

Intracellular localization and trafficking using the ImageStream® imaging flow cytometer

Sherree Friend, Thaddeus George, Brian Hall, Vidya Venkatachalam, Philip Morrissey. Amnis® Corporation, 2505 Third Avenue, Suite 210, Seattle, WA 98121-1480, www.amnis.com

Abstract

Specific ligands or antibody-conjugated drugs can mediate their cellular effects by gaining entry into cells via receptor mediated endocytosis. Once internalized, molecules differ in their preferential endocytic pathway, and drug efficacy is highly dependent on the route of entry and interaction with cellular components. Evaluation of internalization and intracellular molecular trafficking events are traditionally performed using microscopy. These analyses are limited, because manual image acquisition and quantitative image analysis are time consuming processes. Here we describe a method for measuring internalized events using the ImageStream imaging flow cytometer, which automatically collects large numbers of images per data set and provides quantitative image analysis tools. In addition, localization of internalized probes to endosomes and lysosomes is quantified in several model systems. Because the ImageStream collects multiple fluorescent images per cell, internalized marker colocalization to different cellular compartments can be done simultaneously in a quantitative manner.

Introduction

Measurement of the internalization of surface bound proteins is important to the study of cellular processes such as drug uptake and processing by cells, entry of pathogens and toxins, or antigen processing and presentation. Internalization of proteins may occur through clathrin-mediated endocytosis or lipid raft/caveolae pathways and traffic to endosomes that can result in protein sorting and re-cycling to the cell surface or degradation through the lysosomal pathway.

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. This enables the measurement of fluorescence signal in subcompartments of the cell based on imagery and a correlation analysis of the similarity of the images between channels is possible. This has significant utility in the assessment of the localization of proteins.

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.

In this report, Ramos cells were co-stained with a known surface molecule (PE CD45, orange) and a known cytoplasmic marker (Lamp1, purple) or were incubated with labeled transferrin and subsequently stained with anti-CD71. Cellular imagery was acquired on the ImageStream and analyzed for the degree of internalization using the Internalization Score feature. This method can be applied to many other cell surface markers in a quantitative manner and can also be used in combination with the Similarity Bright Detail feature in IDEAS that correlates localization of fluorescence between the images in different channels.

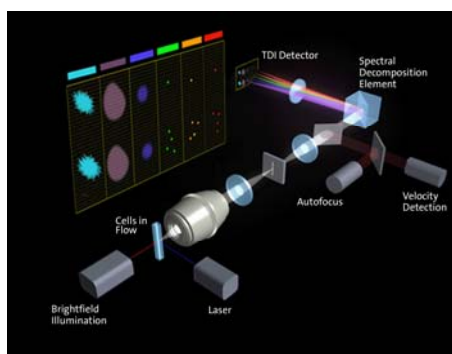


Figure 1: ImageStream Architecture

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 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 registry, 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 pairing 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.

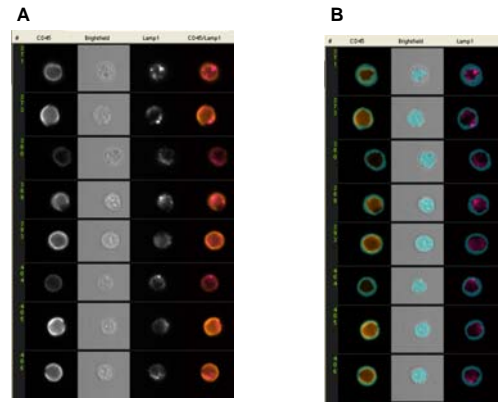


Figure 2: Characterization of the internal and external parts of the cell.

Ramos cells stained on the cell surface with PE anti-CD45, permeabilized and fixed and stained internally with Spectral Red anti-Lamp1 were analyzed on the ImageStream. Panel A shows representative cell images in grey-scale from left to right, CD45, Brightfield, and Lamp1 images, followed by composite images of CD45/Lamp1. Panel B shows the mask used to define the inside of the cell in Brightfield and the membrane portions of the CD45 or Lamp1 images.

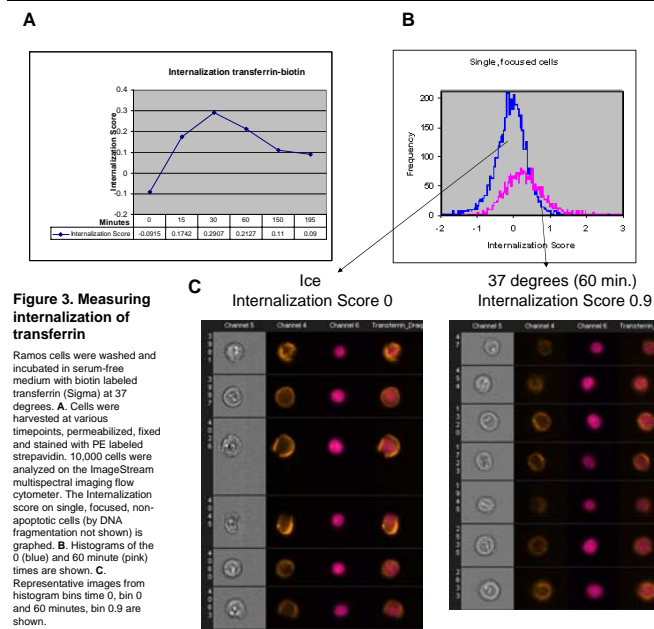


Figure 3: Measuring internalization of transferrin

Ramos cells were washed and incubated in serum-free medium with biotin labeled transferrin (Sigma) at 37 degrees. A. Cells were harvested at various timepoints, permeabilized, fixed and stained with PE labeled streptavidin. 10,000 cells were analyzed on the ImageStream multispectral imaging flow cytometer. The internalization score on single, focused, non-apoptotic cells (by DNA fragmentation not shown) is graphed. B. Histograms of the 0 (blue) and 60 minutes (pink) times are shown. C. Representative images from histogram bins time 0, bin 0 and 60 minutes, bin 0.9 are shown.

Calculation of Internalization Score

The Internalization Score (IS) is defined as the ratio of intensity inside the cell to the intensity of the entire cell. The higher the score, the greater the concentration of intensity inside the cell. For robustness and to accommodate concentrated arc segments, only the upper quartile of pixels (based on intensity) inside the cell and in the membrane are considered for computing the ratio. Also, in order to provide invariance to cell size, the mean of the upper quartile pixel intensities is used to compute the ratio. The ratio is then mapped on a log scale to increase the dynamic range to values between $[-\infty, \infty]$. The thickness of the membrane (in pixels) determines which pixels are used to define the boundary and the membrane portions of the cell. The user supplies an 'internal' mask based on the brightfield image that covers the inside of the cell, the thickness of the membrane in pixels and the fluorescent channel of interest. The cell is divided into 2 regions: External (E) and Internal (I). The user supplies the internal region as the mask. The external region is determined by: 1. Dilating the internal mask by the membrane thickness. 2. Combining 1 with the object mask of the channel of interest. 3. External region equals mask 2 and not the internal mask. Next, the mean intensity of the upper quartile of the pixels in each region is determined. The Internalization Score (IS) is then computed as follows:

$$IS = \log\left(\frac{a}{1-a}\right), \text{ where } a = \frac{m_I}{m_I + m_B} \frac{p_I}{p_B}$$

m_I = Mean intensity of upper quartile pixels in I, m_B = Mean intensity of upper quartile pixels in B, p_I = Peak intensity of upper quartile pixels in I, p_B = Peak intensity of upper quartile pixels in B

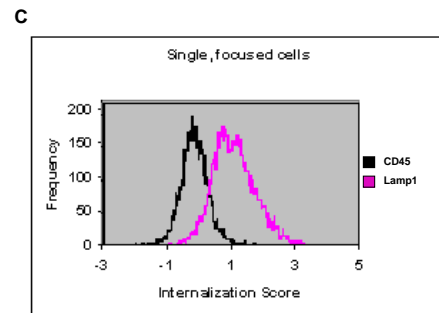


Figure 4: Demonstration of co-localization of transferrin and transferrin receptor

The similarity Bright Detail score is used to identify co-localization of two probes. Ramos cells were incubated for at 37 degrees for 20 minutes with biotin labeled transferrin, permeabilized and fixed and stained with FITC labeled anti-CD71 and PE streptavidin. A. Similarity Bright Detail of the population. B. Selected imagery of high Transferrin/CD71, low Transferrin/Lamp1 or CD71/Lamp1 cells.

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. Non-co-localized proteins may have a high baseline correlation if background antibody staining is significant. To mitigate this problem, the correlation is performed on the open residue image. 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:

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

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 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.

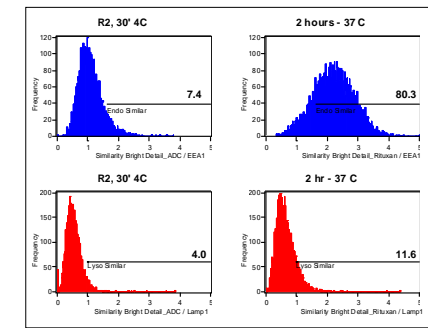


Figure 5: Quantitation of RTX trafficking to subcellular organelles using Similarity Bright Detail (SBD) analysis

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 blue) and RTx / Lamp1 (lower histograms in red) image pairs was calculated and plotted. Images of co-localized markers have high SBD values, and the regions 'Endo Similar' and 'Lys 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.

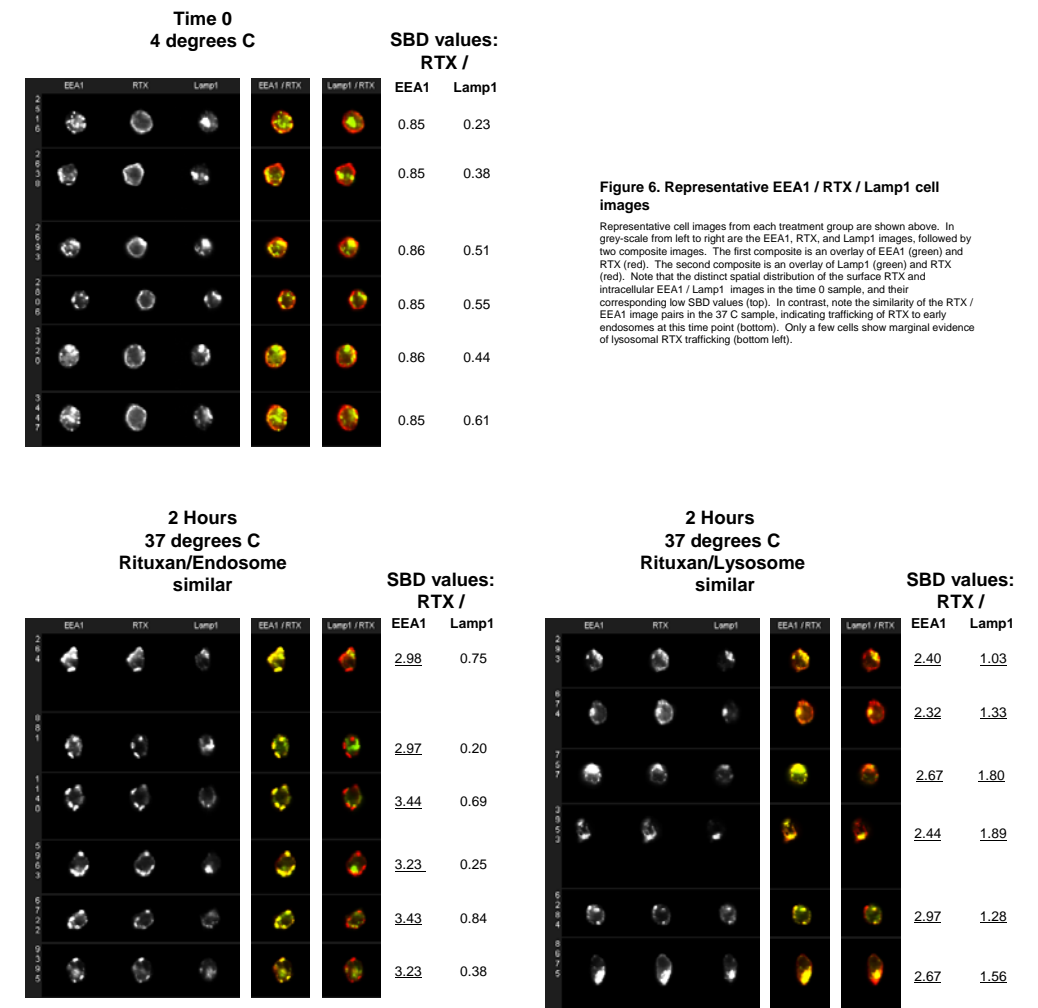


Figure 6: Representative EEA1 / RTX / Lamp1 cell images

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 internalization of proteins and co-localization of proteins of interest to the endosomal and lysosomal cellular compartments. A quantitative method for measuring internalization is described that allows one to extend that analysis to specific localization to intracellular organelles. These data demonstrate the ability of the ImageStream to acquire imagery of cells in numbers required to achieve statistical significance between groups or identify subpopulations with relative ease.

Here data are presented that describes the methodology used to identify the internal and external parts of the cell and the algorithms used for the Internalization Score. A time course of internalization of transferrin is shown. Finally, data 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 internalization and 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.