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Measurement of NF-κB activation in TLR-activated macrophages

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Abstract

Nuclear factor kappa-B (NF-κB) is a key transcription factor in regulation of the innate immune inflammatory response in activated macrophages. NF-κB functions as a homo or heterodimer derived from one or more of the five members of the NF-κB family, and is activated through a well-studied process of stimulus-dependent inhibitor degradation, post-translational modification, nuclear translocation and chromatin binding. Its activity is subject to multiple levels of feedback control both through inhibitor protein activity and through direct regulation of NF-κB components. Many methods have been developed to measure and quantify NF-κB activation. In this chapter we summarize available methods and present a protocol for image-based measurement of NF-κB activation in macrophages activated with microbial stimuli. Using either a stably expressed GFP-tagged fusion of the RelA NF-κB protein, or direct detection of endogenous RelA by immunocytochemistry, we describe data collection and analysis to quantify NF-κB cytosol to nuclear translocation in single cells using fluorescence microscopy.

Keywords

NF-κB; RelA; nuclear translocation; macrophage; transcription factor; GFP; high content imaging

1. Introduction

Nuclear factor kappa-B (NF-κB) is a key pro-inflammatory transcription factor (1), which regulates the transcription of numerous host immune response genes in hematopoietic cell lineages. The expression of many regulatory cytokines, chemokines, receptors and enzymes, such as TNF-α, IL-6 and COX2 (2,3), are dependent on NF-κB activation. In innate immune cells such as macrophages, pathogens are sensed through multiple classes of Pattern Recognition Receptor (PRR) pathways, which invariably induce NF-κB activation. Among these PRR pathways, the Toll-Like receptors (TLRs) have been most widely studied. Furthermore, NF-κB has been shown to coordinate the linkage of inflammation to disease states by inducing expression of inflammatory and tumor promoting cytokines in multiple cell types (4). Thus, activation of NF-κB has a fundamental role in inflammatory regulation and disease.

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The NF-κB family of transcription regulators consists of 5 members: RelA (p65), RelB, c-Rel, NF-κB1 (p50) and NF-κB2 (p52), which all contain an N-terminal REL homology domain required for nuclear localization and DNA binding (2,5). NF-κB family members can form homodimers and heterodimers, and the RelA/p65-p50 complex is considered the prototypical 'NF-κB' heterodimer (6). Only RelA, RelB and c-Rel contain the transactivation domain required for recruitment of transcriptional machinery, so transcription-competent NF-κB dimers typically contain at least one of these Rel proteins. Dimer-specific transactivation, diverse DNA binding motifs and the recruitment of co-transcriptional regulators, all contribute to the selectivity of the NF-κB response (2,5).

NF-κB activation and nuclear translocation constitutes a hallmark of macrophage stimulation by pathogen-associated (PAMPs) or damage-associated (DAMPs) molecular patterns through PRR pathways. In the resting state, NF-κB is sequestered in the cytoplasm by members of the Inhibitor of NF-κB (IκB) family, which mask NF-κBs nuclear translocation signal, thus retaining the transcription factor in the cytoplasm in an inactive form. Upon cellular stimulation, induced by either a pathogen or a stress signal, IκB is phosphorylated by the IκB kinase complex (IKK), which leads to ubiquitin-mediated degradation of IκB by the 26S proteasome (7). NF-κB is released from the cytoplasmic inhibitory complex, further activated by post-translational modifications (PTMs) (8) and translocates into the nucleus where it binds as a dimer to κB sites present at target gene regulatory loci, and induces transcription through the recruitment of co-activators and co-repressors (9).

As NF-κB is arguably one of the most important regulators of pro-inflammatory gene expression, several techniques have been developed to monitor its activity at multiple stages of the NF-κB activation cascade outlined above. For example, NF-κB and IκB proteins can undergo several site-specific PTMs that can be used to measure their activation. Such modifications provide a powerful mechanism to regulate NF-κB transcriptional activation and termination in a context specific manner while recruiting other transcription regulators (8,10-12). PTMs include phosphorylation, acetylation and ubiquitination, all of which can be measured by a western blot using an antibody specific for the modified protein, by gel-based detection of protein mobility changes, or alternatively by protein signal loss in the case of proteasomal degradation.

Another key function of NF-κB that has been routinely measured is DNA binding at the κB enhancer motifs sequences found in NF-κB target genes (13). DNA-protein binding can be measured either by the classical technique, called the electro-mobility shift assay (EMSA) (1,14), or the improved quantitative ELISA format version termed the 'No-Shift assay' (15). Numerous chromatin immunoprecipitation (ChIP) methods have also been developed where specific NF-κB antibodies can be used to isolate NF-κB-DNA complexes which are then characterized by either PCR on an individual gene scale, or by ChIP-seq to provide a genome-scale measurement of NF-κB binding at regulatory loci (16,17). Another common method to measure NF-κB activity at the transcriptional activation level is to use a gene reporter assay, which introduces an exogenous NF-κB consensus promoter sequence linked to a measurable 'reporter gene' such as luciferase (18).

Since IκB degradation releases NF-κB to translocate to the nucleus, monitoring NF-κB movement from the cytoplasm to the nucleus is a popular method to measure NF-κB activity. Two techniques have been developed to test for NF-κB translocation: 1) Cell fractionation, in which cytoplasmic and a nuclear fractions are isolated and NF-κB protein is quantified by western blotting (19-22). 2) Image-based tracking of NF-κB proteins, in which NF-κB dynamics are monitored either by antibody staining, or alternatively, by using NF-κB fused to a fluorescent protein (23). Using the latter option, NF-κB translocation dynamics have been measured and studied in numerous cell types, including innate immune cells (24-29). In macrophage cells activated with TLR ligands, NF-κB translocates to the nucleus within 40 min (Fig. 1A), and shows characteristics of nuclear residence and occupancy that are different from the well-studied oscillatory dynamics observed in fibroblasts (23-25,30-32). This method therefore has the potential to highlight important regulatory aspects for NF-κB activity in innate immune cells, and we describe a detailed protocol for image-based measurement of NF-κB translocation in this chapter.

2. Materials

2.1. Raw 264.7 G9 cells stably expressing RelA-GFP (33) (see Notes 4.1 and 4.2).

2.2. Culture medium: DMEM containing 10% FCS, 20 mM HEPES buffer, 4 mM L-glutamine, penicillin and streptomycin (see Note 4.3).

2.3. Non-treated tissue culture flasks (Nunc, catalog number: 15800), see Note 4.4.

2.4. Phosphate Buffered Saline (PBS).

2.5. 2 mM EDTA in PBS.

2.6. TLR ligand source- 1 mg/ml LPS (Enzo Life Sciences, catalog number: ALX-581-008-L001).

2.7. Clear bottom, black 96-well plate (Falcon, catalog number: 353219).

2.8. Freshly prepared 4% Para-Formaldehyde in PBS.

2.9. Nuclear stain: Hoechst 33342 - NucBlue® Live ReadyProbes® Reagent (Thermo Fisher Scientific, catalog number: R37605).

2.10. Clear microplate-sealing film (Axygen, catalog number: PCR-SP).

2.11. High-content screening (HCS) platform or fluorescence microscope. Method herein describes an imaging and analysis protocol using the CellInsight NXT (Thermo Fisher Scientific).

- For endogenous NF-κB staining also prepare:

2.12. Block/permeabilizing solution: filter sterilized 5% BSA, 0.05% Tween 20 in PBS (see Note 4.5).

2.13. Anti-NF-κB primary antibody (Examples for RelA/p65: Santa Cruz Biotechnology, catalog number: sc-109, or anti-acetylated NF-κB: Cell Signaling Technology, catalog number: 3045).

2.14. Fluorescent-labeled secondary antibody: goat anti-rabbit IgG, Alexa Fluor 488 conjugated (Thermo Fisher Scientific, catalog number: A-11008).

Protocol - Direct measurement of NF-κB translocation to the nucleus by imaging fusion protein of RelA and EGFP

The following detailed technique enables measurement of NF-κB nuclear translocation and single cell analysis using a RAW264.7 macrophage cell line (RAW G9) that constitutively expresses GFP tagged RelA, as was previously described (33). We have also used the method described herein to measure translocation of total- or acetylated-NF-κB in response to TLR stimulation by purified ligands or bacterial infection in immortalized and bone marrow derived macrophages (34,35). The use of a high content image-based method permits the assessment of multiple individual cell properties, which can be missed by cell fractionation methods that only consider averaged properties across a cell population.

Experimental procedure

3.1 Grow RAW264.7 G9 cells in culture medium in a non-treated tissue culture flask and maintain at 37°C in a humidified incubator with 5% CO2. Allow cells to reach 80-90% confluence (see Note 4.1).

3.2 Aspirate and detach the cells by adding cold 2 mM EDTA in PBS for 5 min (see Note 4.4).

3.3 Pipette up and down at least 5 times to break up clumped cells and generate a single cell suspension and collect the cells in a 50 ml tube. Add an equivalent volume of culture medium.

3.4 Centrifuge for 5 min at 400 g.

3.5 Resuspend in growth medium and count the cells.

3.6 Seed 10,000 cells per well in a black, clear bottom 96-well plate in a total volume of 100 μl and incubate overnight (for 384-well plate conditions see Note 4.6).

3.7 The following day, dilute LPS stock (1 mg/ml) to 110 ng/ml in culture medium.

3.8 Treat the cells with 10 μl of diluted LPS for a final concentration of 10 ng/ml LPS, for 0-90 min using 5 to 15 min intervals (see Note 4.7).

3.9 End the experiment by placing the plate on ice, quickly aspirate the medium and fix the cells by adding 4% Para-formaldehyde in PBS for 10 min at room temperature. Keep the plate shielded from light at all times from this point onwards (see Note 4.8).

3.10 Wash twice with PBS (If imaging the NF-κB by antibody staining, continue to 3.1.14).

3.11 Add 1 drop of NucBlue® to 10 ml PBS and add 100 μl per well for 20 min at room temperature.

3.12 Wash twice with PBS.

3.13 Image the plate using a fluorescent microscope, preferably equipped with software for translocation analysis (continue to 3.18).

3.14 If imaging the NF-κB by antibody staining, block the cells and permeabilize using block/permeabilizing solution (5% BSA, 0.05% Tween 20 in PBS) for one hour at room temperature. Note: Include nuclear stain during this step (1 drop NucBlue® per 10 ml block/permeabilizing solution).

3.15 Add 100 μl of a specific NF-κB antibody diluted in block/permeabilizing solution and incubate overnight at 4°C (see Note 4.9 for concentration).

3.16 Wash twice with PBS and add 100 μl of fluorescent-labeled secondary antibody diluted 1:500 in block/permeabilizing solution for two hours at room temperature.

3.17 Wash twice in PBS.

3.18 Seal the plate with a clear film.

3.19 Image for NF-κB localization using a high content imager or suitably equipped fluorescence microscope.

Imaging:

3.20 Acquire images using a 20X, 0.45 NA objective.

3.21 Autofocus using the Hoechst channel (Ch1), excited at 386 nm, and then acquire the image for the GFP channel (Ch2), excited at 485 nm.

3.22 Define the nucleus and the cytoplasm using circle and ring masks as shown in Fig. 1B and1C. The nucleus is outlined by a circle surrounding the Ch1 object, while the cytoplasm is defined by the ring mask that surrounds the nucleus. The ring mask can be defined by either a fixed width or by a threshold-dependent dynamic mask (see Notes 4.10-4.11). For example, Fig. 1C is imaged using a fixed ring mask width of 10 pixels.

3.23 Configure imaging parameters by setting the auto-exposure adjustment to the expected brightest well, reducing the background, and defining the threshold (see Note 4.12).

3.24 Define the number of cells to be imaged and analyzed, ensuring statistical significance. Fig. 1 D-G displays the results obtained for n=200 cells (see Note 4.13).

3.25 Measure the fluorescence intensity of the nucleus (circle) and the cytoplasm (ring) on Ch2 (RelA-GFP) in each of the treated wells of your plate.

Results analysis:

RelA-GFP nuclear occupancy quantification.

3.26 Calculate the well averages of the ratio of nuclear (circle) to cytoplasmic (ring) Ch2 intensities. Note: expect to see an increase in RelA nuclear abundance in response to LPS stimulus in a time dependent manner (Fig. 1D).

3.27 In a modification to the histocytometry method (36), we can either import images or the cell level raw data into FlowJo for further analysis (Fig. 1 E-G and Note 4.14).

3.28 Make sure to also examine the total nuclear intensity, as Noted in 4.15.

Notes/Troubleshooting

4.1. When working with macrophage-like cell lines derived from RAW264.7 cells, we find that cell characteristics are less stable if we exceed 20 passages from a low-passage parental stock. We therefore advise thawing a new cell stock when approaching this passage number.

4.2. RelA-GFP expressing Raw264.7 macrophages are available from our lab upon request. Alternatively, transient transfection of a plasmid expressing any fluorescently tagged NF-κB component can also be used for a translocation assay. In this case, if using RAW264.7 cells we recommend plasmid delivery by electroporation using the Nucleofector™ device by Lonza and the Nucleofector™ transfection kit V (Lonza, catalog number: VCA-1003) to achieve maximal transfection efficiency. However, since transient plasmid transfection can lead to variable expression levels of the reporter protein, we advise generating a stable cell line from which clones can be identified with close to endogenous expression levels (33,32). Primary cells, such as BMDM or peritoneal macrophages, are much harder to transfect than a cell line. We therefore advise to test for NF-κB activation in primary cells by imaging endogenous NF-κB, or to use other methods described in the introduction which doesn't include transfection.

4.3. Macrophages react to the presence of the smallest amount of endotoxin. When working with macrophages, make sure to use endotoxin-free reagents, including endotoxin-free serum and vectors. If transfecting a RelA-GFP vector, purify the vectors using an endotoxin-free prep in order to ensure the macrophages are not exposed to bacterial stimuli prior to the beginning of the experiment.

4.4. We recommend maintaining RAW264.7 cells in non-treated vessels (either flasks or dishes), in order to avoid strong adhesion of the cells to the flask. The cells are easily detached after 5 min treatment with cold 2 mM EDTA in PBS. We do not recommend cell scraping or the use of trypsin, which can contain endotoxin contaminations and additionally can alter properties of macrophage cell surface receptors.

4.5. We recommend using a filter sterilized block/permeabilizing solution, stored at 4°C. Nonspecific background staining is reduced by filtration of auto fluorescent particles. Additionally, we have noted that plate imaging can be done even 4

months after the labeling experiment when using a sterile block/permeabilizing solution for all stages of blocking and staining and stored in the dark at 4°C.

4.6. For a 384-well plate format, seed 1,000 cells per well in a black, clear bottom 384-well plate (Falcon, catalog number: 353962) in a total volume of 40 μl.

4.7. An important note to keep in mind when studying NF-κB signaling kinetics is that the NF-κB kinetics in macrophage can differ from other cells types (37,32), and the oscillatory dynamics observed in fibroblast cells are rarely observed. Thus, when studying NF-κB signaling dynamics in different cell systems, be sure to address signaling kinetics, duration of NF-κB nuclear occupancy and fold-change increases in NF-κB activity (26).

4.8. Shield the plate from light in order to avoid photobleaching of fluorescence signal.

4.9. We have used primary anti NF-κB antibodies to detect the total and the acetylated form of NF-κB antibodies at dilutions of 1:250 and 1:200, respectively. When using a new antibody, or trying new cell lines, we highly recommend testing a serial dilution in order to optimize antibody concentration.

4.10. Adjustment of the ring width should be done based on the spread of the cells to ensure optimal coverage of the cytoplasm while minimizing the background area captured by the ring (Fig. 1B).

4.11. Dynamic thresholding (Fig. 1B6) can also be used to demarcate the cytoplasmic region of a cell, within the fixed width of the ring masks. If we have a cytoplasmic stain, then based on the intensity threshold of the cytoplasmic staining, a region of interest can be auto-demarcated as cytoplasm. This method can also be used based on the GFP staining in the cytoplasm but works best only when the cytoplasm staining is bright. In our hands, when the GFP staining in the cytoplasm is bright, the nucleus/cytoplasm RelA-GFP ratios calculated by the dynamic cytoplasm mask method (Fig. 1B6) vs the fixed mask (Fig. 1B5) are comparable. Additionally based on the cell type and the magnification it might be required to vary the width of the cytoplasm mask to optimize coverage of the cytoplasm.

4.12. Viable nuclei should be defined based on their size and intensity. Gate for nuclei which range in size up to 2 SD of the mean for unstimulated cells, the lower range can be set by visually inspecting the images to decide smallest area of nuclear stain that is acceptable as a nucleus. If the cell culture was not healthy prior to stimulation then there tends to be an increase of sub-threshold staining of nuclear area due to damaged nuclei. Additionally, exclude all cells that are localized to the edges of the imaged field.

4.13. Make sure to sample cells from different fields in each well in order to avoid artifacts. If cell density is high, enlarge the number of cells imaged in order to sample more fields.

4.14. Single cell analysis using FlowJo can help in multifactorial selection of cell populations, e.g. gating of cells for both ratio of nuclear/cytoplasmic NF-κB and the difference in nuclear to cytoplasmic NF-κB (Fig. 1E-G). Depending on the quality of the cell preparation sometimes untreated cells tend to have noisy NF-κB staining or GFP intensities, under such conditions it becomes important to gate cells by comparing untreated vs a known positive control.

4.15. Standard analysis consists of calculating the ratio between NF-κB nuclear abundance and the cytoplasmic abundance in order to observe NF-κB nuclear translocation. Yet, an elevated ratio of NF-κB abundance may not always correlated with reduced abundance of NF-κB in the cytoplasm, due to positive feedback which can elevate the expression of NF-κB components in the cell (32). Therefore, in addition to relative NF-κB nuclear abundance it is highly recommended to compare solely the total intensity of the nucleus (Fig. 1E, F).

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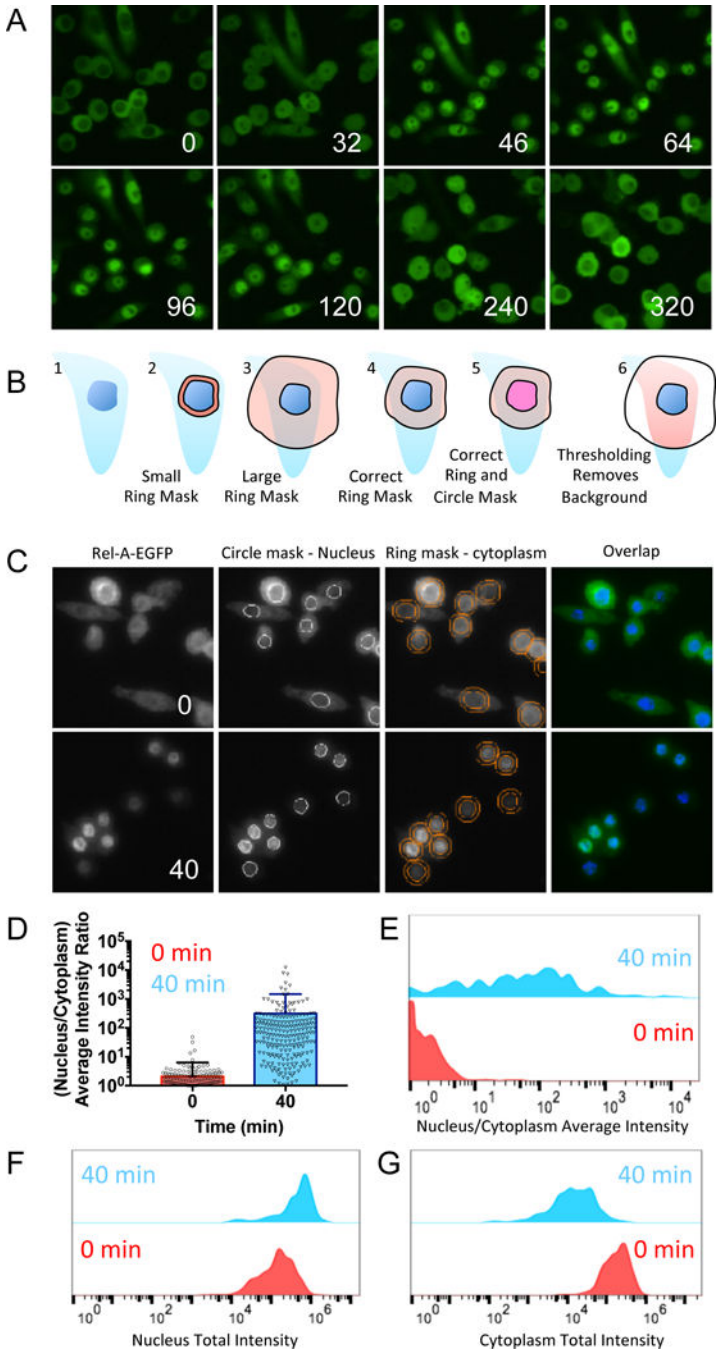

Figure 1: Imaging and analysis of NF-κB nuclear translocation in LPS-activated macrophages.

(A) Time-lapse images taken of the RAW264.7 G9 cells at the specified times (38) after treatment with LPS (10 ng/ml) using confocal microscopy. **(B)** Different options for creating cytoplasmic and nuclear masks for automated image-based calculation of NF-κB translocation. **(C)** RelA-EGFP nuclear translocation analysis at time 0 (top images) and 40 min (bottom images) after 10 ng/ml LPS stimulation, using a high content imager. Images display the nucleus - circle mask in white and the cytoplasm - ring mask in orange. **(D)** Ratio of nuclear to cytoplasmic RelA-EGFP intensity quantification calculated from the

cells shown in (C). **(E-G)** FlowJo-based cell property distributions for the cells shown in (B): nuclear to cytoplasmic ratio **(E)**; total nuclear intensity **(F)**; total cytoplasmic intensity **(G)**.