Quantitation of Nuclear Translocation Events Using ImageStream® Multispectral Imaging Cytometry

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Abstract

Signal transduction pathways regulate most cellular biological processes and have a critical influence on cellular responses to external stimuli. Typically, cell surface receptors trigger a cascade of intracellular signaling events that alter cellular metabolism or gene expression after binding to a specific extracellular mediator, and such changes contribute to the cellular response. The intracellular signaling cascade often involves the translocation of transcription factors or second messengers from the cytoplasm to the nucleus. Historically, nuclear translocation events have been studied microscopically by observing the sub-cellular localization of fluorescent probe-labeled signaling molecules.

Amnis Corporation has developed the ImageStream 100 multispectral imaging flow cytometer. The platform produces high resolution brightfield, darkfield, and fluorescence images with the simplified sample handling and quantitative power of flow cytometry. The IDEAS™ analysis software quantifies over 200 morphometric and photometric parameters for each cell based on its imagery, including parameters that quantify sub-cellular location of probes. Using pixel correlation analysis of images taken on an ImageStream beta prototype, we quantified the nuclear translocation of NFκB in two model systems: TNF-α / IL-1β mediated translocation in an adherent carcinoma line and lipopolysaccharide (LPS) mediated translocation in the non-adherent monocytic cell line THP-1. These data demonstrate the powerful utility of the ImageStream technology in experimental systems that require morphological analysis.

Development of ImageStream technology was partially supported by NIH grants 9 R44 CA01798-02, 1 R43 GM58956-01 and 1R 43CA94590-01.

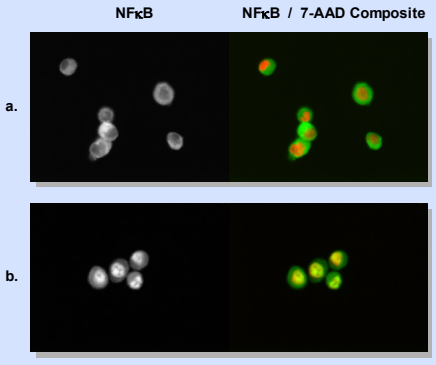


Figure 1: Visualization of NFκB Nuclear Translocation in A549 Cells Using Immunofluorescence Microscopy

TNF-α and IL-1β stimulation initiates a signaling cascade that results in the translocation of NFκB from the cytoplasm to the nucleus of the adherent human carcinoma cell line A549 cells. Untreated A549 cells (a) and A549 cells treated with TNF-α (2 ng/ml) and IL-1β (10 pg/ml) for 1 hour (b) were trypsinized and probed for NFκB expression and nuclear morphology. Briefly, the cells were fixed in 4% paraformaldehyde, permeabilized with 0.1% triton, and incubated with mouse anti-NFκB (p65) + Alexa Fluor® 488 donkey anti-mouse IgG. Cells were washed and resuspended in 1% paraformaldehyde containing 7-AAD, then mixed with an equal volume of antifade and visualized on slides using a Nikon Eclipse E600 fluorescence microscope equipped with bandpass filters appropriate for FITC (535/40 nm) and 7-AAD (630/60 nm) fluorescence. NFκB images in grey are depicted on the left. NFκB (green) / 7-AAD (red) composite images on the right demonstrate the nuclear localization of NFκB following TNF-α / IL-1β treatment.

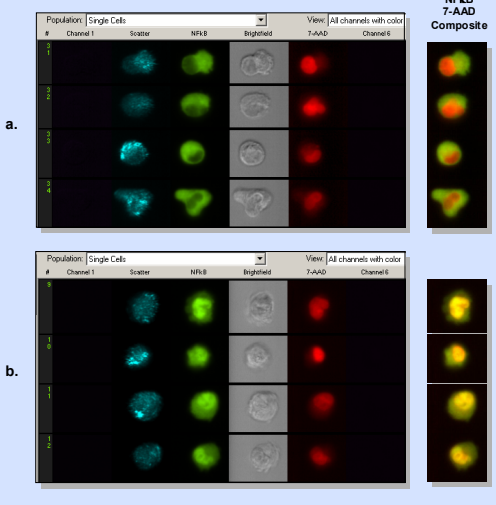
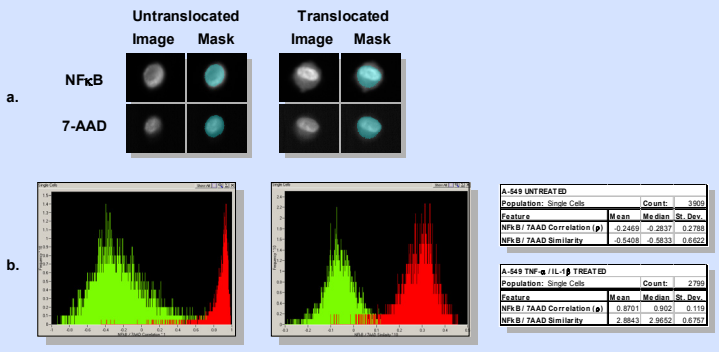


Figure 2: Visualization of NFκB Nuclear Translocation in A549 Cells Using ImageStream Multispectral Imagery

Untreated (a) and TNF-α / IL-1β treated (b) A549 cells were prepared as described in Figure 1 and imaged in flow using the ImageStream 100 instrument. Hydrodynamically focused cells were illuminated from the side with a 488 nm laser and from behind with a brightfield source that was passed through a 577/10 bandpass filter. Four out of a possible six images were used in this experiment. Laser side scatter (pseudocolored blue), NFκB fluorescence (green), brightfield, and 7-AAD fluorescence (red) from the cells were spectrally decomposed and imaged simultaneously onto spatially separated channels on a CCD detector. NFκB (green) / 7-AAD (red) composite images on the right demonstrate the nuclear localization of NFκB following TNF-α / IL-1β treatment.



A-549 UNTREATED

Population: Single Cells			Count:	3909
Feature	Mean	Median	St. Dev.	
NFκB / 7AAD Correlation (ρ)	-0.2469	-0.2837	0.2788	
NFκB / 7AAD Similarity	-0.5408	-0.5833	0.6622	

A-549 TNF-α / IL-1β TREATED

Population: Single Cells			Count:	2799
Feature	Mean	Median	St. Dev.	
NFκB / 7AAD Correlation (ρ)	0.8701	0.902	0.119	
NFκB / 7AAD Similarity	2.8843	2.9652	0.6757	

Figure 3: Quantitation of NFκB Nuclear Translocation Using Image Correlation Analysis

In order to quantitate the extent of NFκB nuclear localization, we analyzed the degree of pixel intensity correlation between the NFκB and nuclear images of each cell within a masked region of interest. The NFκB image of a cell with a high degree of translocation will look qualitatively similar to the nuclear image of that cell, resulting in a high degree of correlation between the two images while the NFκB image of cell without any translocation will have little signal in the nuclear space, resulting in an inverse correlation with the nuclear image. (A) shows the masked areas (turquoise overlay) used for the correlation analysis on an untranslocated and a translocated cell. Two features, the correlation coefficient (ρ) and a logarithmic transformation of ρ (similarity) were calculated, and are represented by the following formulas:

$$\rho = \frac{Cov(X,Y)}{\sigma_X \sigma_Y}$$

$$\text{Similarity} = \ln\left(\frac{1+\rho}{1-\rho}\right)$$

ρ measures the degree to which the spatial distribution of intensities over two separate images is correlated, with a range from –1 (inverse correlation) to +1 (complete correlation). The similarity value ranges from $-\infty$ to $+\infty$, allowing standard statistical comparisons (means and standard deviations) between groups to be made. Histogram overlays of NFκB / 7-AAD correlation (b) and similarity (c) distinguish untreated (green) from TNF-α / IL-1β treated (red) A549 cells.

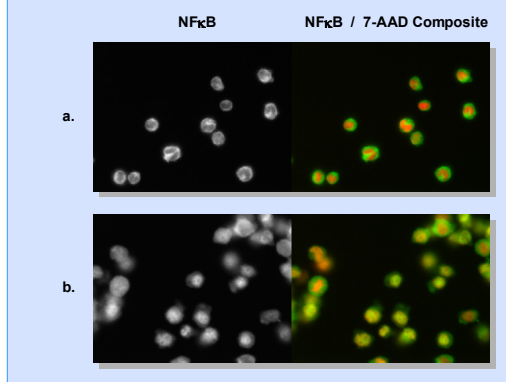
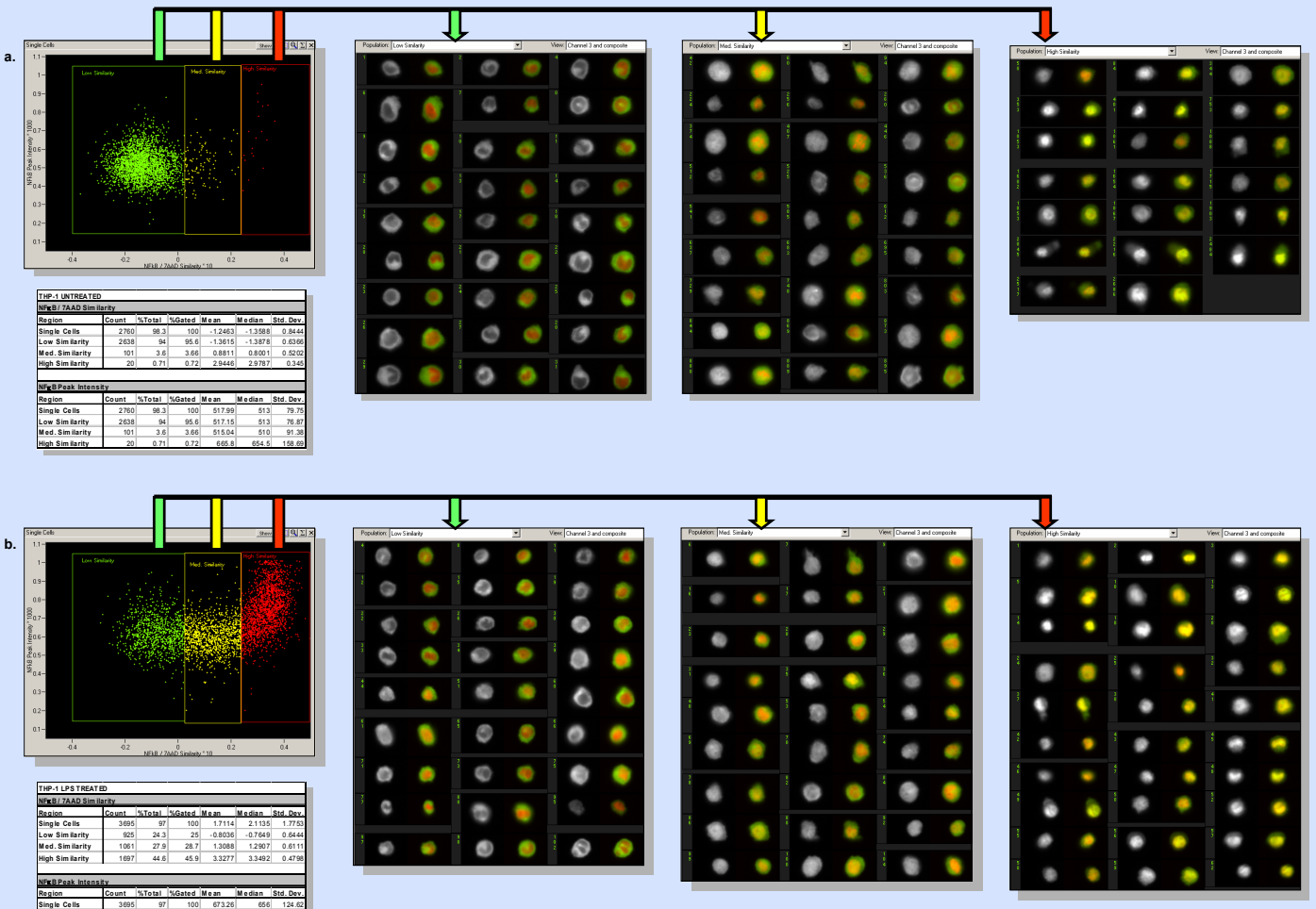


Figure 4: Visualization of NFκB Nuclear Translocation in THP-1 Cells Using Immunofluorescence Microscopy

LPS stimulation initiates a signaling cascade that results in the translocation of NFκB from the cytoplasm to the nucleus of the non-adherent human monocyte cell line THP-1. Untreated THP-1 cells (a) and THP-1 cells treated with LPS (100 ng/ml) for 1 hour (b) were probed for NFκB expression and nuclear morphology. Briefly, the cells were fixed in 4% paraformaldehyde, permeabilized with 0.1% triton, and incubated with mouse anti-NFκB (p65) + Alexa Fluor® 488 donkey anti-mouse IgG. Cells were washed and resuspended in 1% paraformaldehyde containing 7-AAD, then mixed with an equal volume of antifade and visualized on slides using a Nikon Eclipse E600 fluorescence microscope equipped with bandpass filters appropriate for FITC (535/40 nm) and 7-AAD (630/60 nm) fluorescence. Note the relatively thin band of cytoplasm in the untranslocated NFκB images characteristic of this monocytic cell line.



THP-1 UNTREATED

NFκB / 7AAD Similarity

Region	Count	%Total	%Gated	Mean	Median	Std. Dev.
Single Cells	2760	98.3	100	-1.2463	-1.3588	0.8444
Low Similarity	2638	94	95.6	-1.3615	-1.3878	0.6366
Med. Similarity	101	3.6	3.66	0.8811	0.8001	0.5202
High Similarity	20	0.71	0.72	2.9446	2.9787	0.345

NFκB Peak Intensity

Region	Count	%Total	%Gated	Mean	Median	Std. Dev.
Single Cells	2760	98.3	100	517.99	513	79.75
Low Similarity	2638	94	95.6	517.15	513	76.87
Med. Similarity	101	3.6	3.66	515.04	510	91.38
High Similarity	20	0.71	0.72	665.8	654.5	158.69

THP-1 LPS TREATED

NFκB / 7AAD Similarity

Region	Count	%Total	%Gated	Mean	Median	Std. Dev.
Single Cells	3695	97	100	1.7114	2.1135	1.7753
Low Similarity	925	24.3	25	-0.8036	-0.7649	0.6444
Med. Similarity	1061	27.9	28.7	1.3088	1.2907	0.6111
High Similarity	1697	44.6	45.9	3.3277	3.3492	0.4798

NFκB Peak Intensity

Region	Count	%Total	%Gated	Mean	Median	Std. Dev.
Single Cells	3695	97	100	673.26	656	124.62
Low Similarity	925	24.3	25	620.55	617	90.66
Med. Similarity	1061	27.9	28.7	600.23	599	85.66
High Similarity	1697	44.6	45.9	748.04	745	119.38

Figure 5: Visualization and Quantitation NFκB Nuclear Translocation in THP-1 Cells Using ImageStream Multispectral Imagery

Untreated (a) and LPS treated (b) THP-1 cells were prepared as described in Figure 4 and imaged in flow using the ImageStream 100 platform. Plotting NFκB / 7-AAD Similarity vs. NFκB Peak Intensity revealed a range of cells based on similarity that were gated into three populations. Each gated population has an associated image gallery that can be visually inspected. The image galleries here display the NFκB image adjacent to a composite image (green NFκB / red 7-AAD) for each object within a given population. Morphologic inspection of the image galleries revealed that NFκB had translocated to the nucleus in 'High Similarity' but not 'Low Similarity' cells. 'Medium Similarity' cells had a diffuse NFκB staining pattern. Untranslocated and translocated cells had significantly different Similarity values (untreated: -1.36 +/- 0.84 and 2.98 +/- 0.35, respectively; LPS treated: -0.76 +/- 0.64 and 3.35 +/- 0.48, respectively). Treatment with LPS significantly increased the percentage of NFkB-translocated (0.71%, median similarity value 2.98 +/- 0.35 to 44.6%, median similarity value 3.35 +/- 0.48) and Medium Similarity (3.6% to 27.6%) cells.

Conclusion

This study demonstrated the ability of the ImageStream 100 multispectral imaging cytometer to quantitate the extent of NFκB nuclear translocation in adherent (A549 in response to TNF-α and IL-1β) and non-adherent (THP-1 in response to LPS) cell lines. Images taken using immunofluorescence microscopy (40x objective) and with the Imagestream were qualitatively indistinguishable. The extent of NFκB nuclear localization was quantified using the feature 'NFκB / 7-AAD Similarity', a logarithmic transformation of the pixel correlation coefficient (ρ) computed between the NFκB image and a 7-AAD nuclear reference image. By providing population-specific statistical analysis, both the percentage of cells that responded to treatment as well as the extent of response were examined. NFκB translocated to the nucleus in nearly all of the A549 cells that were treated with TNF-α and IL-1β. On the other hand, NFκB translocated to the nucleus in a subpopulation of THP-1 cells after 1 hour of LPS treatment. Furthermore, the fidelity of these classifiers could be validated by inspection of image galleries corresponding to the gated populations. By providing morphology-based information in addition to fluorescence and laser scatter signals, the ImageStream platform allows for discrimination of cells not feasible with standard flow cytometry.

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