Assessment of Protein Co-localization with an Imaging Flow Cytometer and Novel Analytical Method

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Abstract

Co-localization of proteins is frequently assessed in biological systems as inferences can be made regarding potential functional consequences The use of fluorescently labeled monoclonal antibodies (mAb) has been useful in this regard to assess phenomena such as membrane co-capping. Generating this data via manual microscopic assessment of cells is tedious, time consuming and error prone. The ImageStream imaging flow cytometer is capable of acquiring 6 simultaneous images of cells in flow which include brightfield, darkfield and 4 fluorescent images at rates up to 300 cells per second. This technology has been applied to the study of the mechanism of action of Rituxan, a mAb against CD20 which is used in the treatment of B cell lymphomas. The role of complement activation mediated by Rituximab was assessed using the human Raji lymphoma line. Cells were treated with fluorescently labeled Rituximab in the presence of normal human serum as a complement source. An anti-C3b/C3bi mAb was then used to detect the complement fragments, covalently deposited on the cell, and this mAb was labeled with a different fluorochrome. After image acquisition, the data was analyzed by a novel algorithm that measures the correlation of the Rituximab and anti-C3b images on a pixel by pixel basis, allowing a quantitative assessment of the degree of similarity between the Rituximab fluorescent imagery and the C3b complement fluorescent imagery. This analysis revealed a significant co-localization between bound Rituximab and the anti-C3b/iC3b mAb. The results demonstrate that cell-bound Rituximab activates complement and directs deposition of C3 fragments which localize in close juxtaposition with cell-bound Rituximab. Thus, using the ImageStream technology platform, a rapid quantitative assessment of protein co-localization can be made.

Introduction

Rituximab (RTX) is a chimeric, humanized anti-CD20 IgG mAb approved for treatment of B cell lymphomas, and binding of RTX to B cells activates complement and promotes deposition of C3b on cells. Previous fluorescence microscopy studies, based on both in vitro experiments and examination of blood samples from RTX-treated CLL patients, suggest that C3b and/or its breakdown products iC3b/C3b are closely associated with cell-bound RTX. In this study, the ImageStream imaging flow cytometer was used to acquire imagery which was then analyzed via a novel approach that included an image processing step (open residue) and a correlation analysis of the pixel intensities between the image of cells stained with RTX and the image of cells stained with reagents to detect C3b. A quantitative score is then generated that allows statistical comparison of the image data between experimental groups.

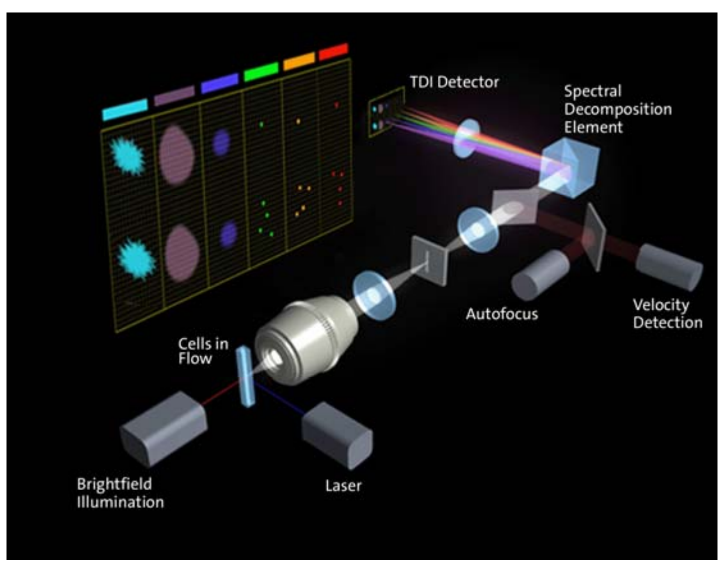

Figure 1: ImageStream Architecture

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.

Materials and Methods

A) Cell Staining

- Raji B lymphoma cells were incubated with AlexaFluor488 RTX at 37 C for 15 minutes to both induce capping of, and to fluorescently label, cell surface CD20.
- In some cases, cells were opsonized with normal human serum as a source of complement.
- After washing, cells were incubated at 4 C for 30 minutes with PE labeled anti-C3b/C3bi (to identify complement fragments), anti-human IgG Fc (to identify cell-bound RTX), or CD45.

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

- Cell imagery was acquired on the ImageStream imaging flow cytometer. Generally, images from 5-10,000 cells per sample were collected.
- Cell images were compensated and analyzed with the IDEAS analytical software package.
- Single cells staining with both fluorochromes 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.

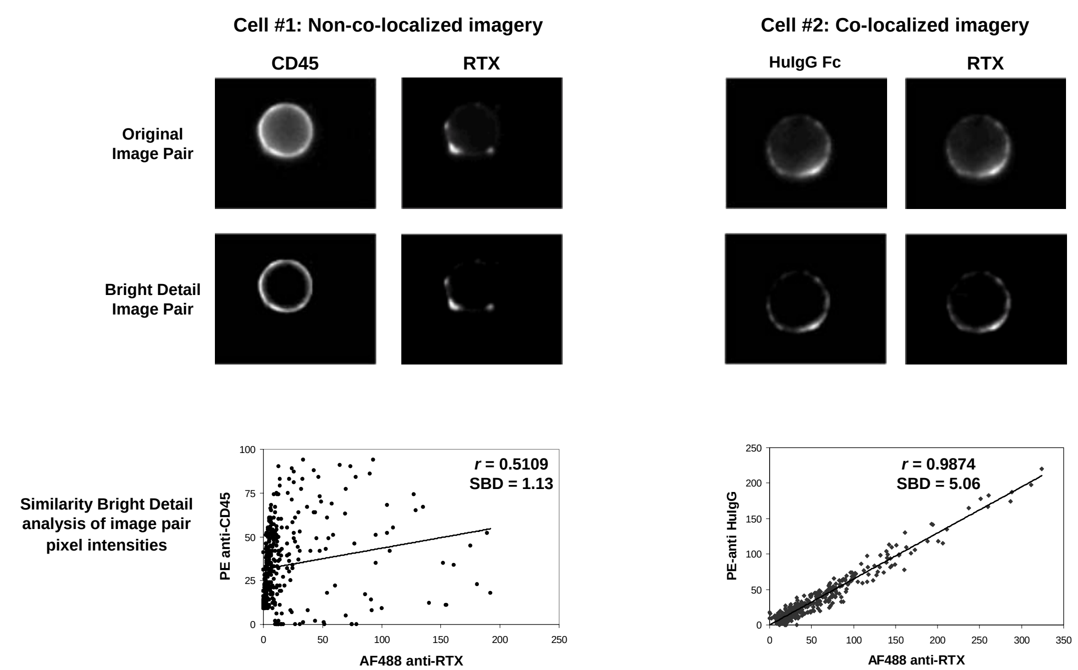

Figure 2. Similarity Bright Detail (SBD) analysis of similar and dissimilar staining patterns post 'open residue' processing.

In order to measure co-localization of surface markers, it is critical to first localize one of the markers. In these experiments, CD20 was localized by staining the cells with AF488 RTX under capping conditions, followed by staining with either PE anti-CD45 (Cell#1) as a negative control for co-localization or PE anti-HuIgG (Cell #2) as a positive control for co-localization. Note that the RTX-HuIgG image pair looks highly similar, while the RTX-CD45 image pair does not. Processing the images to remove background using an opening residue generates new images that retain only the bright details from the original images. The co-localized image pair (cell#2) has a much larger SBD value compared to the non-co-localized image pair (cell #2). A full description of the SBD value is given below:

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;

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

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 surface proteins have a high baseline correlation since both are located on the cell membrane 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.

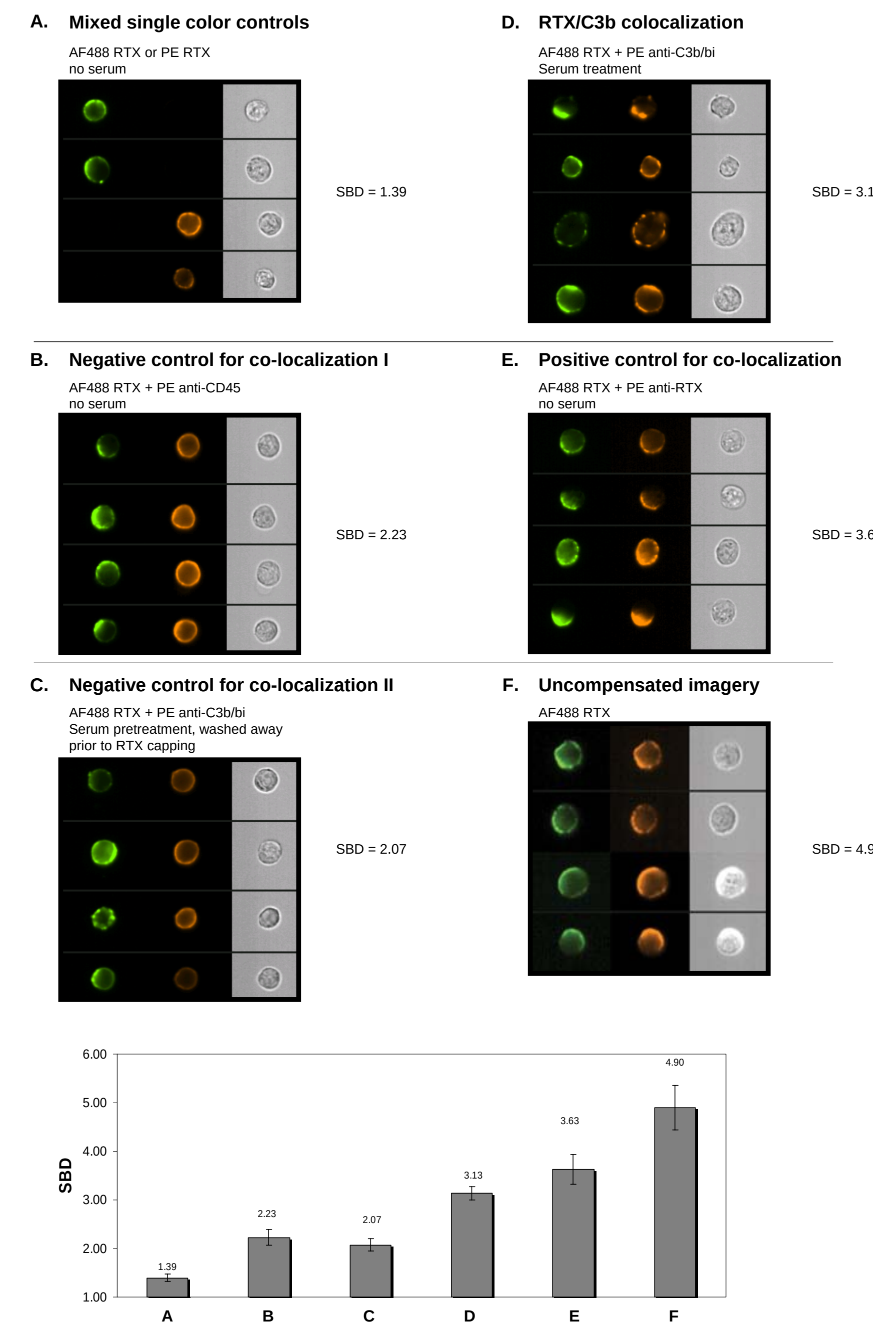

Figure 4. Staining conditions, representative imagery and SBD values for various experimental groups.

Representative AF488 (green), PE (orange), and brightfield images of cells from six experimental groups are shown, along with their SBD_green / orange (A-F). The cells chosen had SBD values equal to the mean SBD value of the entire single cell population (consisting of at least 5000 images). Spectrally compensated single color controls (A) have the lowest values since correlation is performed between a capped surface image and a blank image. Comparing capped RTX to the uniform surface staining of CD45 (B), a receptor known not to localize with RTX, serves as a biologically relevant negative control. Incubation of cells with serum prior to RTX results in diffuse deposition of complement on the cell surface that is not localized to RTX (C). On the other hand, opsonizing RTX-labeled cells with serum results in targeted deposition of complement at the antibody sites, and high SBD values (D). Co-staining cells with AF488 RTX and PE-labeled anti-RTX serves as a positive biological control for co-localization (E). Uncompensated imagery, as expected for mirror image pairs, have the highest values. SBD values (along with SD) for the six groups are plotted in the histogram below the imagery.

Conclusion

This study applies ImageStream technology to quantify surface protein co-localization from fluorescence images of cells captured in flow to study complement fixation by Rituxan. The metric for co-localization, Similarity Bright Detail, assigns a numeric value to the similarity between an image pair for each cell. The images of a co-localizing marker looks similar to the RTX image: these cells have high SBD values. On the other hand, a non-co-localizing marker looks different from the RTX image: these cells have low SBD values. Because the sample sizes are large and because each image pair is scored with an objective value, robust statistical analysis can be performed on this historically qualitative assessment of co-localization. This method of analysis can be applied to other systems for protein co-localization where suitable reagents exist. The benefits of a large sample size in such analyses would be to achieve statistical robustness between experimental groups as well as being able to identify and analyze with confidence subpopulations with unique characteristics.

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