

Quantitative Analysis of Therapeutic Monoclonal Antibody Localizations to Endosomes and Lysosomes Using the ImageStream® Imaging Flow Cytometer



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Abstract

Monoclonal antibodies (mAb) have recently become effective drugs for the treatment of a variety of human diseases. A number of these bind to cell surface determinants and thus are subject to cellular mechanisms of membrane clearing and internalization. The efficacy of therapeutic mAbs could be influenced by the rate of membrane clearing and progression to the degradative lysosomal compartment. One such mAb used clinically, binds to CD20 which is expressed on chronic lymphocytic leukemia cells. Interestingly, not all patients have a therapeutic response when treated with anti-CD20 (Rituximab). The difference may be due to the clearance and catabolism of the mAb. Thus it would be of interest to determine the cellular fate of the mAb in established tumor cell lines and eventually in patient samples.

A study to track antibody drug conjugate trafficking has been performed utilizing the ImageStream imaging flow cytometer which acquires 6 channels of imagery simultaneously from cells in flow including bright field, dark field and 4 channels of fluorescent imagery.

The Ramos cell line was incubated with fluorochrome labeled anti-CD20 for various times and then fixed and stained with fluorochrome labeled markers for endosomes and lysosomes. Cellular imagery was acquired and analyzed. The data demonstrated an initial capping of the anti-CD20, followed by association with the endosome marker and later with the lysosome marker. This was observed in sample sizes of 10,000 cells with a quantitative score which allows for statistical analysis.

Thus this approach should be useful clinically in analyzing potential differences in intracellular fates of mAbs between responder and non-responder populations.

Introduction

Cell surface proteins are subjected to a continual process of membrane clearing through shedding or internalization, which can occur once proteins have bound ligand. Internalized proteins enter into intracellular pathways in the endosomes that can result in protein sorting and re-cycling to the cell surface or degradation through the lysosomal pathway.

Determining the fate of a cell surface bound protein relies on the co-localization of the protein with markers of the endosomal or lysosomal compartment often with immunofluorescent microscopy or by biochemical methods such as co-isolation or co-precipitation.

The ImageStream imaging flow cytometer acquires 6 channels of imagery including brightfield, darkfield and 4 channels of fluorescent imagery using a CCD camera in which the pixels in each channel are in spatial registry. Thus, a correlation analysis of the similarity of the images between channels is possible. This has significant utility in the assessment of the co-localization of proteins.

In this report, Ramos cells were labeled with fluorochrome labeled anti-CD20, incubated for various times and then fixed, perm'd and labeled with antibodies to markers for the endosomal (anti-EEA1) and lysosomal (anti-Lamp1/CD107a) compartments. Cellular imagery was acquired on the ImageStream and analyzed for the degree of correlation of the anti-CD20 fluorescent imagery with the endosomal and lysosomal markers. Through the use of the non-mean normalized Pearson's correlation coefficient, a quantitative score could be assigned to each time point which allows for a statistically valid representation of the degree of co-localization of the antibody with the endosome and lysosome compartments. This method can be applied to many other cell surface markers in a quantitative manner.

Materials and Methods

A) Cell Staining

- Ramos cells were labeled with RTX on ice for 30 minutes, washed and cultured for the times indicated at 37 degrees. Time 0 cells were not cultured, but processed immediately for additional staining.

- Cells were harvested, washed, resuspended in Cytotix/Cytoperm solution (BD Biosciences) and incubated for 20 min at RT.

Note: After each step below, cells were washed twice in PermWash buffer (BD Biosciences).

- Cells were incubated for 20 minutes on ice with the following reagents in the order listed:

- PE-goat anti-human IgG (to stain RTX).

- Murine anti-human EEA1 mAb.

- AF488-goat anti-mouse IgG (to stain EEA1).

- Biotinylated anti-Lamp-1 (CD107a) antibody.

- Cyochrome-Streptavidin.

- Fix cells in 1% paraformaldehyde in PBS.

B) Image Acquisition, Image Processing and Calculation of the Similarity Bright Detail Score

- Acquire images from 5-10,000 cells per sample with the ImageStream.

- Cell images were compensated for spectral crosstalk and analyzed with the IDEAS analytical software package.

- Single cells staining with RTX were selected by gating.

- The images in the single cell population were subjected to image processing (an opening residue operation was performed that minimizes dim background, leaving behind a Bright Detail image).

- Similarity Bright Detail Score between the relevant fluorescent image pairs was then calculated on a per-cell basis using IDEAS.

Calculation of SBD.

The Similarity Bright Detail score is derived from the non-mean normalized Pearson's correlation coefficient (r) calculated for pairs of values taken from different data sources. In this case the values are pixel intensities and the different data sources are the different channels of fluorescent imagery. The formula is given below:

$$r = \frac{\sum X \times Y}{\sqrt{\sum X^2 \times \sum Y^2}}$$

The data pairs (X, Y) are simply the pixel intensities at the same location in each fluorescent image channel. This correlation coefficient produces values that range from 0 (no correlation) to 1 (perfect correlation). Interpretation of r as a metric for surface co-localization is limited because a) non-co-localized proteins may have a high baseline correlation if background antibody staining is significant and b) distributions of r values at the high end of the range are compressed. To decompress the high end of the range, we calculate SBD, which is a transformation of r using the following formula:

$$\text{Similarity Bright Detail (SBD)} = \ln \left(\frac{1+r}{1-r} \right)$$

Unlike r , SBD is unbounded and produces normal distributions over large sets of measurements. As a result, the "dynamic range" of SBD is higher and correlates more closely to qualitative judgments of visual distinctiveness.

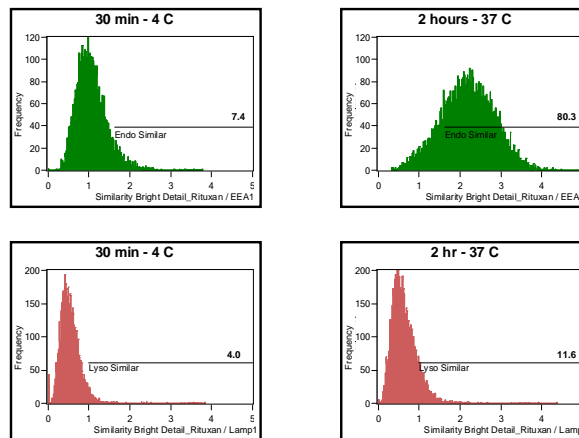


Figure 2. Quantitation of RTX trafficking to subcellular organelles using Similarity Bright Detail (SBD) analysis.

Cells labeled with PE-RTX were probed with AF488 anti-EEA1 to visualize early endosomes and CyChrome anti-Lamp1 to visualize lysosomes either before (30 min - 4 C) or after (2 hours - 37 C) RTX internalization. For each cell, SBD of the RTX / EEA1 (top histograms in green) and RTX / Lamp1 (lower histograms in red) image pairs was calculated and plotted above. Images of co-localized markers have high SBD values, and the regions 'Endo Similar' and 'Lyso Similar' were defined based on visual inspection of the imagery in each bin on the histogram. The data show that the RTX / EEA1 similarity, but not RTX / Lamp1 dramatically increases after 2 hour incubation at 37 C, indicating that at this time point, RTX has trafficked to the early endosomal compartment.

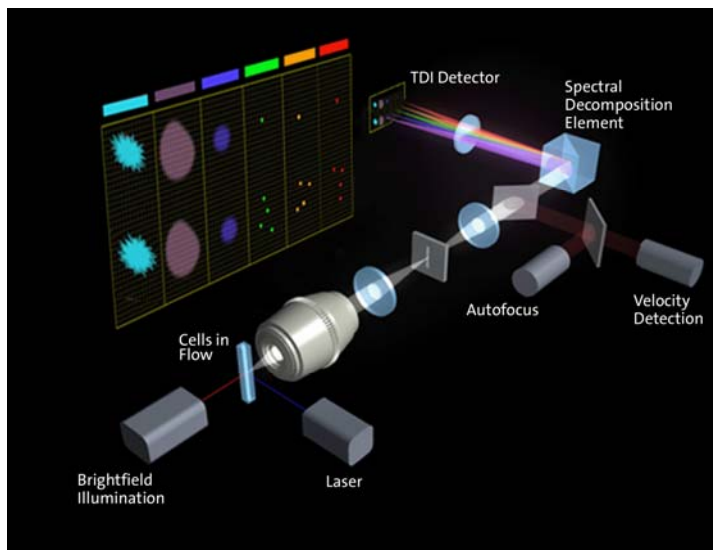


Figure 1: ImageStream Architecture

ImageStream is a novel technology designed to image rapidly-moving objects in flow or on substrates 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 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.

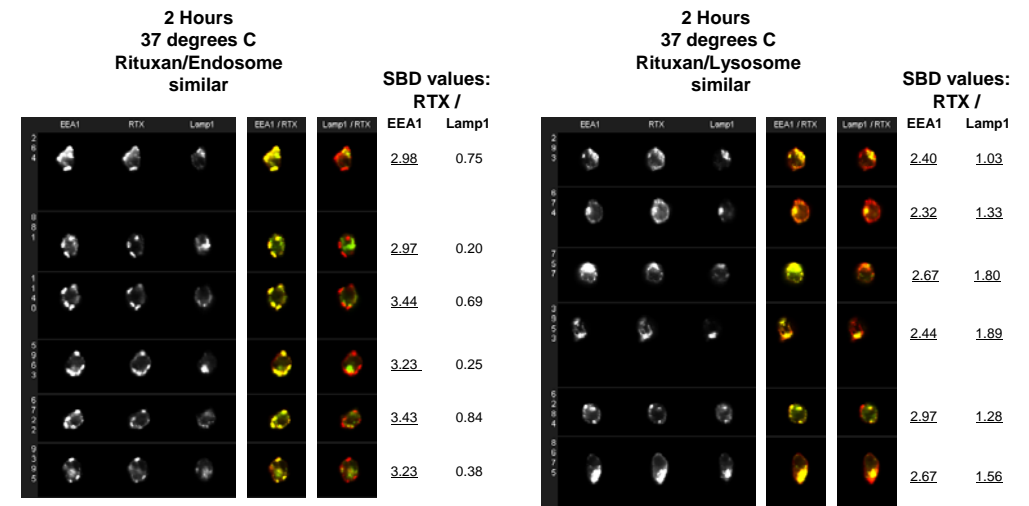
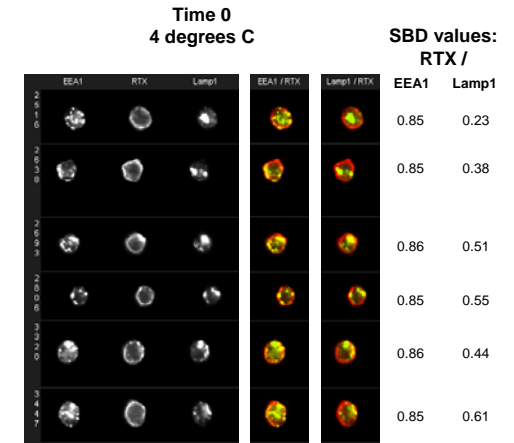


Figure 3. Representative EEA1 / RTX / Lamp1 cell images.

Representative cell images from each treatment group are shown above. In grey-scale from left to right are the EEA1, RTX, and Lamp1 images, followed by two composite images. The first composite is an overlay of EEA1 (green) and RTX (red). The second composite is an overlay of Lamp1 (green) and RTX (red). Note that the distinct spatial distribution of the surface RTX and intracellular EEA1 / Lamp1 images in the time 0 sample, and their corresponding low SBD values (top). In contrast, note the similarity of the RTX / EEA1 image pairs in the 37 C sample, indicating trafficking of RTX to early endosomes at this time point (bottom). Only a few cells show marginal evidence of lysosomal RTX trafficking (bottom left).

Conclusion

This study demonstrates the feasibility of using the ImageStream technology to measure co-localization of proteins of interest to the endosomal and lysosomal cellular compartments. A quantitative methodology for assessing co-localization is described that allows statistically robust analysis on groups of thousands of cells. The ability of the ImageStream to acquire imagery at rates of 300 cells per second allows the accumulation of event numbers required to achieve statistical significance between groups or identify subpopulations with relative ease.

Here data are presented for a single time point (2 hr) in a kinetic study regarding the cellular fate of Rituximab bound to CD20 on the cell surface. After 2 hours at 37 degrees C, approximately 80% of the Rituximab signal co-localizes with the endosomal marker. There is a smaller percentage that co-localizes with the lysosomal marker at this time. This percentage increases over time but not to the extent of the endosomal co-localization (data not shown). The use of the fluorochrome, PE, as the marker for Rituximab may influence this proportion as PE is known to denature/quench in the lysosomal environment.

This co-localization methodology can also be combined with a nuclear stain that would allow simultaneous assessment of apoptosis by assessing nuclear fragmentation (data not shown). In the study of therapeutic drug antibody combinations this methodology may allow for a more accurate assessment of drug candidate efficacy and mechanism of action by evaluating the rate and proportion of endosomal/lysosomal localization and the induction of apoptosis.