Luminex®

Application Note

Quantitating NF-κB Translocation Using the Amnis® ImageStream® System and Optimized Reagent Kit

Introduction

Nuclear Factor kappa B (NF-κB) is a transcription factor that plays a central role in regulating many critical processes in mammalian cells, including proliferation, inflammation, immune, and stress responses. NF-κB is held in an inactive state in the cytoplasm by its inhibitor, IκB. Upon activation, IκB is targeted for ubiquitination, allowing phosphorylation of NF-κB and facilitating its translocation into the nucleus.[1] Classical biochemical techniques are semi-quantitative in nature and do not provide per-cell translocation measurements. Manual microscopy allows visual identification of nuclear translocation on a per-cell basis, but an objective and statistically rigorous assessment is difficult to obtain.

To overcome these problems, we used the Amnis® ImageStream® Imaging Flow Cytometry System, which combines the quantitative power of flow cytometry with the spatial information provided by microscopy in one system. Here, we present three case studies demonstrating the capacity of the Amnis® NF-κB Translocation Kit (Part No. ACS10000) for monitoring NF-κB translocation in THP-1 human acute monocytic leukemia cells, peripheral blood monocytes, and HL-60 human promyelocytic leukemia cells.

Quantitation of NF-κB translocation

In these experiments, the ImageStream® Imaging Flow Cytometer, equipped with a 488 nm laser and using the MultiMag option, was used to assess the nuclear translocation of NF-κB in THP-1 human monocytic leukemia cells treated with 1 µg/mL lipopolysaccharide (LPS) (**Figures 1 and 2**), peripheral blood monocytes stimulated with 10 µg/mL LPS (**Figure 3**), and HL-60 human promyelocytic leukemia cells treated with 0-10 ng/mL tumor necrosis factor (TNFα).

The Amnis NF-κB Translocation Kit was used to fix, permeabilize, and label the cells with Alexa Fluor® 488-conjugated anti-Human NF-κB and nuclear dye 7AAD. Images from 10,000-50,000 cells were acquired for each sample at 40X or 60X magnification. Image analysis was then performed using the Nuclear Localization Wizard available in the IDEAS® Image Analysis Software package.

Figure 1. Analysis of NF-κB translocation in THP-1 cells using the Nuclear Localization Wizard

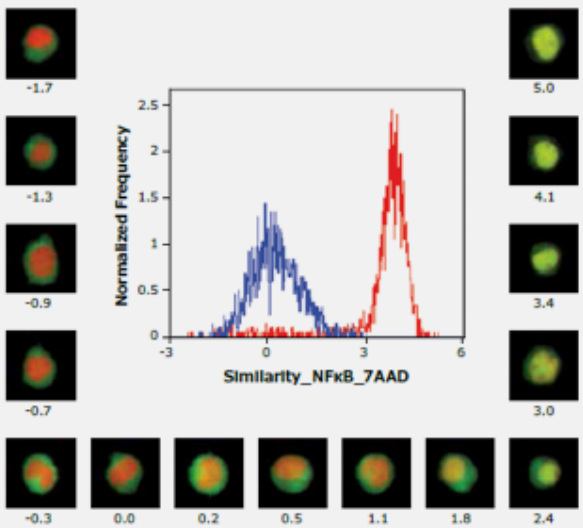


Figure 1. The Nuclear Localization Wizard assigns each cell a score from which population statistics can be generated. Histograms of control (blue) and 1 µg/mL LPS-treated (red) THP-1 cells are shown, along with 60X composite images of NF-κB (green) + 7AAD (red). The Similarity scores demonstrate non-translocated, partially translocated, and fully translocated cells.

Analysis of NF-κB translocation using the Nuclear Localization Wizard

The Nuclear Localization Wizard in the IDEAS Software creates an analysis template for measuring the nuclear localization of a probe—in this case, the NF-κB antibody. The Nuclear Translocation Wizard uses the Similarity feature, which does a pixel-by-pixel correlation between the channel containing the NF-κB image and the channel with the nuclear image. The wizard gives each cell a score that represents how similar the nuclear 7AAD image is to the Alexa Fluor® 488-conjugated anti-Human NF-κB image. A high Similarity score indicates the two images are similar, where low scores indicate that the images are dissimilar (**Figure 2**). The Similarity score has been validated to measure NF-κB translocation on the ImageStream Platform.[1]

Quantifying differences in nuclear translocation of NF-κB in THP-1 cells treated with LPS

LPS stimulation activates several intracellular signaling pathways, including the NF-κB pathway. In this study, THP-1 human acute monocytic leukemia cells were treated with 1 µg/mL LPS for 1 hour to induce NF-κB translocation.

Figure 2. Quantifying differences in nuclear translocation of NF-κB in THP-1 cells treated with LPS

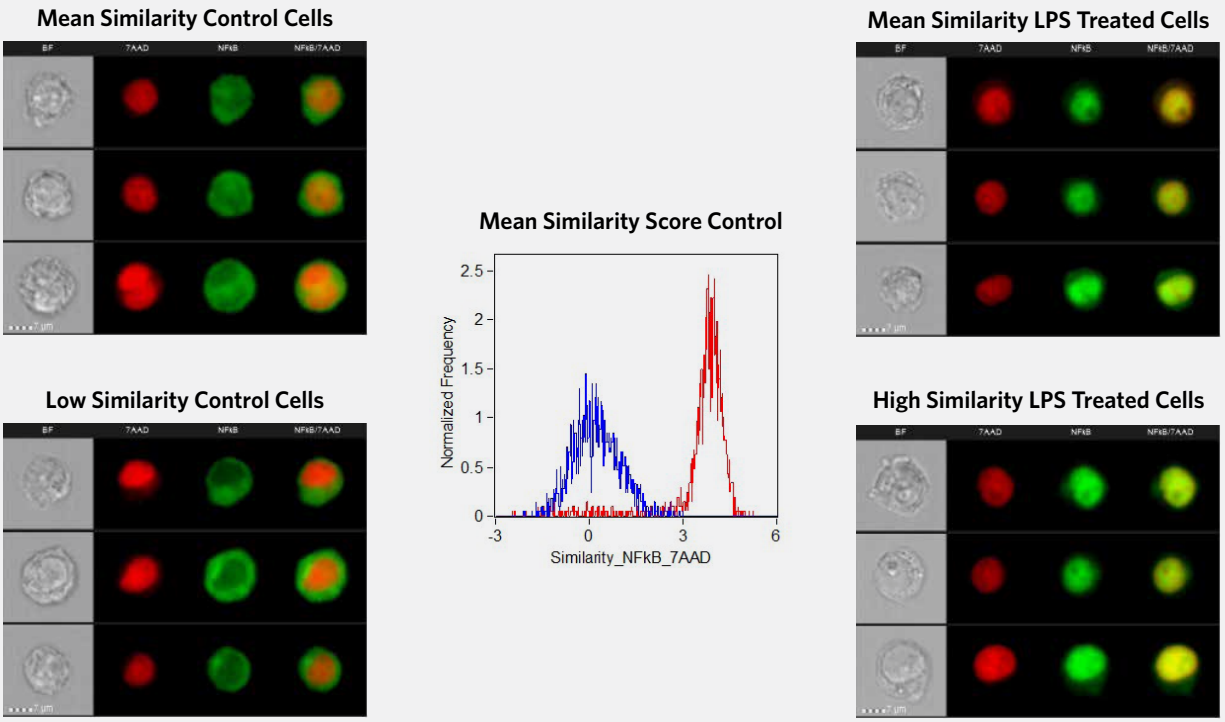


Figure 2. Histograms for control (blue) and 1 µg/mL LPS-treated (red) THP-1 cells are shown, along with example 60X images of the selected regions. The brightfield, 7AAD (red), anti-Human NF-κB Alexa Fluor® 488 (green), and composite anti-Human NF-κB Alexa Fluor® 488 + 7AAD images are shown.

NF-κB translocation and immunophenotyping

In this study, peripheral blood was stimulated with 10 µg/mL LPS for 1 hour to induce NF-κB translocation. LPS stimulation of monocytes activates several intracellular pathways, including the NF-κB pathway. Human monocytes were identified using the surface marker CD14. For an internal control, eosinophils were differentiated using CD45 expression as well as high side scatter (SSC) levels.

Histograms for the control (blue) and 10 µg/mL LPS-treated (red) cells for both the monocytes and eosinophils showed that the CD14+ monocytes had a clear shift in their mean Similarity score with respect to LPS treatment, whereas the LPS-treated eosinophils did not respond to LPS and did not have a significant shift in their mean Similarity score (**Figure 3**).

Figure 3. NF-κB translocation in LPS-responsive monocytes and non-responsive eosinophils

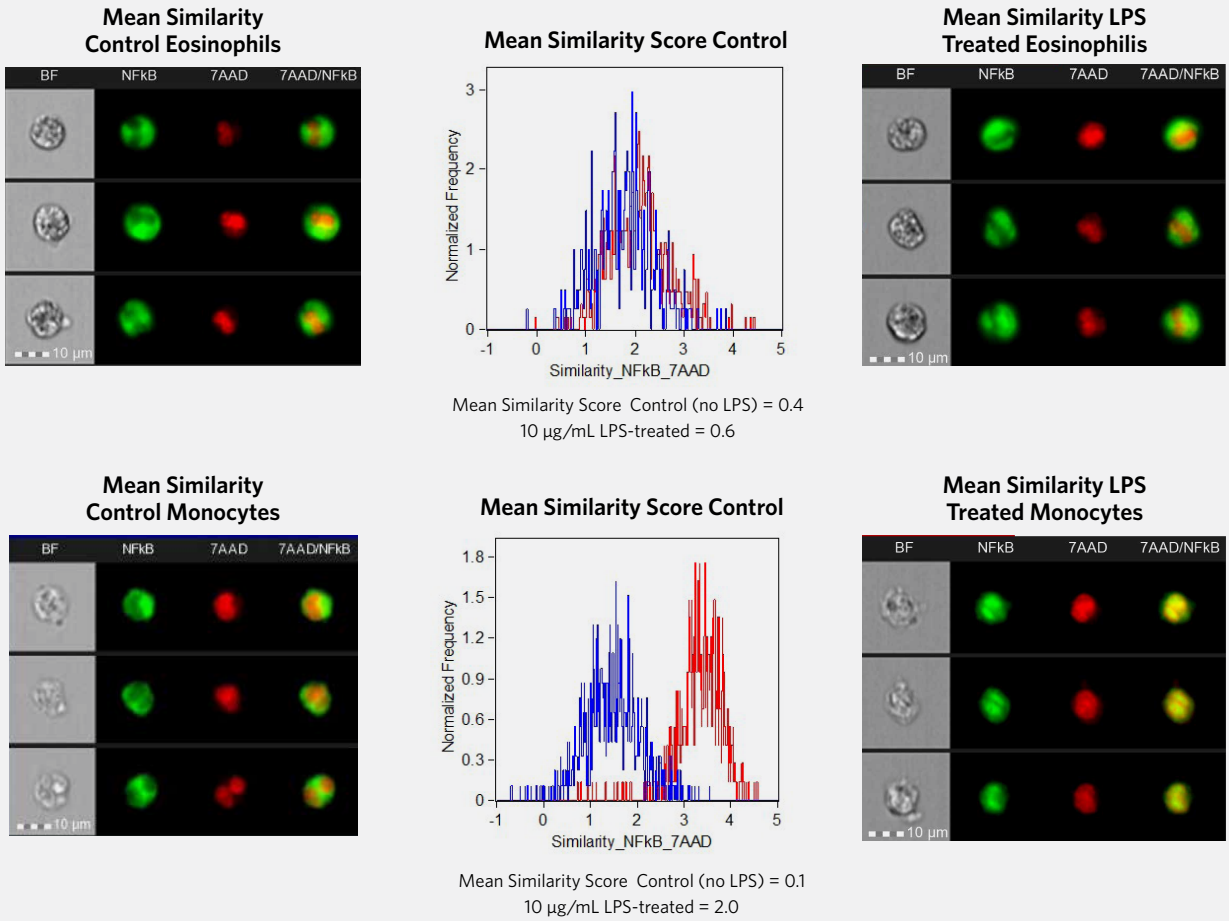
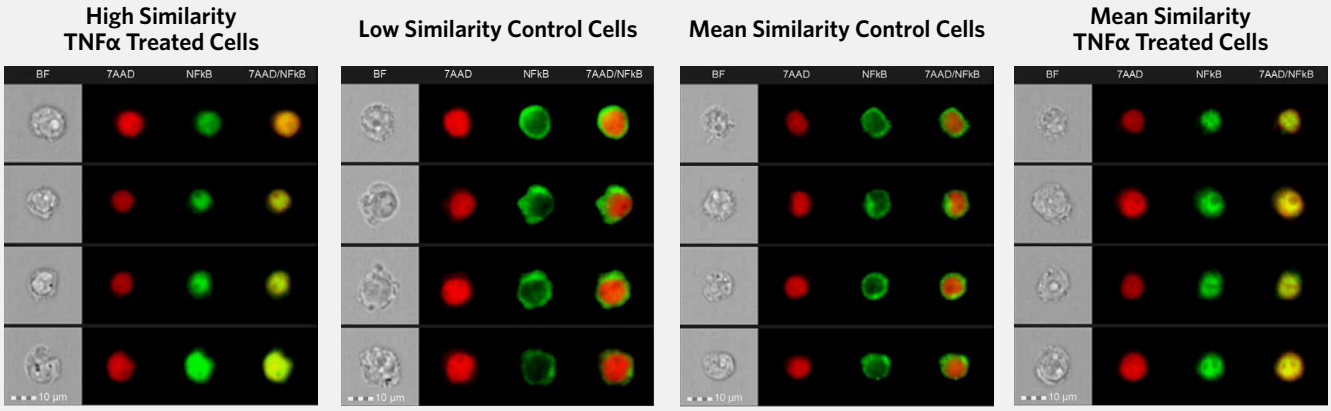


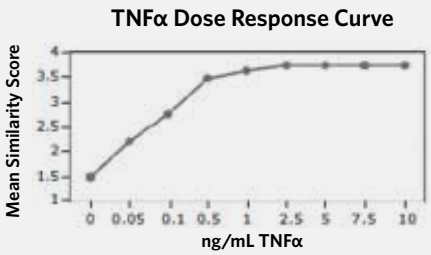
Figure 3. Histograms for control (blue) and 10 µg/mL LPS-treated (red) cells for both the monocytes and eosinophils are shown. Example 60X images of the brightfield, 7AAD (red), anti-Human NF-κB Alexa Fluor® 488 (green), and composite anti-Human NF-κB Alexa Fluor® 488 + 7AAD are shown of cells with mean Similarity scores.

Dose-dependent TNFα induced NF-κB translocation in HL-60 cells

HL-60 cells were incubated with 0-10 ng/mL TNFα for 30 minutes to stimulate NF-κB translocation. Histograms of the 0 ng/mL TNFα and 10 ng/mL TNFα treated cells show a clear shift in mean Similarity score (**Figure 4**). As the dose of TNFα increases, so does the mean Similarity score, until it reaches a maximum mean Similarity score at a dose of 2.5 ng/mL TNFα, as shown in the TNFα dose-response curve (**Figure 4**).

Figure 4. Dose-dependent TNFα-induced NF-κB translocation in HL-60 cells





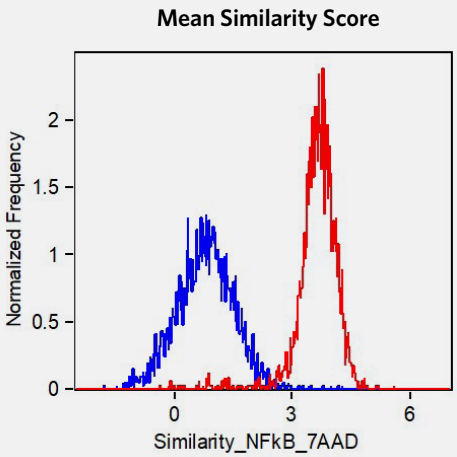


Figure 4. Histograms for the 0 ng/mL (blue) and the 10 ng/mL TNFα-treated (red) HL-60 cells are shown, along with example 40X images of cells with mean Similarity scores. The brightfield, 7AAD (red), anti-Human NF-κB Alexa Fluor® 488 (green), and composite anti-Human NF-κB Alexa Fluor® 488 + 7AAD images are shown of cells with mean Similarity scores. The TNFα dose-response curve shows that as TNFα increases, so does the mean Similarity score.

Conclusions

The immune response involves numerous cell interactions that can be regulated by translocation of transcription factors and subsequent gene expression. Full understanding of immune signaling requires the quantitative analysis of large numbers of cells. The Amnis ImageStream Imaging Flow Cytometer is uniquely suited to measure NF-κB nuclear translocation and other translocating proteins, since it combines the quantitative power of large sample sizes (common to flow cytometry) with the information content of microscopy.

These studies also demonstrate the capacity of the Amnis NF-κB Translocation Kit using the ImageStream^X Imaging Flow Cytometry System to measure nuclear translocation of NF-κB in an objective and statistically robust manner. As a result, it is possible to measure nuclear translocation on a per-cell basis efficiently. Given the ease of detecting several markers simultaneously, this platform opens the door to multiplexing and evaluating subpopulations of complex biological samples.

REFERENCES:

1. Maguire O, Collins C, O'Loughlin K, et al. Quantifying nuclear p65 as a parameter for NF-κB activation: Correlation between ImageStream cytometry, microscopy, and Western blot. Cytometry A. 2011 Jun;79(6):461-9.

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