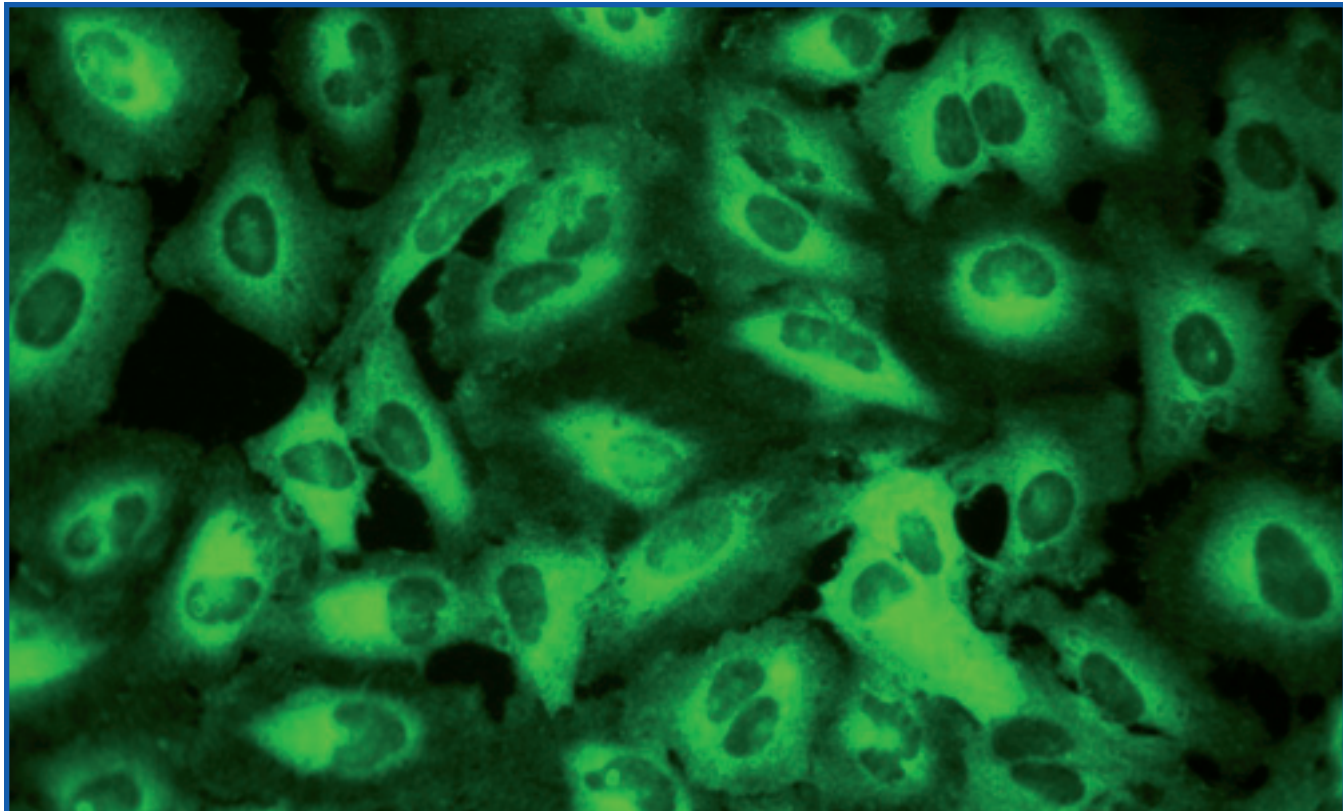


BD Biosciences

Translocation of NF- κ B in HeLa Cells in Response to TNF α



Introduction

The transcription factor NF- κ B has been shown to translocate from the cytoplasm into the nucleus upon activation by, for example, cytokines, in a variety of cell lines (Baldwin, 1996). The translocation event itself is upstream of the DNA binding and transcription initialization step, and the movement of the receptor therefore forms an attractive target for both basic biomedical and drug research. Numerous factors have been identified that modulate the translocation rate, such as [Ca²⁺] oscillations (Dolmetsch *et al.*, 1998, Hu *et al.* 1999) and BCL-3 (Verma *et al.* 1995). High-resolution fluorescence microscopy has offered the ability to study the process in unprecedented cellular detail.

BD Bioscience has developed the BD Pathway™ Bioimager, a sophisticated automated cellular imaging platform for advanced academic research and assay development. The BD Pathway Bioimager uses lamp excitation and can take advantage of the entire visible, near-UV, and near-infrared spectrum for excitation of multiple probes. The system's proprietary confocal disk can be switched in and out of the light path by the user in order to accommodate virtually all imaging needs.

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Methods

Cell Culture

Cells (HeLa, American Type Culture Collection, CCL-2) were grown and maintained in Minimum Essential Medium, Eagle's, Dulbecco's Modification (DMEM, Biofluids, P104G) containing 10% Fetal Bovine Serum (Gibco, 26140-079), 1% Penicillin / Streptomycin (Biofluids, 303), 1% Non-Essential Amino Acids solution (Biofluids, P332). For experimentation, 5000 cells / well were seeded into BD Matrigel™-coated 96 well plates and grown overnight at 37°C, 5% CO₂/95% Air.

Fixation and Staining

After drug treatment, cells were removed from the incubator and fixed using 3.7 % pre-warmed para-formaldehyde in Phosphate-buffered Saline Solution (PBS). Following a ten minute incubation, the cells were washed with PBS and permeabilized with 0.1% Triton X100 for 15 minutes. After discarding the permeabilization buffer, cells were washed twice with PBS and incubated with the primary antibody against NF-κB (Santa Cruz Biotechnology, SC-372, 1:250) for one hour. Removal of the primary antibody was followed by addition of detergent buffer (0.01% Tween 20) and two PBS wash-cycles. The labeled, secondary antibody (AlexaFluor 488, Molecular Probes A11008, 1:400) was added together with Hoechst 33342 (Molecular Probes, H-3570 at 1µg/mL) and the plates were incubated light-protected for one hour. Secondary staining solution was discarded; the cells rinsed in detergent buffer and then washed twice with PBS to remove fluorescent aggregates.

The plates were sealed and stored light-protected at 4°C until imaging.

Concentration Response Curve to TNFα

Cells were incubated with TNFα (Calbiochem, 654205, 10pg/mL – 30ng/mL) for 30 minutes in DMEM. The treatment plate layout allowed for triplicate wells with vehicle (H₂O) controls.

Z determination to Estimate HTS Performance

To assess whether the assay is robust enough for automated cellular screening, the widely quoted Z' (Zhang *et al.* 1999) was used. Cells were seeded and treated with TNF (30ng/mL) for 40 minutes and fixed as described above. The treatment followed the standard protocol of using n = 47 wells for each control and drug. The Z' was calculated using the equation:

$$Z'=1-\frac{(3*SD_{sample} + 3*SD_{control})}{(|Mean_{sample} - Mean_{control}|)}$$

Scanning and Image Analysis

The cells were imaged on a BD Pathway™ 855 Bioimager system in a non-confocal mode using BP360/10nm excitation, 435Lp emission filters for Hoechst 33342, BP488/10nm excitation, and 515LP emission filters for AlexaFluor488. For these experiments, a 20X U-Apo 340 Objective (Olympus, NA 0.75) was used. Images were binned 2x2 and montaged at 2x2. Real-time analysis greatly accelerated the process from reading the sample to reviewing the data using sophisticated pre-defined analysis protocols (macros). Data was analyzed in the BD Image Data Explorer (a proprietary Add-in for Microsoft™ Excel™), which allows the generation of concentration-response curves, EC₅₀ and single-cell analysis using advanced gating and thresholding techniques.

The degree of NF-κB translocation from the cytoplasm to the nucleus is presented in two ways:

- On a cell-by-cell basis, the cytoplasmic fluorescence intensity is subtracted from the intensity found in the nucleus (nuc – cyt). Traditionally, this method generates a greater assay window but is prone to higher variability since the fluorescence intensity of individual cells often varies.

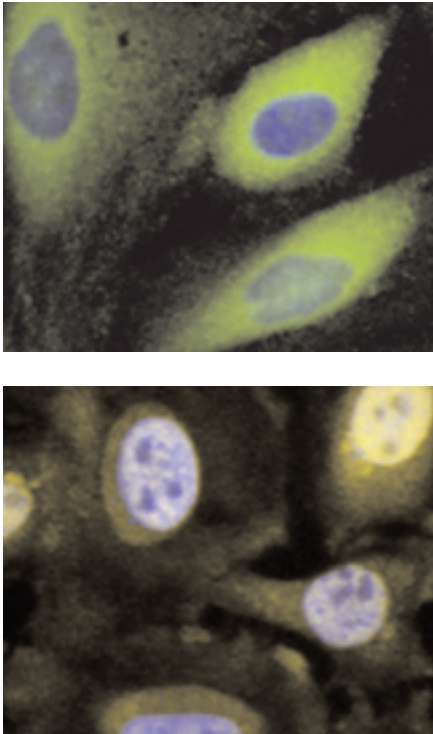


Figure 1 NF-κB Translocation in Response to TNFα in HeLa Cells (20x objective)

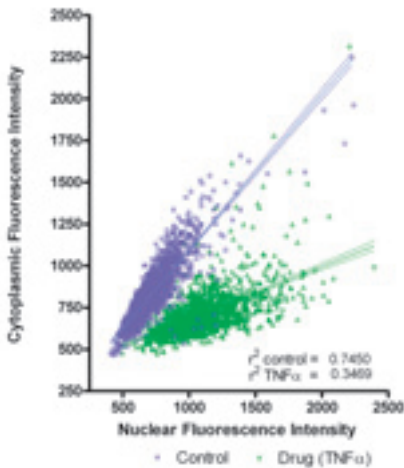


Figure 2 Scattergram of Cytoplasmic vs Nuclear Intensity in Cells Treated with TNFα

b) On a cell-by-cell basis, the nuclear fluorescence intensity is divided by the cytoplasmic fluorescence intensity (nuc/cyt). This method generates a smaller assay window but, due to a tighter distribution of errors (see Figure 4), the resulting Z' is generally higher in cellular assays.

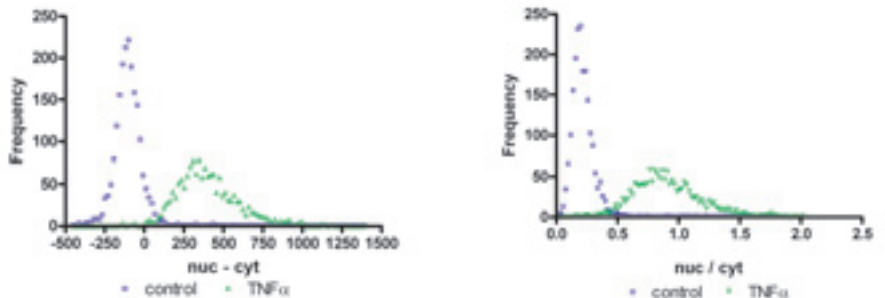
Results

The cellular phenotype of NF-κB translocation is shown in Figure 1. The NF-κB stain is shown in green and is overlaid with a blue image of the DNA-intercalating dye Hoechst 33342. The upper panel shows the untreated cells with their typical cytoplasmic distribution of NF-κB. The lower panel shows cells that were exposed to TNFα at 30ng/mL for 30 minutes. The difference in the cytoplasmic and nuclear staining intensity is evident. The figure presents only a small portion of the entire image in order to illustrate the cellular response at high resolution. Notice the fine detail of the cytoplasmic NF-κB staining (upper panel) suggesting a heterogeneous distribution of the transcription factor at the basal level.

Distribution of Data

Using the data generated by the Z' experiment, the cytoplasmic NF-κB fluorescence intensity is plotted against the nuclear NF-κB fluorescence intensity on a cell-by-cell basis. The control group contained 1950 cells, the NF-κB treated group slightly fewer cells (1545).

The scattergram (Figure 2) illustrates that although the two cell populations (control



and TNFα-treated) are well separated, a significant number of outliers is evident in either group. This is typical of a heterogeneous non-synchronized cell population. The proprietary Excel Add-In, BD Image Data Explorer, can be used to constrain the data allowing better separations between the groups. However, to test the naive performance of the assay, for all subsequent analysis steps, the data were left unchanged.

Figure 3 Frequency Histogram of Nucleus – Cytoplasmic Fluorescence Intensity
 Good statistical separation between control and TNFα-treated cells was achieved. The kurtosis values for each histogram reveal that the nuc/cyt calculation generates a better separation of the two groups (control – TNFα treated) by increasing the “peakedness” of the control values. Curiously, the kurtosis values of the nuc/cyt histogram of the drug treated group are slightly lower than in the nuc-cyt calculation.

Table 1 Kurtosis Values for NF-κB Treated Cells

	nuc - cyt	nuc / cyt
Control	8.15	22.8
TNFα	1.53	0.77

Concentration Response Curve

The concentration-dependent effect of TNFα on NF-κB translocation is shown in Figure 4

The generated EC₅₀ values of 0.25ng/mL and 0.31ng/mL (for nuc-cyt and nuc/nuc respectively) are slightly lower than those in published reports (Ding *et al* 1998) possibly indicating that under the present experimental conditions a higher sensitivity can be achieved. The data shown are the mean ± S.D. and the tight errors exemplify the quality of the image analysis routines. In fact, if Z' is calculated from the three control wells versus the three highest concentrations, a value of Z' = 0.61 (nuc – cyt) and Z' = 0.72 (nuc/cyt) is generated (see inset).

Determination.

For traditional Z' determination, a 96 well plate is divided into two treatment sectors (control and maximal concentration of a known agonist). This experimental layout assumes that by increasing “n” (the number of wells as the most basic statistical unit), the Z' will increase. This approach is justified if well/well variations are great due to sample treatment, autofocus unreliability or other image or hardware-related limitations.

The following data (Figure 5) were acquired using 47 wells/treatment condition. The Z's generated on the BD Pathway Bioimager were 0.64 (nuc–cyt) and 0.67 for nuc/cyt. Both values are considered highly significant and are fully within the constraints of an HTS assay. All images proved to be perfectly focused and devoid of image artifacts.

The high Z' values obtained in dedicated experiments as well as during routine concentration-response curves indicate that the imaging platform itself introduces very little variability due to its superior autofocus and objective-positioning system. This precision eliminates the need (at least for this assay) for a full-scale Z' determination, thus significantly saving time and expense.

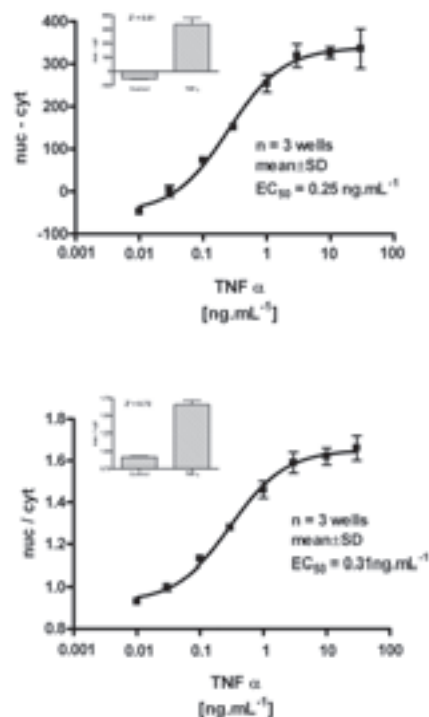


Figure 4 Concentration-response Curves for TNF

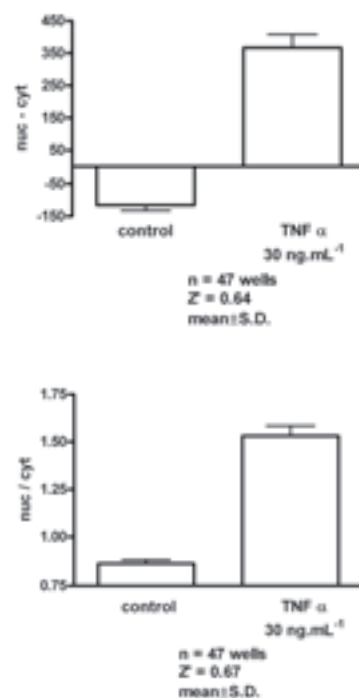


Figure 5 Measurement of Z' in control and TNFα-stimulated HeLa Cells

Discussion

The well-known nuclear transcription factor NF- κ B reliably translocates from the cytoplasm to the nucleus following TNF α stimulation. The cells were not synchronized and exhibited a heterogeneous response, very typical for cells in assay development and screening environments.

The results show that the BD Pathway™ Bioimager system is a robust cellular imaging tool with high precision and reproducibility. In combination with AttoVision, a sophisticated and flexible image analysis software platform and the BD Image Data Explorer, a user-friendly, yet powerful Microsoft™ Excel™ Add-In, the system generates superior data quality ($Z' \approx 0.61-0.72$) and aids assay development and screening efforts.

References

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