Analysis of the Cellular Fate of a Drug-Conjugated Antibody
With the ImageStream® 100 Multispectral Cell Imaging System

Summary
This report demonstrates the use of the ImageStream 100 to study the effects of binding of a drug conjugated to a monoclonal antibody (a drug-conjugated antibody, or DcA) specific for a cell-surface molecule expressed on the RAMOS B-lymphoblastoid line, and shows time-dependent internalization and intracellular processing of the mAb. The high optical resolution achieved by the ImageStream system combined with novel image correlation algorithms built into the IDEAS™ data analysis software allow accurate intracellular co-localization of the mAb to endosomes and lysosomes over multiple time points in a large population of treated cells, thereby permitting statistically valid analysis.

Introduction
The recent advent of use of monoclonal antibodies in the treatment of a wide variety of disease processes offers exciting possibilities for the development of therapies with substantially increased specificity compared to conventional chemotherapies. Examples include mAb specific for cell-surface molecules such as CD20 (Rituximab™), Her-2/Neu (Herceptin™) and EGFR (Erbitux™) in the treatment of non-Hodgkin’s lymphomas, breast and colorectal cancers, respectively, and Vla-4 (Tysabri™) and TNF-R1 (Remicade™) in the treatment of multiple sclerosis, inflammatory bowel disease and rheumatoid arthritis. Antibodies have also been conjugated to either radioisotopes (eg Zevalin™ and Bexxar™) or toxins (eg, Mylotarg™) for treatment of certain cancers. A vital aspect in the development of these, and other potentially therapeutic molecules, concerns the mechanism of action of these molecules. For example, it is important to determine in any disease model whether a given therapeutic requires internalization or is capable of mediating its function at the cell surface. The unique capabilities of the ImageStream system are well suited to provide valuable information about the mechanism of action (MOA) of such molecules.

ImageStream System Capabilities
The ImageStream 100 simultaneously acquires six high resolution digital images of each cell in flow, using three imaging modes: brightfield, darkfield, and fluorescence. Cells are imaged at rates up to 100 cells per second, producing a standard 10,000 cell data file in about two minutes. The IDEAS image analysis software package calculates over 200 features for each cell, including all standard intensity-based features used in flow cytometry, while also quantitating numerous morphologic features such as cell area, perimeter, aspect ratio, texture, nuclear size, spot counts, and others. The IDEAS package also includes several built-in algorithms that quantitate the ‘similarity’ between images of the same cell, enabling quantitative co-localization studies without the need for FRET or other techniques.

Experimental Design
The experiment described here was performed over a 24 hour period with RAMOS cells that had been incubated with DCX-123 mAb for varying time periods. The mAb was visualized using a phycoerythrin-conjugated secondary antibody and analysis software package calculates over 200 features for each cell, including all standard intensity-based features used in flow cytometry, while also quantitating numerous morphologic features such as cell area, perimeter, aspect ratio, texture, nuclear size, spot counts, and others. The IDEAS package also includes several built-in algorithms that quantitate the ‘similarity’ between images of the same cell, enabling quantitative co-localization studies without the need for FRET or other techniques.

Figure 1. Cell images from each time point, as indicated. Channel 1 shows endosome staining; Channel 2, DCX123-mAb; Channel 3, Brightfield imagery; and Channel 4, lysosome staining.

Figure 2. Histograms of cell populations. Each histogram represents a population of cells at the time point indicated, stained with DCX-123 mAb and with either an endosomal or a lysosomal stain.

Figure 2.
intracellular endosomes and lysosomes were visualized with distinct secondary antibodies labeled with AlexaFluor 488™ and Cy-Chrome™ (a PE-Cy5 conjugate), respectively. Dead cells were identified based on their darkfield laser side scatter image characteristics and verified by visual inspection. The cellular location of the DCX-123 mAb DcA was determined using a quantitative similarity algorithm contained in the IDEAS data analysis package that cross-correlates DCX-123 DcA imagery with the endosomal and lysosomal imagery acquired from the same cell.

**Results and Data Analysis**

Ramos B cells were incubated with the DCX-123 mAb DcA for either 30 min at 4°C (to act as a control for binding of antibody in the absence of the cells’ ability to cap and internalize) or at 37°C for 2, 4, 8 or 24 hours. Cells from these groups were fixed, permeabilized and stained for the mAb (using a HuIg-specific secondary antibody), early endosomes (anti-EEA-1) and lysosomes (anti-CD107a). Cell images were acquired on an ImageStream 100 using standard protocols and processed with the IDEAS image analysis software package.

Representative images from cells incubated with the labeled antibodies are shown in Figure 1. Cells incubated for 30 min at 4°C had principally circumferential binding of the CDX-123 mAb with little or no distinct capping or localization. As the incubation time at 37°C increased, an association (co-localization) between the CDX-123 mAb and endosome or lysosome markers became increasingly apparent.

Single cells (ie, cells positively stained for all three antibodies) were selected for visualization and further analysis using the IDEAS ‘virtual sorting’ capability. The degree of co-localization of DCX-123 mAb at each time point with either early endosome or lysosome markers was quantified using the Bright Detail Similarity algorithm feature contained in the IDEAS program. A gate identifying cells in which some association had occurred was set by reference to the control sample (incubation at 4°C for 30 minutes). In the control sample this threshold corresponded to a percent associated of 5% of the population. The fraction of cells in which the DCX-123 mAb DcA had associated with either endosomes or lysosomes through the time course is shown graphically in Figures 2-4. It can be seen that the fraction of the cell population exhibiting endosomal association increased over two hours, peaking at 73.3% and decreasing thereafter. Peak association of the DCX-123 mAb DcA with intracellular lysosomes was lower in magnitude, and lagged slightly behind, peaking at 15% of cells at four hours. Analysis of histograms depicting laser scatter intensity versus cell size also permitted estimation of the proportion of dead cells in each population. A time course cell death resulting from treatment with the DCX-123 mAb DcA is shown in Figure 5.

**Conclusions**

The ImageStream 100 was used to provide important insight into the intracellular fate of an anti-DCX-123 drug-antibody conjugate and could lead to a better evaluation of its potential therapeutic efficacy in killing RAMOS B-lymphoblastoid cells in vitro. Since it combines the visual power of microscopy with the statistical power of a flow cytometer, the ImageStream system is uniquely well suited to this kind of analysis. In sum, the data generated in this study suggest that after initially binding to determinants on the cell surface, the DCX-123 mAb DcA is internalized to intracellular endosomes, followed by a transfer to lysosomes, where it is rapidly degraded. Although the fate of the drug initially conjugated to the DCX-123 mAb was not followed in these experiments, the increased proportion of cell death seen after 24 hours of culture suggests that the drug is still in a form that is capable of performing its cytotoxic function subsequent to the internalization and processing functions described above.