Immunology

Measurement of nuclear translocation in primary T cells using correlation analysis of images obtained on the ImageStream®

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Abstract

Molecular translocation of transcription factors from the cytoplasm to the nucleus is a pivotal event in many processes critical to cellular activation, differentiation, and host defense. Existing methods used to measure nuclear translocation have significant limitations. Classical biochemical techniques are semi-quantitative in nature, and do not provide per-cell translocation measurements. Per-cell scoring can be achieved using direct visual observation with a fluorescence microscope, but the variation in extent of nuclear translocation presents a significant challenge to objective image-based measurement, and because manual image acquisition and quantitative image analysis are time consuming processes, such analyses of nuclear translocation have remained largely qualitative. Furthermore, image-based quantitation of the nuclear localization of probes has historically relied on the use of adherent cells with large cytoplasm to nuclear ratios; most primary immune system cells, on the other hand, have very small cytoplamic compartments, and are more amenable to flow-based instrumentation.

We have recently shown that nuclear translocation events within non-adherent tumor cells can be quantified by correlating transcription factor and nuclear images collected in flow using the ImageStream imaging flow cytometer. This instrument automatically acquires up to six different spatially registered images (brightfield, darkfield, and four fluorescent) per cell at rates on the order of 1000's of objects per minute using a digital CCD camera. The digital imagery obtained is analyzed using the IDEAS statistical image analysis program which provides tools for the objective numerical scoring and discrimination of cells based on the characteristics of their imagery. The ability to numerically score large numbers of automatically acquired images is ideally suited to the analysis of nuclear translocation within primary immune system cells.

Here we extend the quantitative technique to the study of nuclear localization within primary human or murine T cells in the following systems: 1) nuclear localization of T-bet in murine CD4+ cells; 2) antigen-specific translocation of NF-kB in CD8+ thymocytes involved in conjugates with APC presenting cognate peptide, and 3) IL-12 or IFN-α-driven phosphorylation and nuclear localization of STAT4 in human peripheral blood CD4+ cells following cytokine stimulation. These data demonstrate image-based measurement of nuclear translocation within primary cells with large nuclear to cytoplasmic area ratios using ImageStream technology.

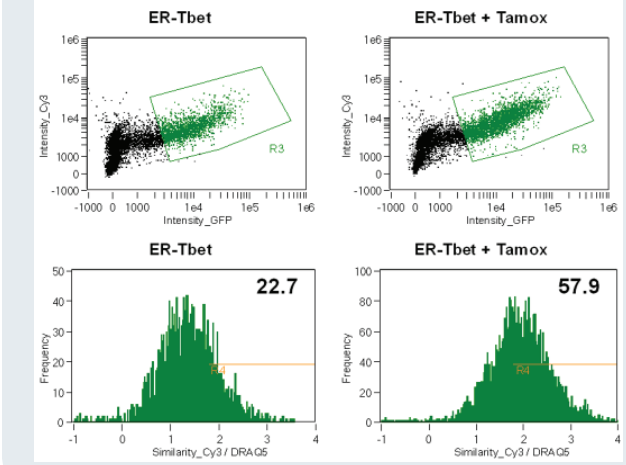


Figure 1.

Nuclear Localization of T-bet

Purified CD4 cells transduced with empty GFP vector, T-bet-RV, or T-bet-ER-RV were cultured with and without tamoxifen, then stained for T-bet expression and with DRAQ5 to visualize nuclei. In-focus single cells were identified (data not shown). The upper panels in Figure 1 show examples of gating GFP+ transduced cells. The lower panels show the method for determining nuclear localization of T-bet, which employs a calculation of the similarity in distribution of staining between DRAQ5 (nucleus) and T-bet.

Figure 2.

Confirmation of the Translocation Gating Strategy

As described in Figure 1, cells in which T-bet had translocated to the nuclues in response to tamoxifen treatement were gated using a metric unique to the ImageStream system. The Similarity Score measures the degree of similarity between the distributions of two stains in the cell image. The greater the similiarity in distribution of the labels, the higher the Similarity Score.

The upper panel of cell images in this figure shows four cells in which translocation has not occurred significantly. Column 1 shows the distribution of Cy3-T-bet; the second column shows the distribution of DRAQ5 and the third column shows a composite of the two images. Distributions of the Cy3 label (green) and the DRAQ5 lavel (red) are clearly distinct, indicating that T-bet is located in the cytoplasm of these cells and yielding a low overall Similarity Score (See Figure 1). In contrast, in the lower panel the distributions of the Cy3 T-bet label and the DRAQ5 label are very similar, indicating that most or all of the T-bet in these cells has translocated to the nucleus. This population of cell images yields a significantly higher Similarity Score.

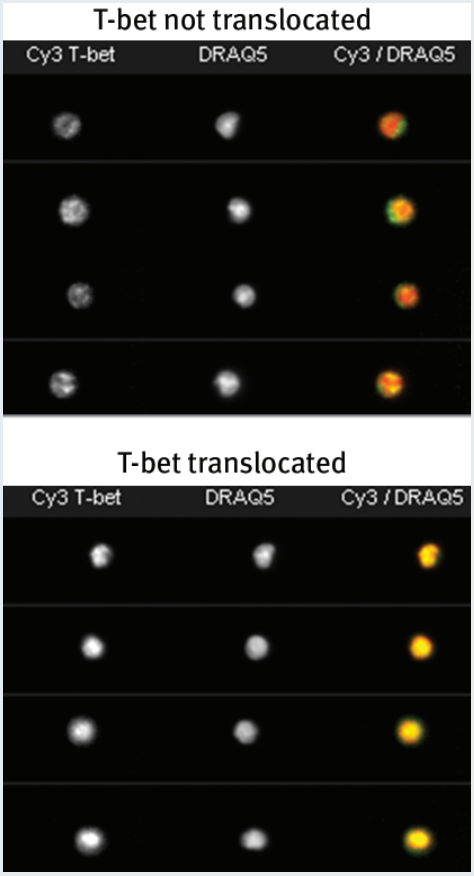


Figure 3.

Nuclear localization of phosphorylated STAT4 in human CD4+ T cells following IFN-a or IL-12 stimulation

Cytokine stimulation of CD4+ T cells can activate signaling pathways that result in phosphorylation of STAT4 and subsequent translocation to the nucleus. Here, human PBMC were treated with IL-12 or IFN-α, followed by staining for pSTAT4, CD4, CD45RA and DRAQ5. In-focus single cells were identified and from these lymphocytes (low SSC / low area cells) were then gated (data not shown). The histogram plots a quantitative metric of nuclear localization, the Similarity Score (Similarity_ pSTAT4/DRAQ5; see legend to Figure 2) for pSTAT4+ (red) and pSTAT4- (black) cells. Resentative cell images from the non-translocated population (black) and the translocated population are shown. Each panel shows several images of two cells. Column 1 shows the brightfield image, Column 2, the pSTAT4 image, Column 3, the nuclear image (DRAQ5) and Column 4, a composite of the pSTAT4 and nuclear images.

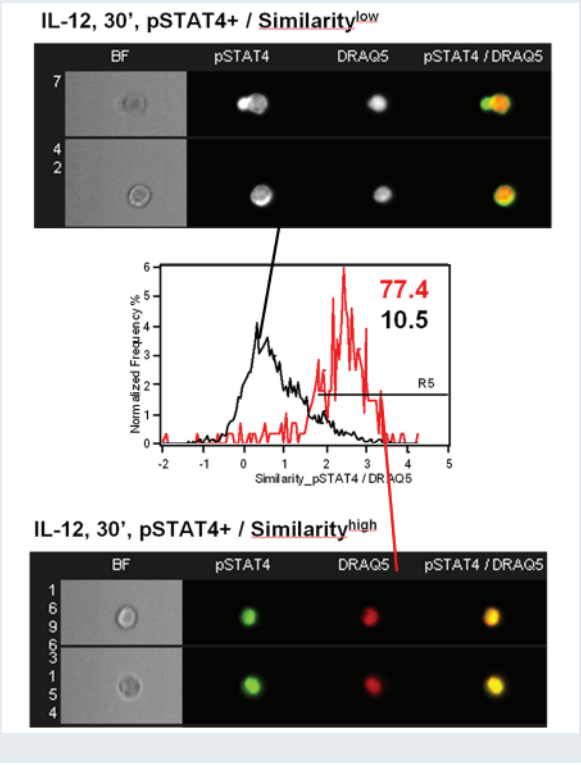


Figure 4.

Antigen-specific NF-κB translocation in T cell conjugates with APC

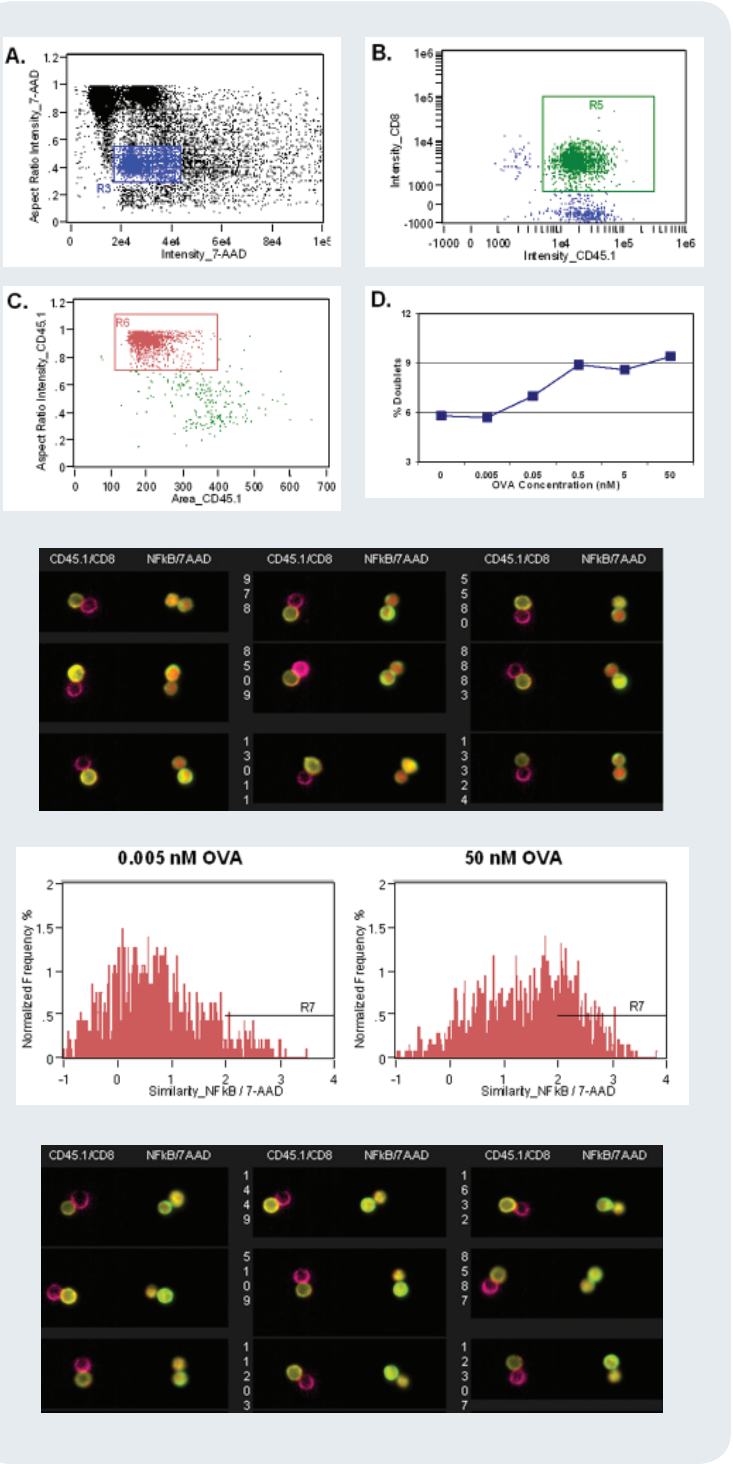
Stimulation of the TCR with cognate ligand/MHC complexes presented by APC results in activation and nuclear translocation of NF-κB. In this experiment, Tap1 -/- OT-1 thymocytes were co-cultured with OVA-pulsed B6 spleen cells to induce T:APC conjugate formation.

First, 7AAD+ cells with low to intermediate side scatter area were gated to eliminate debris and clumps from the analysis and in-focus cells with high 7AAD contrast were selected (data not shown). Doublets were distinguished from singlets by gating (R3) on events with 4N DNA content and low nuclear aspect ratios (A). Aspect ratio is width divided by height, and circular images have a values near 1; doublets have values close to 0.5. CD8+ cells conjugated to a B6-derived cell (CD45.1+) were gated (R5) in (B).

CD8:APC conjugates were distinguished from B6 doublets on the basis of their higher CD45.1 aspect ratios and lower CD45.1 areas (indicating single B6 cells) in (C), and the OVA dose-dependent increase in conjugate formation (R6 as a percentage of cells) is plotted in (D).

Here we calculated the Similarity score specifically within the T cell of the conjugate. This was done using the '7-AAD and not CD45.1' mask, which masks the non-CD45.1 T cell nucleus (E). Conjugate events whose T cells have nuclear localized NF-κB are gated (R7) on the Similarity plots, and the percentage of these events (R7 as a percentage of R6 conjugates) at different OVA doses is plotted.

Representative composite image pairs from the 5pM and 50nM samples are shown: The first composite is CD45.1 (yellow) / CD8 (pink) and the second is NF-κB (green) / 7-AAD (red).



Conclusions

This study demonstrates application of ImageStream technology to the measurement of nuclear translocation in primary human and murine T cells in a diverse range of experimental systems. 1) nuclear translocation of retrovirally induced T-bet in murine T cells: In this system, both baseline expression of T-bet in the absence of tamoxifen as well as induced expression is measurable by western blot and traditional flow cytometry; however, imaging is required to determine whether T-bet signal is localized to the nucleus, and we demonstrate here that induction is required for robust translocation. 2) Antigen-specific NF-κB translocation within T cells conjugated to APC: this study demonstrates tools that enable specific measurement of translocation within one cell of a conjugate; this ability is critical to measuring activation while antigen-specific T cells are actively engaged with APC presenting cognate ligand; 3) temporal relationship between STAT4 phosphorylation and nuclear translocation in CD4 cells responding to cytokines; this study provides a more complete understanding of transcription factor activation and compartmental localization. Interestingly, many events considered too dim for pSTAT4 fluorescence had significant nuclear localization, and CD4+CD45RA+ cells do not robustly translocate pSTAT4 in response to IL12. Thus, the ImageStream is ideally suited the measurement of nuclear translocation in a wide variety of primary immune cells and experimental systems.

ImageStream^X Specifications

Excitation Sources

Laser (nm)	Example Dyes
405	DAPI, Pacific Blue™
488	FITC, PE, ECD, PE-Cy5
560	Alexa Fluor® 546, Cy3
592	Texas Red®, Alexa Fluor® 594
658	Cy5, Alexa Fluor® 647, APC, APC-Cy-7

Imaging Performance

Magnification	20X	40X	60X
Numeric Aperature	0.5	0.75	0.9
Field of View (μm)	120 x 512	60 x 256	40 x 170
Imaging Rate (cells/sec)	2,000	1,000	600

Instrument Capabilities

Images per Cell	Up to 12
Imaging Modes	Brightfield, SSC, and fluorescent
Sample Throughput	1 sample/min nominal
Automated Processes	Startup, shutdown, and self-calibration