APPLICATION NOTE

Detecting Subcellular Changes in NF-κB Using Araceli Endeavor®

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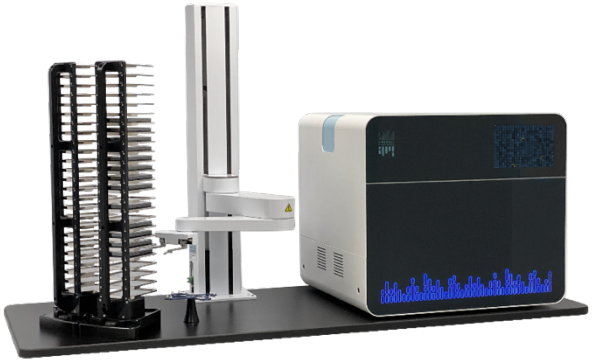
How can you screen thousands of compounds for changes in subcellular protein localization in a single day, with single cell sensitivity? By visualizing the expression of transcription factor NF-κB and quantifying its post-activation translocation into the nucleus, we show that compounds regulating cellular stress can be readily identified at high throughput speeds. Araceli Endeavor® high content imaging system delivers an unmatched combination of speed and resolution that allows you to capture the highest quality data of every cell, in 96- 384- and 1536- well high content plates, in under 10 minutes. This application note illustrates the capabilities of Araceli Bioscience's high content technology to measure nuclear translocation, showing a clear effect of cytokine treatment on protein localization from the cytoplasm to the nucleus.



Interrogating Cellular Immune Response with Two NF-κB Activators

Nuclear factor kappa B (NF-κB) is the cell's frontline response to stress. It waits, inactivated, in the cytoplasm to respond to a wide range of cellular stressors, from reactive oxygen species and heavy metals to UV irradiation and viruses (Ghosh et al 1998). Once activated, this conserved family of transcription factors translocates to the nucleus, where they promote the expression of a host of different inflammatory, anti-apoptotic, and stress-related factors. With well-characterized up- and down- stream targets, NF-κB is ideal for interrogating immune activity and inflammation and plays a critical role as a central inflammatory mediator. NF-κB and its associated pathways are targets of several common drugs such as aspirin and glucocorticoids (Liu et al 2017) and continue to be the subject of much drug discovery research. With a reliable, transcription- and translation- independent nuclear activation response, NF-κB screening offers a paradigmatic example of a high content protein translocation assay (Trask 2012).

In this application note, we use two known NF-κB activators, IL-1β and TNF-α (Ding et al 1998), to interrogate immune function in a relevant, druggable model, showing a clear switch between inactive cytoplasmic NF-κB to an active nuclear transcription factor after cytokine treatment. Our data recapitulate published dose response curves, matching established EC50 values. This is a challenging high content assay because it must be sensitive enough and have sufficient detail to detect the translocation of the transcription factor from the cytoplasm to nucleus versus visualizing a simple upregulation of NF-κB. Endeavor's sub-micron resolution, full well coverage, and precise optical path with high-powered LEDs deliver crisp, clear, even and in-focus images that are capable of detecting subcellular protein localization in 96-, 384-, or 1536-well microplates in 10 minutes or less.

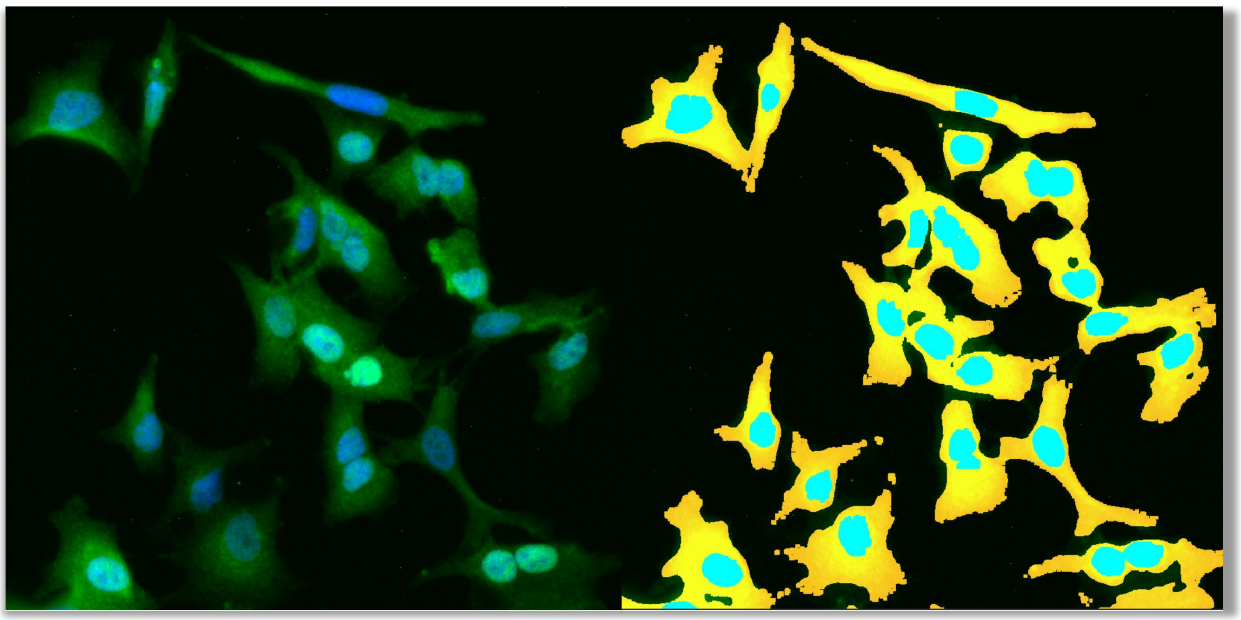


Figure 1: Segmentation results with cytosol masks (yellow) based on NF-κB staining (green) and nuclear masks (cyan) derived from Hoechset stain (blue).

Methods

In 96-well high content plates, human lung epithelial cells (A549) were challenged with one of two cytokines, IL-1β or TNF-α in a 1:2 dilution series and incubated for 35 minutes, then stained with an antibody against p65, the most abundant subunit of NF-κB (Noursadeghi et al 2008) as well as Hoechst 33342 stain to visualize nuclei.

After imaging, nuclei and cytosol were segmented (Figure 1) using Araceli's internal high content image analysis software, measuring intensity in both to generate a nucleus:cytosol ratio for NF-κB expression. See extended methods for details.

Imaging

All imaging was done on Araceli Endeavor® high content imaging system The assay was first assessed with an initial preview run, with a single 1.18x1.18 field of view (FOV) imaged in two channels at full resolution for all 96 wells in <90 seconds. From there, exposure times and focal plane were optimized (15ms for blue, 200ms green), and full well data (Figure 2) imaged in 2 channels at full well resolution for the 96 well plate: 4x4 FOV for 22.6mm^2/well with 0.27 micron pixel size in <10 minutes. Full plate scan times of <10 minutes for 384- and 1536- well plates ensure this assay can be scaled up if higher throughput is needed. This resolution allows for the interrogation of subcellular protein expression in detail.

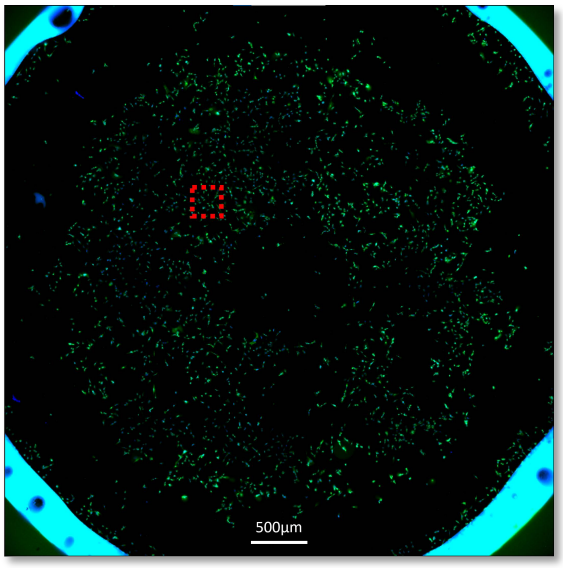


Figure 2: Full well image with Hoechst (blue) and NF-κB (green). FOV in red is the 'intermediate dose' image in Figure 3.

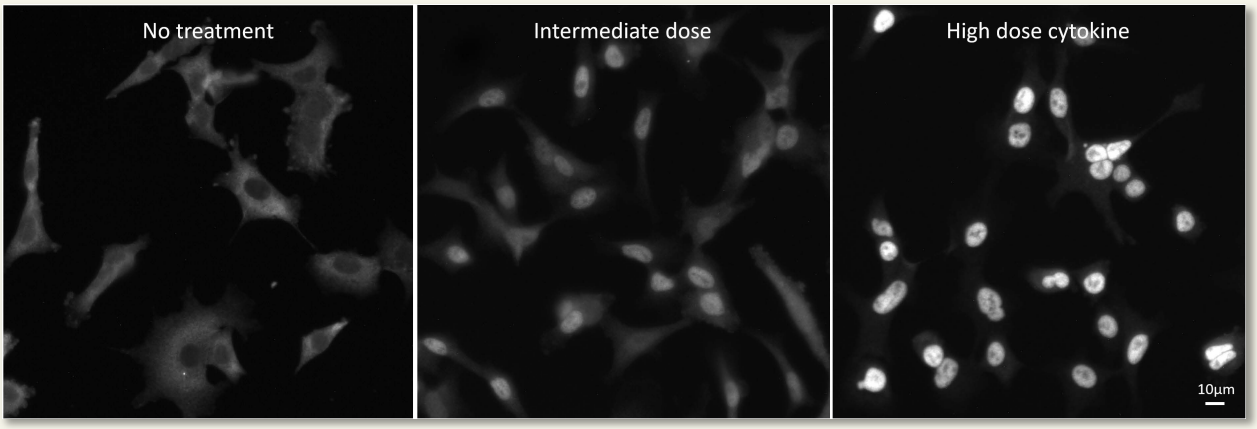


Figure 3: NF-κB staining after no, intermediate (.3 ng/mL) or high (50ng/mL) IL-1β treatment for 35 minutes. Note cytoplasmic staining in untreated cells, becoming more nuclear as dosage increases.

Results

We found that application of cytokines IL-1β and TNF-α for 35 minutes elicited translocation of the primary subunit of NF-κB, p65, from the cytoplasm to the nucleus (Figure 3). Nuclear translocation was dose-dependent and followed a logarithmic titration curve for both cytokine treatments (Figure 4). These data are well-represented by a non-linear dose response curve, R^2=0.96 for IL-1β and R^2=0.92 for TNF-α, with EC50 values of 0.64 µM and 0.12 µM, respectively. These data reliably recapitulate cytokine-dependent nuclear translocation that is described in the literature (e.g. Noursadeghi et al 2008)), with EC50 values in line with previous reports from other cell types (Ding et al 1998, Trask 2012).

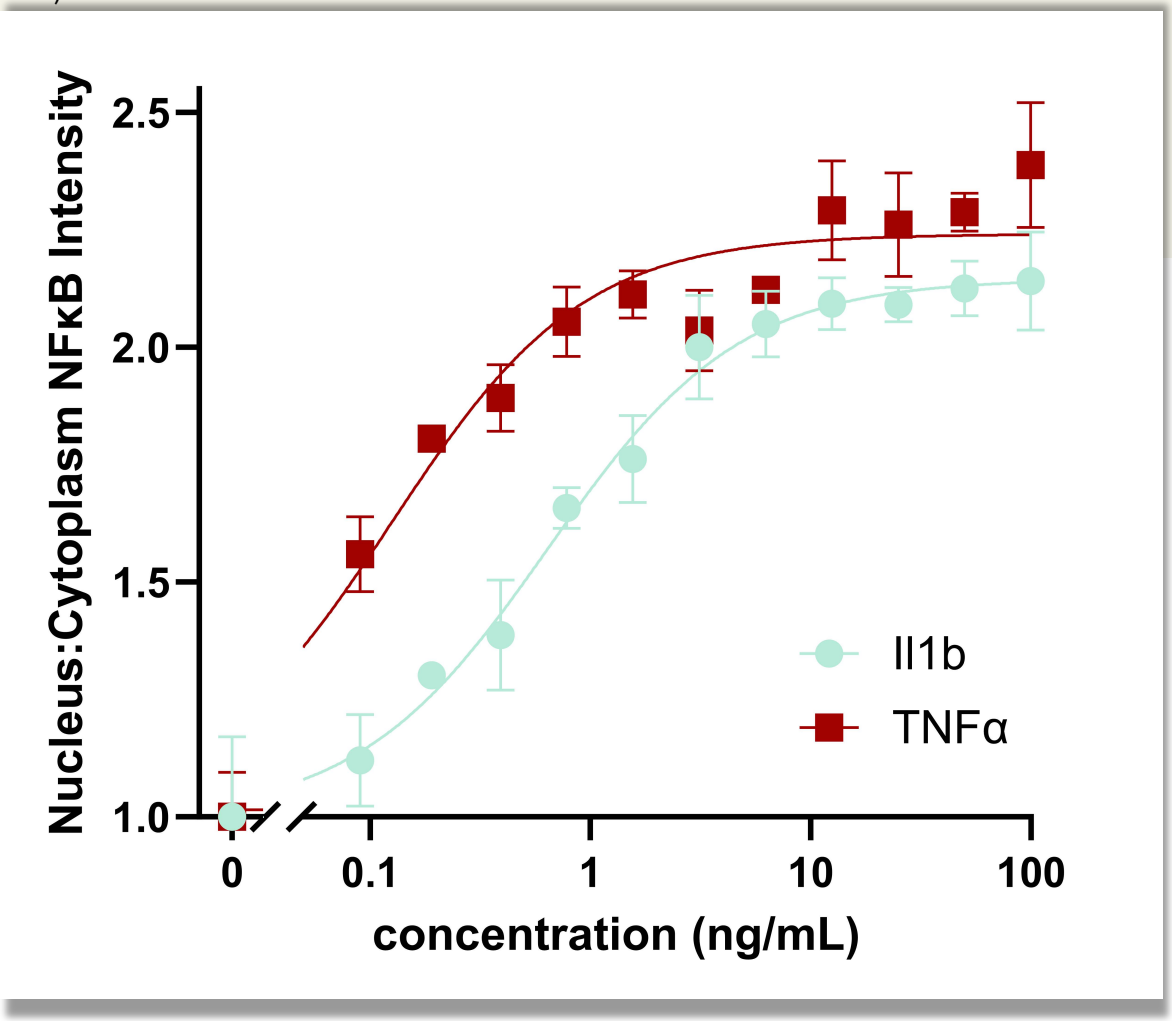


Figure 4: NF-κB (p65) staining nuclear/cytoplasmic intensity ratio, normalized to no cytokine control, after 35-minute application of either IL-1β (blue) or TNF-α (red), error bars +/- standard deviation, nonlinear fit (Hill slope=1)

Conclusion

This application note demonstrates Endeavors ability to interrogate cellular immune response by measuring the translocation of NF-κB from the cytoplasm to nucleus. The results identified robust nuclear translocation of NF-κB after cytokine treatment, with corresponding dose response curves and EC50 matching the literature (Figure 4). Full well imaging of an entire microplate by Endeavor and single cell analysis took less than 30 minutes.

While this Application Note details an assay to detect subcellular changes specifically in NF-κB, it may be broadly applied to a range of translocation assays, from interrogating the well-known tumor suppressor p53 (Liontas and Yeger 2004), to detecting the efficacy of gene therapy (Shin et al 2022). Most transcription factors undergo cytoplasm to nucleus translocation upon activation (Liu et al 2018). With over 1600 transcription factors in the human genome (Lambert et al 2018), translocation then can be an effective readout for hundreds of different potential druggable targets covering a vast array of disorders. The combined speed of Endeavor and our internal high content image analysis software allows luminescence-based assays such as estrogen receptor perturbation screens, which rely on localized fluorescent estrogen binding (Huang et al 2014), or many other similar toxicology assays to be performed at high throughput. This allows for more information to be gleaned from each experiment and the ability to do detailed post-hoc analysis, without sacrificing time or accuracy.

References

Ding GJ, Fischer PA, Boltz RC, Schmidt JA, Colaianne JJ, Gough A, Rubin RA, Miller DK. Characterization and quantitation of NF-κappaB nuclear translocation induced by interleukin-1 and tumor necrosis factor-alpha. Development and use of a high capacity fluorescence cytometric system. J Biol Chem. 1998 Oct 30;273(44):28897-905. doi: 10.1074/jbc.273.44.28897. PMID: 9786892.

Ghosh S, May MJ, Kopp EB. NF-κappa B and Rel proteins: evolutionarily conserved mediators of immune responses. Annu Rev Immunol. 1998;16:225-60. doi: 10.1146/annurev.immunol.16.1.225. PMID: 9597130.

Huang R, Sakamuru S, Martin MT, Reif DM, Judson RS, Houck KA, Casey W, Hsieh JH, Shockley KR, Ceger P, Fostel J, Witt KL, Tong W, Rotroff DM, Zhao T, Shinn P, Simeonov A, Dix DJ, Austin CP, Kavlock RJ, Tice RR, Xia M. Profiling of the Tox21 10K compound library for agonists and antagonists of the estrogen receptor alpha signaling pathway. Sci Rep. 2014 Jul 11;4:5664. doi: 10.1038/srep05664.

Lambert SA, Jolma A, Campitelli LF, Das PK, Yin Y, Albu M, Chen X, Taipale J, Hughes TR, Weirauch MT. The Human Transcription Factors. Cell. 2018 Feb 8;172(4):650-665. doi: 10.1016/j.cell.2018.01.029.

Liontas A, Yeger H. Curcumin and resveratrol induce apoptosis and nuclear translocation and activation of p53 in human neuroblastoma. Anticancer Res. 2004 Mar-Apr;24(2B):987-98. PMID: 15161054.

Liu Y, Li P, Fan L, Wu M. The nuclear transportation routes of membrane-bound transcription factors. Cell Commun Signal. 2018 Apr 3;16(1):12. doi: 10.1186/s12964-018-0224-3.

Liu T, Zhang L, Joo D, Sun SC. NF-κB signaling in inflammation. Signal Transduct Target Ther. 2017;2:17023–. doi: 10.1038/sigtrans.2017.23

Noursadeghi M, Tsang J, Haustein T, Miller RF, Chain BM, Katz DR. Quantitative imaging assay for NF-κappaB nuclear translocation in primary human macrophages. J Immunol Methods. 2008 Jan 1;329(1-2):194-200. doi: 10.1016/j.jim.2007.10.015

Shin CH, Park SC, Park IG, Kim H, An B, Lee C, Kim SH, Lee J, Lee JM, Oh SJ. Cytosolic microRNA-inducible nuclear translocation of Cas9 protein for disease-specific genome modification. Nucleic Acids Res. 2022 Jun 10;50(10):5919-5933. doi: 10.1093/nar/gkac431.

Trask OJ Jr. Nuclear Factor Kappa B (NF-κB) Translocation Assay Development and Validation for High Content Screening. 2012 Oct 1. In: Markossian S, Grossman A, Brimacombe K, Arkin M, Auld D, Austin C, Baell J, Chung TDY, Coussens NP, Dahlin JL, Devanarayan V, Foley TL, Glicksman M, Gorshkov K, Haas JV, Hall MD, Hoare S, Inglese J, Iversen PW, Kales SC, Lal-Nag M, Li Z, McGee J, McManus O, Riss T, Saradjian P, Sittampalam GS, Tarselli M, Trask OJ Jr, Wang Y, Weidner JR, Wildey MJ, Wilson K, Xia M, Xu X, editors. Assay Guidance Manual [Internet]. Bethesda (MD): Eli Lilly & Company and the National Center for Advancing Translational Sciences; 2004–. PMID: 23035273.

Extended Materials and Methods

Assay: Human lung epithelial cells (A549, ATCC) were seeded at 10,000 cells/well in a 96-well microplate (Perkin Elmer ViewPlate, #6005182) and grown overnight at 37^{0}C in 100µL supplemented F-12 media. Cells were then washed 1x in 100µL phosphate-buffered saline (PBS) and challenged for 35 minutes with one of two cytokines, IL-1β (Sigma-Aldrich H6291) or TNF-α (Invitrogen RTNF-AI) applied in a 1:2 dilution from 0.1 to 100 ng/mL in supplemented F-12 media. Final volume of each well was 100µL and four biological replicates at each concentration were prepared. After 35 minutes cells were washed 2x with 100µL PBS, fixed for 15 minutes with 10% neutral buffered formalin, permeabilized for 10 minutes with 0.1% Triton X-100 in PBS, and blocked with 3.3% bovine serum albumin (BSA) in PBST for 1 hour. Cells were stained for 1 hour with primary antibody, 1:1000 rabbit anti-P65 (Cell Signaling Technology mAb 8242) in PBST with 0.33% BSA, followed by 1 hour of incubation with 1:1000 Alexa 488-conjugated anti-rabbit secondary antibody and 1:1500 20mM Hoechst 33342 stain in PBST with 0.33% BSA. Wells were then washed with 100µL PBS and stained cells were stored in 300µL PBS and kept at 4^{0}C until imaging. Full well imaging of the entire microplate was performed using Endeavor and took a little over 10 minutes. Data from the blue channel (Hoescht 33342 stain) and green channel (NF-κB) (Figure 1) were used to identify the nucleus and cytosol, respectively. Our internal high content image analysis software then segmented nuclei and cytosol in the images and measured their intensities to generate a nucleus: cytosol ratio for NF-κB expression.

Image Analysis: Araceli's internal image analysis workflow provides a fast, straightforward analysis pipeline for the assay with minimal modifications beyond specifying objects (i.e. cytosol and nucleus) and defining the channels to be measured. To ensure each image was free from aberrations (such as dust or pipet tip strikes) or focal issues, an automatic image quality control was run on the blue channel which uses a proprietary Fourier transform-based algorithm to compare each image to a user-defined paradigmatic example. Any field of view failing to reach a minimum quality threshold was excluded from the analysis. To measure intensity within the cytosol and nuclei, images were first segmented using AI-based segmentation to define masks for the NF-κB in the cytosol (green channel) and masks for the nuclei using the blue channel (Hoechst 33342 stain) (Figure 1). Then, background subtraction and vignetting correction were automatically applied to eliminate staining artifacts and isolate relevant staining. Within the areas defined by the nuclear and cytoplasmic masks, pixel intensity was then summed and ratioed and averaged across the well. Graphing and statistics were done in Graphpad Prism, using [agonist] vs response for a nonlinear curve fit and to calculate EC50 values.