfield imagery in Channel 5). Since no functional association is expected between CD20 and CD45, the staining patterns should show little or no similarity. Simple visual inspection suggests there is no significant similarity in the PE and AF488 staining patterns, as would

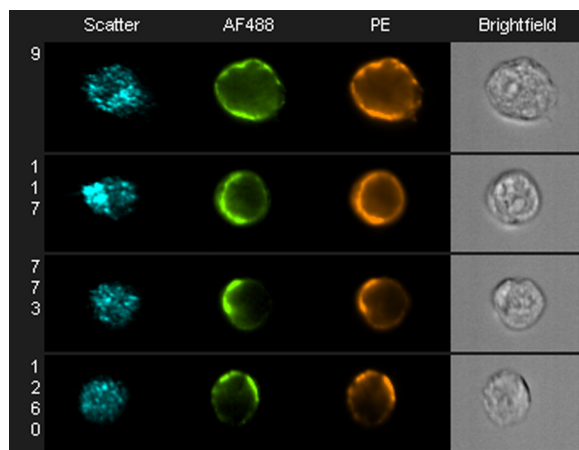


Figure 3. ImageStream Image Gallery Analysis. Channel images displayed include laser side scatter; RTX-AF488, mAb 7C12 (PE) and Brightfield.

be expected. To extend the visual observations, a statistical analysis of similarity in the staining patterns was performed (Figure 2). The similarity value was calculated using an algorithm for co-localization based on the relative intensities in individual pixels of the cells. Since each pixel has a spatial registry, pixel intensity values in different channels (e.g. AF488 and PE) can be compared and evaluated. The value obtained by this method for the population of cells is low, indicating that CD20 and CD45 do not associate on the cell membrane. By contrast, results obtained using RTX- and C3b-specific probes show much greater similarity. Shown in Figure 3 are representative images from cells incubated with RTX-AF488 in normal human serum, as in the control, but, in this case, stained with the C3b-specific mAb 7C12 (PE-labeled). Visual inspection of the displayed images indicates a somewhat higher degree of co-localization than observed in the control, but the comparison of these few images does not reveal dramatic differences. However, statistical analysis of the entire cell population utilizing the co-localization algorithm (Figure 4) demonstrates that there is, in fact, a substantially greater degree of co-localization between RTX and C3b than observed in the control (RTX and CD45).

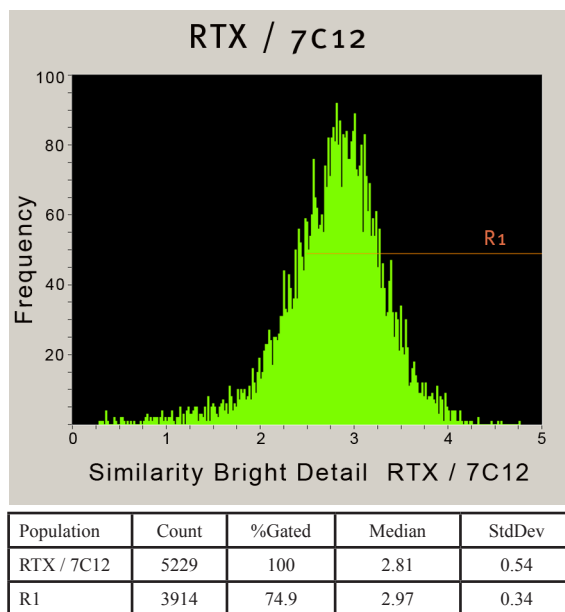


Figure 4. Population analysis of the similarity in cell-surface distribution between RTX-AF488 and mAb 7C12 (PE).

## CONCLUSIONS

The ImageStream cell analysis system provides high resolution, high sensitivity image analysis of cells in flow. Using a similarity algorithm, we were able to quantify the degree of similarity between distributions of two molecules – RTX and C3b – on the surfaces of large populations of Raji and B cells. The results provided a valuable method for measuring the degree of co-localization of these molecules on both individual cells and cell populations that is not possible by subjective visual inspection techniques. Quantitative analysis of the population of cell images significantly improves the scope and reliability of the conclusions in contrast to the analysis of a few individual cells alone.