Application Note

Cell Signaling

Agilent
Trusted Answers

Nuclear Translocation of RelA in Stimulated Macrophages

Using image analysis to quantitate protein movement from the cytoplasm to the nucleus

Author

Paul Held, PhD
Agilent Technologies, Inc.

Abstract

As critical components of the innate immune system, macrophages respond to microbes by recognizing molecules, such as the gram-negative bacteria product lipopolysaccharide (LPS), via Toll-like receptors. Receptor activation stimulates a complex signaling network that involves, among others, the NF-κB pathway. This application note quantitates the nuclear translocation of the protein RelA, a component of the NF-κB transcription factor complex, in macrophages using image-based analysis.

Introduction

Innate immunity is the first line of host defense against infection and is critical for the development of adaptive immunity. While all white blood cells play a role in the immune response, macrophages serve as the most efficient phagocytes and can phagocytose substantial numbers of cells, bacteria, or other microbes.[1] The binding of bacterial molecules to receptors on the surface of a macrophage triggers it to engulf and destroy the bacteria through the generation of a respiratory burst, causing the release of reactive oxygen species. Pathogens also stimulate the macrophage to produce chemokines, which summon other cells to the site of infection[2] and cause inflammation.[3] These cell-surface receptors, called Toll-like receptors (TLRs), are pattern recognition receptors that recognize conserved structures of microbial-derived molecules or pathogen associated molecular patterns (PAMPs), such as bacterial cell wall components, bacterial DNA, and viral RNA. One such component that binds TLRs is lipopolysaccharide (LPS), which is an outer membrane component of gram-negative bacteria and a potent activator of monocytes and macrophages. LPS has been demonstrated to trigger the abundant secretion of many cytokines from macrophages including IL-1(4), IL-6(5), and TNF-α(6) as a result of changes in transcription via the NF-κB complex. RelA, or p65, is a subunit of this complex that normally resides in the cytoplasm, but translocates into the nucleus after stimulation.

The movement of proteins into the cell nucleus is a tightly controlled process that involves nuclear pore complexes (NPCs). Although small molecules can enter the nucleus without regulation, macromolecules, such as proteins, require association with transport factors, called importins, to enter the nucleus.[7] Proteins that must be imported to the nucleus from the cytoplasm carry nuclear localization signals (NLS) which act as a tag to bind to importins. They are diverse in their composition and are commonly hydrophilic, consisting of one or more short sequences of positively charged lysines or arginines exposed on the protein surface. Importins, along with their associated protein cargo, interact with nuclear pore complexes to transition from cytoplasm through the nuclear membrane into the nucleus. This process is regulated by small Ras-related GTPase, Ran, which binds the importin-cargo complex inside the nucleus and causes importin to lose affinity for its cargo.[8]

RelA is part of the heterodimer complex, NF-κB. Together with p50, RelA interacts with various proteins in both the cytoplasm and in the nucleus during the process of classical NF-κB activation and nuclear translocation (Figure 1). In the inactive state, RelA/p50 complex is mainly sequestered by IκBα (Inhibitor of κB) in the cytosol. TNFα, LPS, and other factors serve as activation inducers, followed by phosphorylation at residue 32 and 36 of IκBα, leading to its rapid degradation through the ubiquitin-proteasomal system and subsequent release of RelA/p50 complex.[9] IκBs are proteins that contain multiple copies of a sequence called ankyrin repeats, which by virtue of theses ankyrin repeat domains, mask the nuclear localization signals (NLS) of NF-κB proteins and keep them sequestered in an inactive state in the cytoplasm.[10] With the loss of IκBα, the RelA nuclear localization signal is now exposed and rapid translocation of the NF-κB occurs. After NF-κB nuclear localization, the RelA/p50 heterodimer functions as a transcription factor, binding to many gene promoters involved numerous biological processes, including leukocyte activation/chemotaxis, negative regulation of TNFIKK pathway, cellular metabolism, and antigen processing.[11]

Several tools, such as RAW 264.7 G9 cells (RAW G9), have been developed to study protein nuclear translocation. These cells are a modified mouse macrophage cell line that constitutively expresses GFP-tagged RelA using a lentiviral transfection system.[12] The fusion protein partitions to the cytoplasm in unstimulated cells and translocates to the nucleus after activation with LPS.

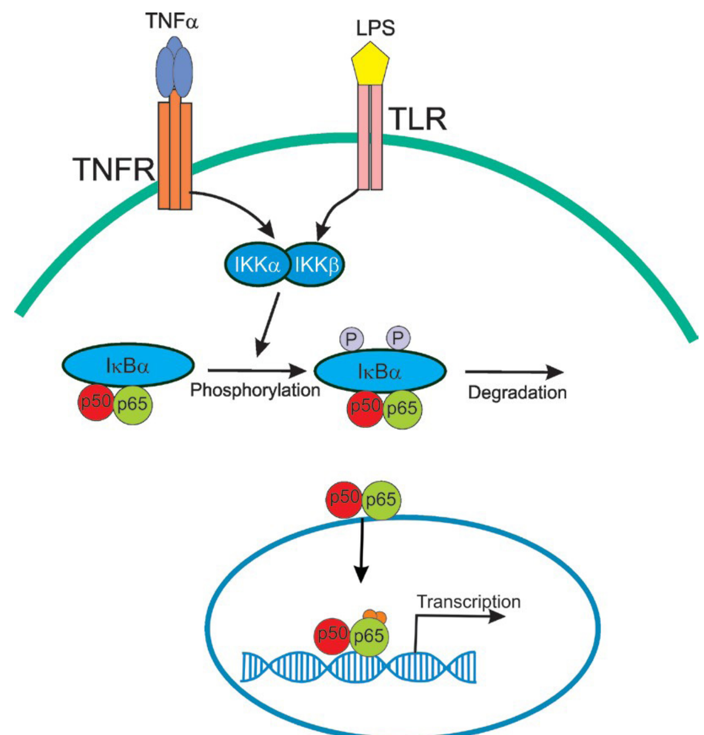

Figure 1. Schematic of RelA (p65) regulation and translocation.

Materials and methods

Cells

RAW 264.7 G9 cells stably expressing RelA-GFP were generous gift from Iain Fraser (NIAID). DMEM (part number 12430), Phenol red free DMEM (part number 31053), Fluorobright/DMEM (part number A18967), FBS, and Glutamine, Pen/Strep were from Life Technologies (Carlsbad, CA), Black-sided, clear-bottom 96-well (part number 3904) microplates were from Corning (Corning, NY). LPS solution (part number ALX-581-008-L001) was obtained Enzo Life Sciences (Farmingdale, NY). Nontreated T-75 tissue culture flasks (part number 15800) were from Nunc (Roskilde, Denmark).

Cell culture

RAW G9 cells were grown in DMEM supplemented with 10% FBS, 2 mM glutamine culture medium in nontreated tissue culture flasks and maintained at 37 °C in a humidified incubator with 5% CO_2. Cultures were routinely passaged at 70% confluence. Cells were detached by adding cold 2 mM EDTA in PBS for five minutes.

Fixed and stained experimental process

For fixed-cell experiments, cells were seeded into black-sided, clear-bottom 96-well plates at 10,000 cells per well using DMEM media in a total volume of 100 µL and incubated overnight. The next day, various concentrations of LPS were added at 2x final concentration in a volume of 100 µL. After activation, cells were washed 2x with PBS and then fixed for 15 minutes using 4% paraformaldehyde in PBS at RT. After fixation, cells were washed 2x with PBS and stained with Hoechst 33342 (10 µM) and Texas red-phalloidin (6.6 µM) in PBS (with 0.1% Triton X-100) for 30 minutes at RT. Cells were then washed 2x with PBS and 200 µL of PBS added to maintain hydration during imaging.

Live cell experiments process

For live cell kinetic experiments, cells were seeded into black-sided, clear bottom 96-well plates at 10,000 cells per well using phenol red free media in a total volume of 100 µL and incubated overnight. The next day, cells were stained with Hoechst 33342 (10 µM) for 20 minutes at 37 °C. After nuclear staining, cells were washed 1x with Fluorobright/DMEM media then 100 µL of Fluoro bright media was added. Imaging exposure settings for the DAPI and GFP channels were adjusted. Once imaging settings were ascertained, LPS was added at various concentrations in 100 µL of Fluorobright/DMEM media and kinetic imaging begun immediately.

Imaging

Experimental cultures were imaged using an Agilent BioTek Lionheart FX automated microscope configured with DAPI, GFP, and TR light cubes. LED light sources in conjunction with bandpass filters and dichroic mirrors provide appropriate wavelength light. The DAPI light cube uses a 337/50 excitation filter and a 447/60 emission filter, the GFP light cube uses a 469/35 excitation filter and a 525/39 emission filter, and the TR light cube uses a 586/15 excitation filter and a 647/57 emission filter. Live cell images were captured kinetically using a 20x objective every 7.5 minutes for three hours. Temperature control (37 °C) and gas control (5% CO_2) were enabled during the imaging process.

Table 1. Imaging and preprocessing parameters.

Imaging	
Parameter	**Value**
Channel	DAPI 377, 447 GFP 469, 525
Focus	Laser auto focus
Objectives	20x
Z-stack	No
Montage	No
Preprocessing	
Image Set	GFP 469, 525 DAPI 377, 447
Background	Dark
Background Flattening	Rolling ball 124 µm 150 pixels

Analysis

The basis of the translocation assay is the movement of fluorescence signal from the cytoplasm to the nucleus as a result of cellular stimulation (Figure 2). These experiments use a RelA-GFP chimera that is inherently fluorescent. The relative fluorescence between the nucleus and the cytoplasm can be used as a means to assess stimulation. However, the localization of RelA is a dynamic phenomenon that is not all or nothing. In unstimulated cells RelA is found predominantly in the cytoplasm, but not exclusively. With LPS binding of the Toll receptors, RelA is allowed to translocate and concentrate in the nucleus.

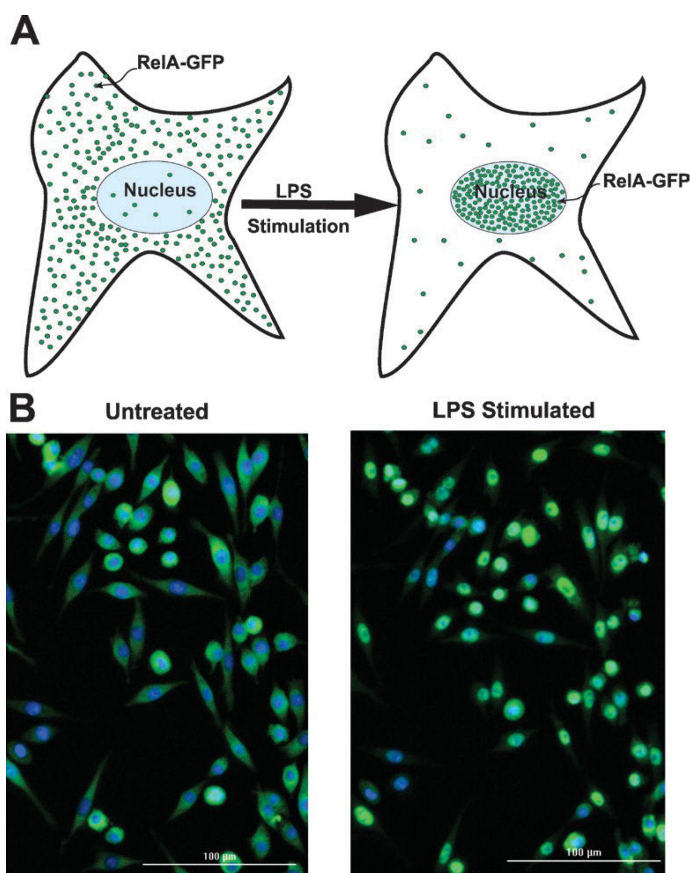

Figure 2. Comparison of untreated and stimulated RAW G9 cells. (A) Cartoon depicting the migration of fluorescent moieties from the cytoplasm to the nucleus as a result of receptor mediated stimulation. (B) Live RAW G9 cells were stained with Hoechst 33342 and imaged before and after stimulation with 10 ng/mL LPS for 60 minutes.

The model uses the fluorescent signal of the RelA-GFP chimera to quantitate localization through dual mask analysis. Cells (live or fixed) are stained with Hoechst 33342 dye and imaged in the DAPI and GFP channels (Figure 3A). The primary or nuclear mask identifies the cell nucleus using threshold analysis of the DAPI channel (Figure 3B). The secondary mask identifies the cytoplasm. This mask is denoted by extending the nuclear mask outward from the outer rim of the primary mask (Figure 3C). It is further refined to exclude background fluorescence using a threshold on the GFP signal of the extended region (Figure 3D).

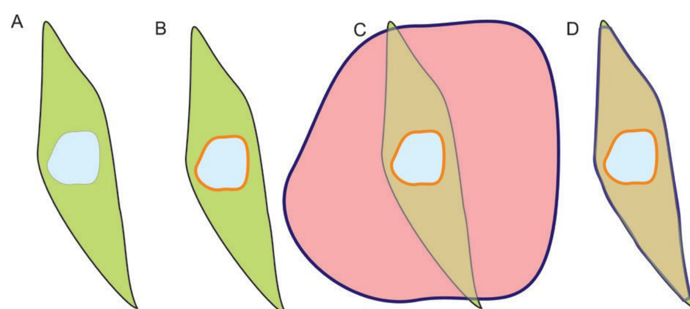

Figure 3. Determination of cytoplasmic and nuclear masks for automated image-based calculation of NF-κB translocation. (A) Live cells expressing RelA-GFP chimera stained with Hoechst 33342 are imaged in the DAPI and GFP channels. (B) Primary (nuclear) mask is identified by object threshold of the DAPI signal. (C) The secondary mask (cytoplasmic) is determined by expanding the primary mask. (D) Threshold analysis on the GFP signal is also applied to the secondary mask.

After background flattening, dual mask image analysis was used to identify stimulated cells. Cellular nuclei were first identified as the primary mask using fluorescence threshold and size exclusion on the DAPI signal as selection criteria (Table 2). Once the primary mask objects had been identified and counted, the outline of the mask was expanded to denote the secondary mask representing the cytoplasm (Table 3). The secondary of cytoplasmic mask was further refined based on a threshold of GFP fluorescence.

Table 2. Nuclear mask parameters.

Primary Object Selection	
Parameter	**Value**
Channel	Tsf [DAPI 377, 447]
Threshold	
Value	5,000
Background	Dark
Split Touching Object	Yes
Fill Holes in Masks	Yes
Advanced Options	
Background Flattening	
Rolling Ball	25 µm
Smoothing	1
Background	5% of lowest pixels
Object Size Selection	
Minimum	6.5 µm
Maximum	30 µm
Include Edge Objects	Yes
Entire image	Yes

Table 3. Cytoplasmic mask parameters.

Secondary Mask Selection	
Parameter	**Value**
Channel	Tsf [GFP 469, 525]
Background	Dark
Measure within a Primary Mask	Yes
Reduce Primary Mask	0.5 µm
Measure within a Secondary Mask	Yes
Distance from Primary Mask	0.5 µm
Ring Width	45 µm
Threshold	3,000
Method	Propagate mask
Fill Holes in Mask	Yes

With nuclear (primary) and cytoplasmic (secondary) masks identified, cells positive for RelA nuclear translocation can be identified. A custom metric can be calculated using the ratio of the mean GFP signal from the nuclear mask to the Mean GFP signal from the cytoplasm (Table 4). With an increase in translocation GFP fluorescence will migrate from the cytoplasmic region to the nuclear region of cells. This is reflected in a change in the Translocation (TL) ratio.

Table 4. Image-based analysis.

Calculated Metrics	
Data	**Name**
Cell Count	Cell count
Object Mean [Tsf[GFP 469,525]]	Nuclear GFP mean
Object Mean_2 [Tsf[GFP 469,525]]	Cytoplasmic GFP mean
Custom Metrics	
TL Ratio Mean	
Metric	**Name**
Nuclear GFP Mean	M1
Cytoplasmic GFP Mean	M2
Formula	M1/M2
Sub-Population Analysis	
Parameter	**Value**
TL Pos Cells	
TL Ratio Mean	Untreated mean +2σ

Cells positive for translocation can be identified using subpopulation analysis. The TL ratio of untreated cells can be used to define cells positive for translocation. The mean TL ratio plus two times its standard deviation was then used as the minimum value for a translocation positive cell (Figure 4). This value was typically close to 2.5 for most experiments. Cells with TL ratio values above this value were denoted as positive and those below negative. The relative percentages of positive cells are then calculated using the primary mask count (cell count) as the total number of cells imaged.

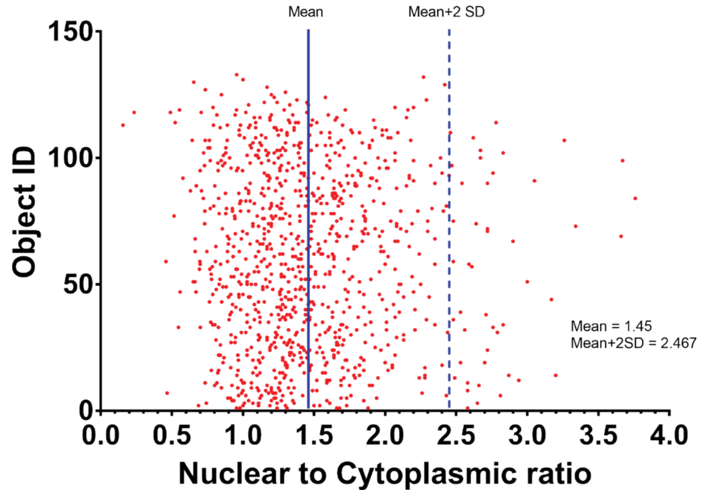

Figure 4. Determination of positive cell threshold. Threshold criteria for positive cells was determined from the nuclear to cytoplasmic signal ratio of untreated control wells. The mean plus two standard deviations.

Results and discussion

These data demonstrate that LPS can stimulate the translocation of a RelA-GFP chimera from the cytoplasm to the nucleus in RAW G9 cells that stably express the protein.

As shown in Figure 5, RAW G9 cells have a spindle shape, as depicted by the phalloidin stained cytoskeleton. In untreated cells RelA-GFP is primarily localized in their cytoplasm as GFP fluorescence can be observed the length of the cells. A halo of fluorescence surrounds the nucleus of many cells with relatively low amounts of GFP in the nuclear region. Stimulation with 3 ng/mL LPS for 60 minutes results in the movement of GFP fluorescent signal from the cytoplasm to the nucleus. Bright GFP fluorescence is now observed in the nuclear region, with a diminishment of signal in the cytoplasmic region, as observed from the composite images. This consolidation is not the result of changes in cell morphology as the cytoskeleton exhibits the same spindle shape with LPS stimulation. There is a certain degree of stimulation in untreated cells, with a small proportion (typically <10%) exhibiting substantial nuclear GFP fluorescence. Not all cells seem to be stimulated with LPS treatment, with approximately 15% seeming to be refractive.

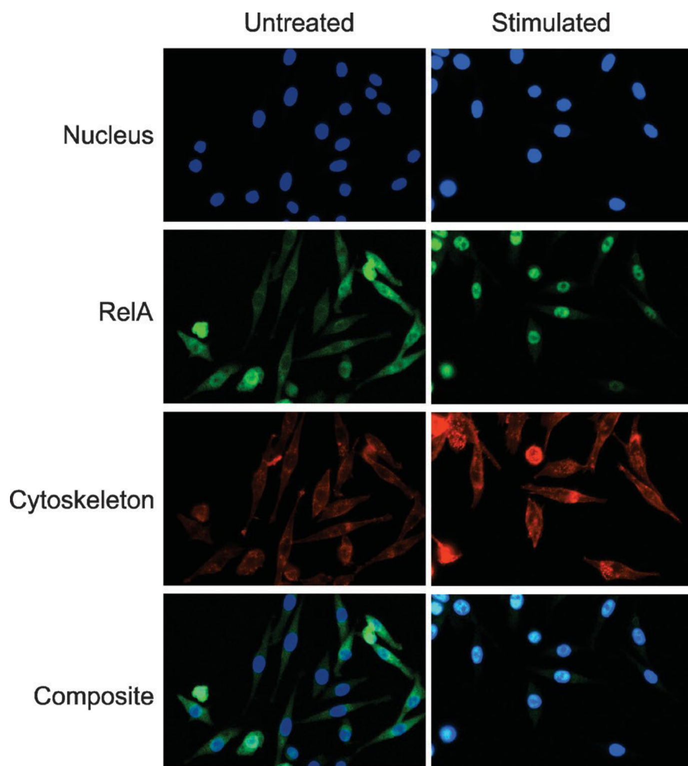

Figure 5. Localization of RelA-GFP in untreated and stimulated RAW G9 cells. RelA-GFP expressing cells were either stimulated with 3 ng/mL LPS for 60 minutes or left untreated before fixation with 4% paraformaldehyde. After fixation cells were stained with Hoechst 33342 and TR-phalloidin for 30 minutes. After staining, cells were imaged using a 20x objective in the DAPI, GFP, and TR channels. Images represent an optically zoomed portion of the image area. Composite images depict overlaid GFP and DAPI channels.

Live cell imaging can also be used in this application using the same image capture, processing, and analysis metrics. Figure 6 demonstrates the dynamic movement of RelA-GFP to the nucleus with LPS stimulation in live cells. Over time, significant amounts of the observed GFP signal move from the cytoplasm to the nucleus. After 60 minutes of LPS exposure, most cells have GFP fluorescence mostly confined to the nucleus. With continual stimulation, RelA remains in the nucleus. Nearly all RelA-GFP fluorescence is observed in the nucleus of cells stimulated for 120 minutes.

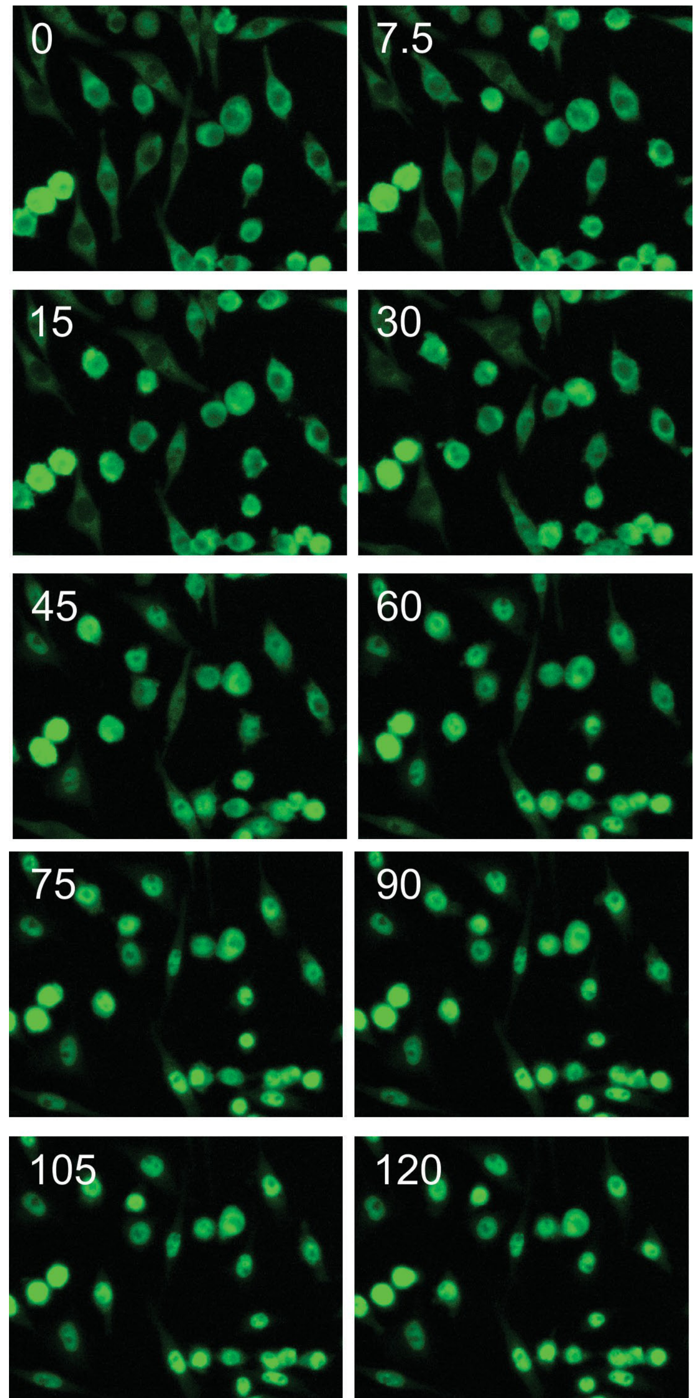

Figure 6. Time-lapse images of RAW G9 cells stimulated with 30 ng/mL LPS.

Image analysis can be used to quantify the degree of stimulation over time. Using the ratio of mean GFP fluorescence in the nucleus to that of the cytoplasm as the defining criteria, cells can be denoted as positive or negative for RelA translocation. The cut-off value is determined from unstimulated controls. As demonstrated in Figure 7, approximately 15% of the untreated cells exhibit some degree of nuclear translocation. The percentage of cells positive for nuclear translocation rapidly increases to over 80% within 45 minutes of exposure to 100 ng/mL LPS. Once maximal stimulation is achieved, there is no observed decrease over the time of exposure. Cells treated with lower concentrations of LPS exhibit a lower rate of increase in the percentage of positive cells and a lower maximal percentage (Figure 7).

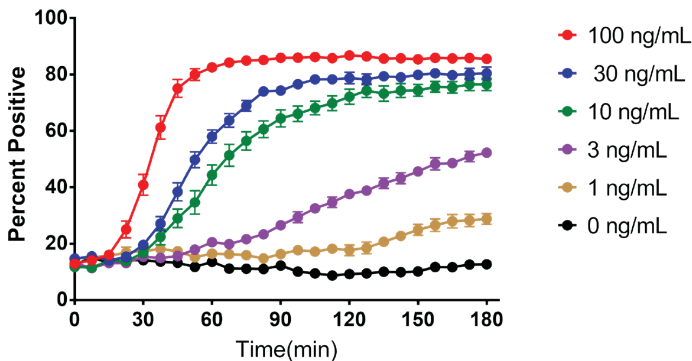

Figure 7. Kinetic effect of LPS on RelA translocation. RAW G9 cells were stimulated with different concentrations of LPS and imaged kinetically for three hours. Image analysis identified translocation positive cells and plotted the percentage of positive cells against elapsed time. Data points represent the mean of eight determinations.

Agilent BioTek Gen5 microplate reader and imager software can be used to provide data reduction from the kinetic data generated after image analysis. As shown in Figure 8, Gen5 microplate reader and imager software can determine the area under the curve (AUC) or integral for each LPS concentration. The AUC calculation can be performed for the entire extent of the experiment or some portion of the total, based on time or image cycle as desired by the investigator.

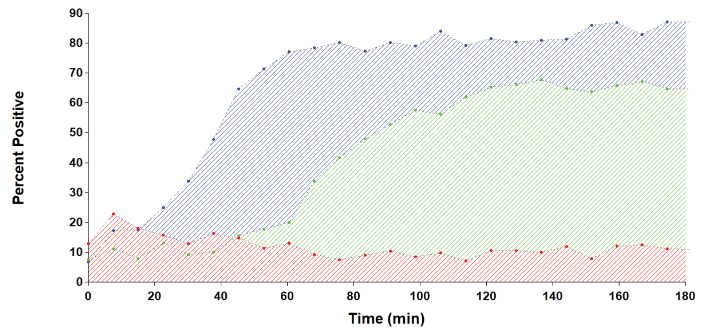

Figure 8. AUC of percent positive kinetic plots. The area under the curve (AUC) or integral of the kinetic plots for each well are automatically calculated and plotted against LPS concentration. The blue striping represents the AUC for 100 ng/mL LPS, while the green and red represent 10 ng/ml and 0 ng/mL, respectively.

The resultant AUC values can be used to show the effect of LPS concentration on RelA nuclear translocation. As demonstrated in Figure 9, LPS has a concentration-dependent relationship with RAW G9 cell activation, as measured by RelA translocation, when cells are imaged kinetically over a period of three hours. There seems to be a concentration threshold, as little translocation is observed at LPS concentrations less than 1 ng/mL.

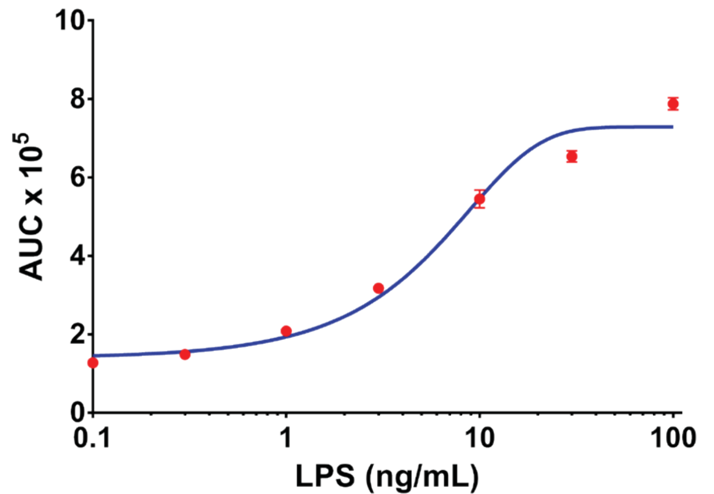

Figure 9. Effect of LPS concentration on kinetic RelA translocation. The AUC for each LPS concentration was plotted.

RAW G9 cell activation can also be measured as an end-point determination rather than kinetically in either live cells or after fixation with paraformaldehyde. As observed in Figure 10, activation of RAW G9 cells with various concentrations of LPS for 120 minutes results in similar titration curves. Fixation often results in a variable attenuation of GFP fluorescence making exact comparisons of results difficult.

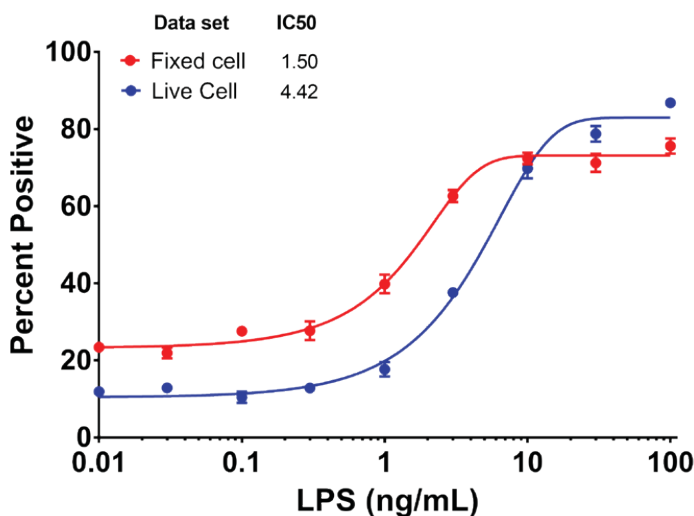

Figure 10. Comparison of fixed cell and live cell LPS titration. The percentage of positive cells at 120 minutes of exposure to LPS for each concentration was plotted from live cell imaging and fixed cell imaging experiments. Data points represent the mean and standard deviation of eight determinations.

Temporal experiments can be performed on fixed and stained cells by exposure to LPS for differing durations before fixation. Using the same analysis criteria, Figure 11 demonstrates the delay between exposure and measurable translocation with fixed cells, which appears to be roughly the same as that for live cells seen in Figure 7.

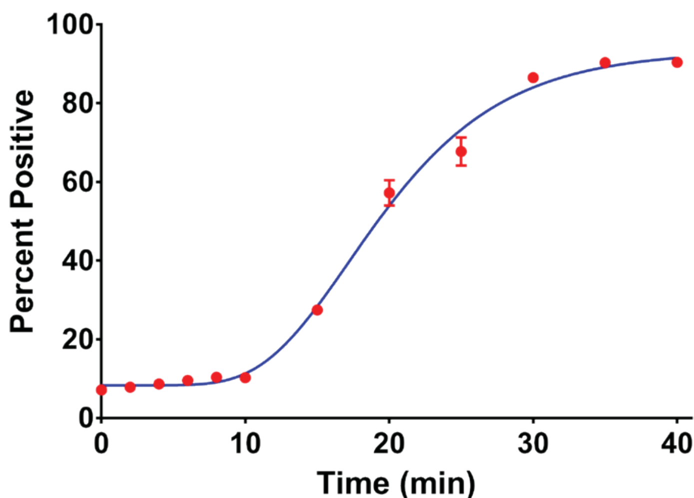

Figure 11. Time course for LPS activation of RAW G9 cells. RAW G9 cells were treated with 30 ng/mL of LPS for various periods of time before the fixing with 4% PFA. After fixation cells were stained with Hoechst 33342 and imaged as described previously. Data represent the mean and standard deviation of eight determinations.

Similarly, LPS titrations with different activation times can be performed with fixed cells. Figure 12 demonstrates the results from image analysis of fixed cells. Like kinetic experiments, where live cells are imaged, fixed cells demonstrate a time delay between initial activation and measurable translocation that cannot be overcome with increasing drug concentration. As with live cell kinetic experiments, there exists both an LPS threshold and a time delay as RelA translocates from the cytoplasm to the nucleus.

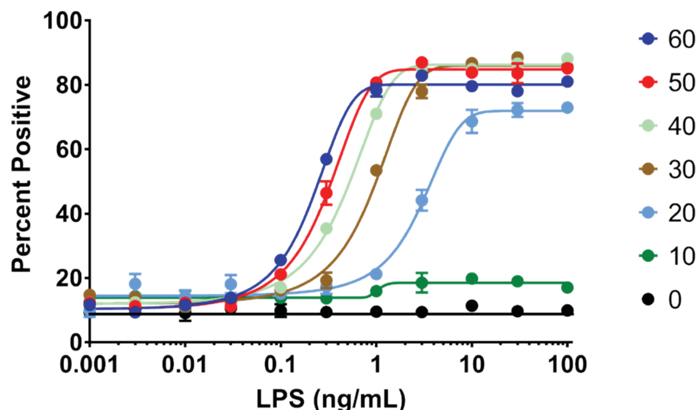

Figure 12. Effect of exposure time and LPS concentration on RAW G9 activation with fixed and stained cells. RAW G9 cells were exposed to various concentrations of LPS for different amounts of time (minutes). The titration results for different activations times were then plotted and compared. Each data point represents the mean and standard deviation of four determinations.

Conclusion

The regulation of translocation of proteins into or out of the nucleus is not completely understood. The creation of GFP tagged proteins such as RelA in the cells used in these studies allows for a rapid analysis of this phenomenon in live cells. Prior experiments required the use of specific antibodies to identify the location of proteins. As such, cells had to be fixed, permeablized, and stained before imaging.

RAW G9 cells can be used to monitor RelA translocation in live cells and after fixation. Both methodologies have strengths and weaknesses. Live cell experiments require the use of environmental control, such as temperature, humidity, and CO_2. These experiments provide large amounts of data per well, as wells can be imaged multiple times to provide kinetic information. However, there is a requirement to provide some sort of nuclear marker or stain, in order to denote the nuclear mask. These stains have the potential to be deleterious to cell viability in long-term experiments. Fixed cell

experiments, which use a nuclear stain after the fixation step, are not subject to this issue. Once fixed, these cells are not subject to any time constraints regarding imaging and can be interrogated with labeled antibodies specific for protein other than RelA. These experiments often require more samples for temporal experiments as any one well can only be used for a single time-point.

The image capabilities of the Agilent BioTek Lionheart FX automated microscope or the Agilent BioTek Cytation cell imaging multimode readers, in conjunction with the Agilent BioTek Gen5 microplate reader and imager software, provide a solution that makes automating translocation experiments easy. The images used were captured using a 20x objective in order to provide sufficient subcellular resolution. If necessary, montage imaging combined with image stitching can be used to increase cell counts when looking for rare events. The Gen5 microplate reader and imager software has the necessary tools to adequately quantitate subcellular localization of GFP-tagged RelA by using dual-mask analysis. A nuclear stain is used to provide a total count of cells in the field of view by creating a primary mask around stained nuclei using a fluorescent signal threshold and size exclusion. Gen5 generates the secondary mask by extending the primary mask outward and in conjunction with signal thresholding denotes the cytoplasm. Once identified, these two regions are used to designate populations of cells positive for RelA translocation.

References

1. Janeway, C. *et al.* Immunobiology (Fifth ed.) New York and London: *Garland Science.* ISBN 0-8153-4101-6, **2001**.
2. Alberts, B. Essential Cell Biology (Second ed.) *Garland Science Pub.* pp. 504–506. ISBN 978-0815334811, **2004**.
3. McDonald, E. M. *et al.* Bacterial Stimulation of Toll-Like Receptor 4 Drives Macrophages To Hemophagocytose. *Infect. Immun.* **2016** Jan, *84(1)*, 47–55, PMCID: PMC4693988, PMID: 26459510, doi: 10.1128/IAI.01149-15
4. Dinarello, C. A. Biologic Basis for Interleukin-1 in Disease. *Blood*. **1996**, *87*, 2095–2147. PMID: 8630372
5. Akira, S.; Taga, T.; Kishimoto, T. Interleukin-6 in Biology and Medicine. *Adv. Immunol.* **1993**, *54*, 1–78. PMID: 8379461.
6. Tracey K. J.; Cerami, A. Tumor Necrosis Factor: a Pleiotropic Cytokine and Therapeutic Target. *Annu. Rev. Med.* **1994**, *45*, 491–503. PMID: 8198398 DOI: 10.1146/ annurev. med.45.1.491.
7. Alberts, B. Essential Cell Biology (Second ed.) *Garland Science Pub.* pp. 504–506. ISBN 978-0815334811 **2004**.
8. Alberts, B. *et al.* Molecular Biology of the Cell (Forth ed.) *Garland Science.* **2002**.
9. Chen, L. F.; Greene, W. C. Shaping the Nuclear Action of NF-kappaB, *Nature Reviews in Molecular Cell Biology* **2004**, *5(5)*, 391–401. Doi:10.1038/nrm1368. PMID 15122352.
10. Jacobs, M. D.; Harrison, S. C. Structure of an IκBα/NF-κB Complex, *Cell* **1998**, *95(6)*, 749-758. Doi:10.1016/S0092-8674(00)81698-0. PMID 9865693.
11. Nowak D. E. *et al.* RelA Ser276 Phosphorylation is Required for Activation of a Subset of NF-kappaB-dependent Genes by Recruiting Cyclin-Dependent Kinase 9/cyclin T1 Complexes. *Molecular and Cellular Biology.* Jun **2008**, *28(11)*, 3623–38 doi:10.1128/ MCB.01152-07. PMC 2423290. PMID 18362169.
12. Li, N. *et al.* Development of a Cell System for siRNA Screening of Pathogen Responses in Human and Mouse Macrophages. *Sci. Rep.* **2015**, *5*, 9559. doi:10.1038/ srep09559 [PubMed: 25831078]

www.agilent.com/lifesciences/biotek

For Research Use Only. Not for use in diagnostic procedures.

RA44412.537337963

This information is subject to change without notice.

© Agilent Technologies, Inc. 2019, 2022

Printed in the USA, June 23, 2022
5994-3399EN

Agilent

Trusted Answers