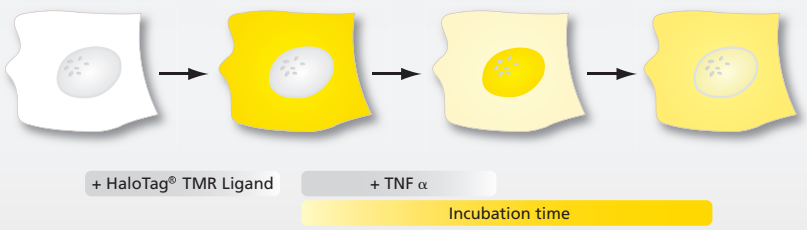

APPLICATION NOTE

Cellular Imaging and Analysis

Key Features

- Fluorescent labeling of living cells for a high content assay using HaloTag® technology.
- Live cell imaging with the Operetta system equipped with live cell chamber.

Live Cell Imaging: Studying NF-κB Signaling Dynamics Using the Operetta System

Background

The NF-κB family of transcription factors was identified more than 20 years ago and today it is well established that NF-κB plays a pivotal role in all aspects of immunity and cellular response to pathogens [Hayden and Ghosh, 2008]. Aberrant activation of NF-κB signaling is associated with inflammatory diseases such as rheumatoid arthritis [Criswell, 2010] and asthma [Broide *et al.*, 2005]. More recently, low-grade inflammation and NF-κB activation have been associated with the initiation and propagation of metabolic disease [Baker *et al.*, 2011]. NF-κB signaling therefore remains an attractive target in the development of drugs against inflammation-associated diseases.

The NF-κB family of transcription factors consists of five members that act as homo- or heterodimers. In its inactive state in most cell types, dimeric NF-κB is sequestered in the cytoplasm by the endogenous inhibitor of NF-κB signaling, IκB. The NF-κB pathway is activated by numerous stimuli that all result in the activation of IκB kinase (IKK). Phosphorylation of IκB by IKK leads to polyubiquitination of IκB, which causes the proteasomal degradation of the inhibitory protein. Nuclear import of the released NF-κB dimer is then mediated via unmasked nuclear localization signals (NLS) on both NF-κB subunits. Once inside the nucleus, NF-κB binds to different target genes that encode various inflammatory cytokines, transcription factors, cell adhesion molecules and the IκB protein. The latter provides a negative feedback mechanism to terminate NF-κB signaling.

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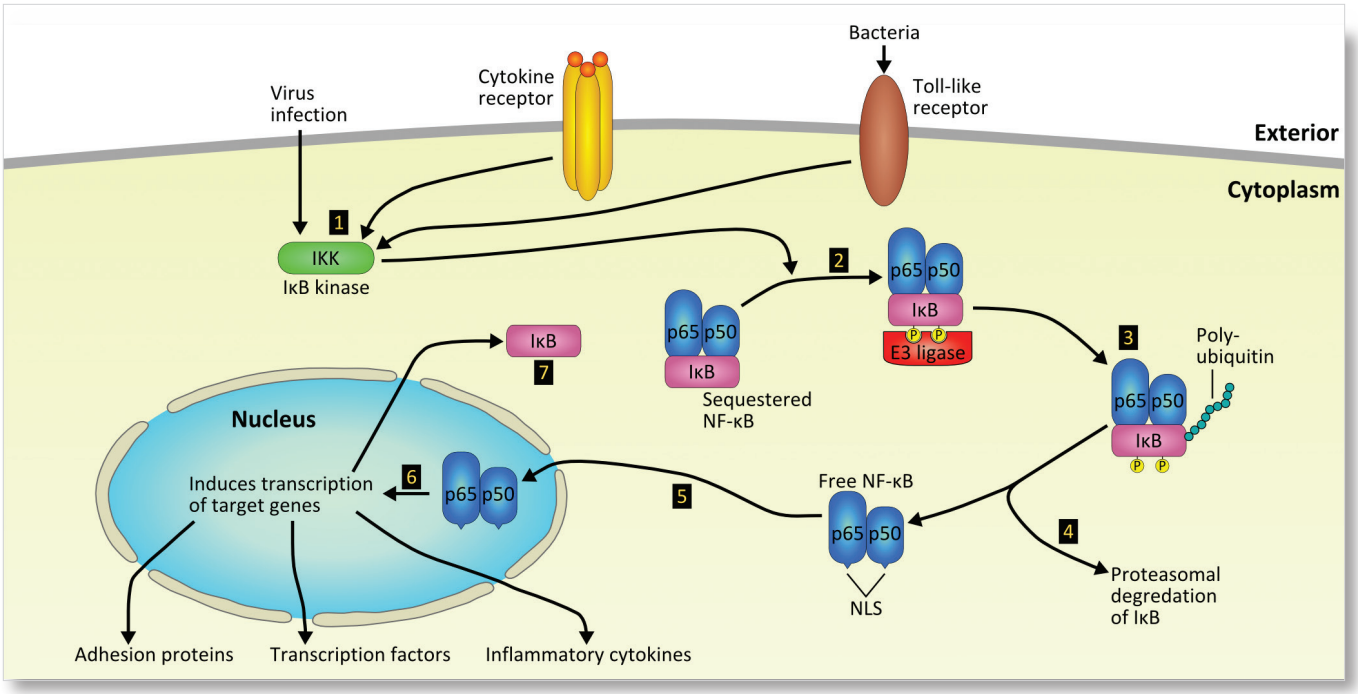

Figure 1. The NF-κB signaling pathway. (1) Different stimuli activate the IκB kinase (IKK). (2) Active IKK phosphorylates the inhibitor of NF-κB signaling (IκB). Phosphorylation results in the recruitment of an E3 ubiquitin ligase. (3) The E3 ubiquitin ligase facilitates the polyubiquitination of IκB. (4) Polyubiquitinated IκB is degraded by the proteasome. (5) The free NF-κB dimer has exposed nuclear localization signals (NLS) that accomplish nuclear import. (6) NF-κB binds to the κB region of the promoters of specific target genes that are predominantly involved in pro-inflammatory processes. (7) The expression of IκB results in a negative feedback, which terminates NF-κB signaling.

To study the nuclear import and export of NF-κB in living cells, the Promega HaloTag® technology was applied. The HaloTag® protein is a mutant hydrolase protein that forms a site-specific covalent bond to the HaloTag® ligands, which are available with different fluorophores and as membrane permeable or impermeable versions. The HaloTag® can be N- or C-terminally fused to any protein of interest and, due to the prokaryotic origin of the hydrolase protein, only a small amount of background is observed in fluorescently stained eukaryotic cells. The ability to selectively label a protein of interest at the cell surface or intracellularly, with a variety of different colors, and in living cells, renders the HaloTag® technology a highly attractive alternative to fluorescent protein tags [Los *et al.,* 2005].

High content imaging provides a rapid and flexible tool to quantitatively study the intracellular localization of fluorescent markers on a single cell basis. Here, a live cell imaging approach is presented for studying the nuclear translocation of NF-κB using the Operetta® High Content Imaging System. In contrast to fixed endpoint assays, this allows the negative feedback of NF-κB signaling, which results from NF-κB-induced expression of IκB (Figure 1), to be analyzed.

Application

To study NF-κB translocation, a stable HEK293 cell line expressing the NF-κB family member p65 c-terminally fused to a HaloTag® (p65-HT) was used. Cells were seeded at a density of 30,000 cells per well into a 96-well CellCarrier™ microtiter plate (PerkinElmer, 6005550) freshly coated with 5 µg/cm² collagen I (BD Biosciences, 354236). After overnight cultivation in serum-supplemented medium, cells were stained with Hoechst 33342 (1.62 µM) and HaloTag® TMR Ligand (5 µM) for 15 min, followed by washes with PBS and serum-free, phenol red-free, medium. Subsequently, cells were serum-starved for 5 hr in phenol-red free medium to render them highly susceptible to stimulation with tumor necrosis factor α (TNFα), which activates the NF-κB pathway via a cytokine receptor.

Live cell imaging was performed using an Operetta High Content Imaging System equipped with a live cell chamber. The environmental control unit was set to 37 °C and 5% CO_2, and images were acquired using a LWD 40X objective in widefield fluorescence mode. The Harmony® High Content Imaging and Analysis Software's time series settings allow a break during a measurement for the addition of compounds. Therefore, it was possible to record two baseline time points prior to stimulating the cells with different concentrations of TNFα (Calbiochem®, 654205). After the addition of TNFα, cells were imaged every 10 min, for a time period of 3 hr, to monitor the translocation of p65-HT to the nucleus (Figure 2).

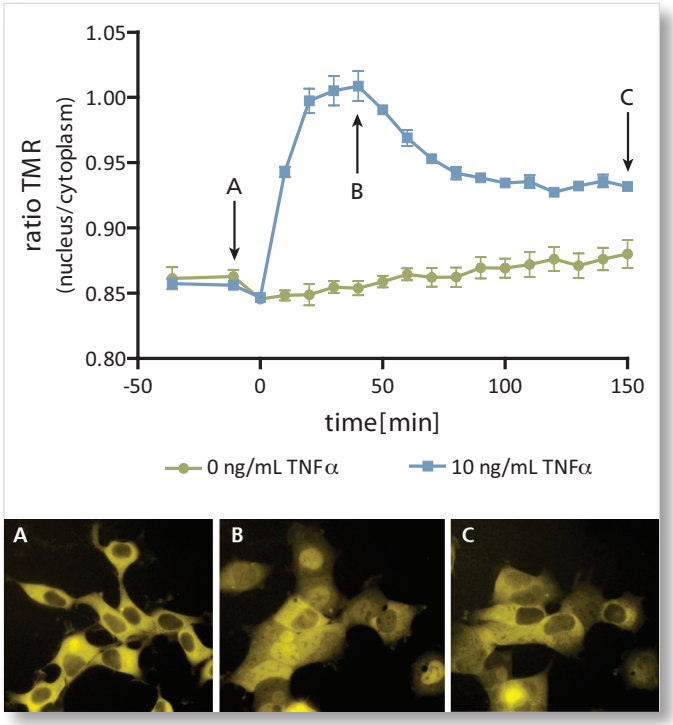

Figure 2. Time course of NF-κB translocation. Top: The fraction of nuclear p65-HT rapidly increases to a maximum level after 30 min before slowly decreasing and reaching a steady-state between nuclear import and export (N = 3 wells, at 30 min Z'=0.7). Bottom: Representative images are shown for the 3 time points indicated above. (A) before stimulation, (B) maximum response, (C) steady-state.

To segment the individual cells, the *Find Nuclei* Building Block of the Harmony software was applied to the Hoechst channel image. Subsequently, a small ring region of 1 px width was generated around the nuclei to measure the TMR intensity in the cytoplasm. The ratio of the mean TMR intensity in the nuclear and ring regions was then calculated as the final readout parameter. A quantitative analysis of the acquired time series revealed that the cellular response to TNFα stimulation reaches a maximum level at 30 min post stimulation before decreasing again towards a steady-state between nuclear import and export (Figure 2).

The 30 min time point was chosen to calculate a dose-response curve for TNFα stimulation of the HEK293 p65-HT cell line, which resulted in an EC_{50} of 0.5 ng/mL (Figure 3A). Furthermore, NF-κB signaling was inhibited by treating cells with Ro106-9920, an irreversible inhibitor of IκB ubiquitination. Ro106-9920 treatment leads to lower levels of proteasomal IκB degradation and, consequently, to lower levels of NF-κB translocation. Cells were pre-incubated with different concentrations of Ro106-9920 for 20 min and subsequently stimulated with 10 ng/mL TNFα. Due to high cytotoxicity of the inhibitor, only concentrations up to 5 µM could be used, which reduced the nuclear translocation of p65-HT by ~45% compared to non-inhibited control cells (Figure 3B).

Conclusion

The high content imaging application presented here investigates different aspects of the NF-κB signaling pathway in living cells. The live cell approach chosen allows the kinetics of the nuclear import of NF-κB to be studied, as well as the subsequent negative feedback triggered by the NF-κB-induced expression of IκB. Importantly, this allows screening for compounds that affect either the nuclear import or nuclear export kinetics, both of which have implications for gene regulation, in a single experimental setup. The applied HaloTag® technology allows the labeling of proteins in living cells with fluorophores that are freely selectable depending on the required spectral and physicochemical properties. The low background and the interchangeable labeling possibilities make the HaloTag® an ideal tool for multiplexing in high content assays. The image analysis strategy applied in this example resulted in excellent Z' scores, demonstrating the relevancy of the assay for screening applications. The cells used here did not exhibit any detrimental effects, showing that the environmental control unit of the Operetta system provides optimal conditions for live cell imaging. In summary, the use of Halo-tagged proteins on the Operetta system allows sensitive and flexible live cell assays to be performed for diverse high content screening applications.

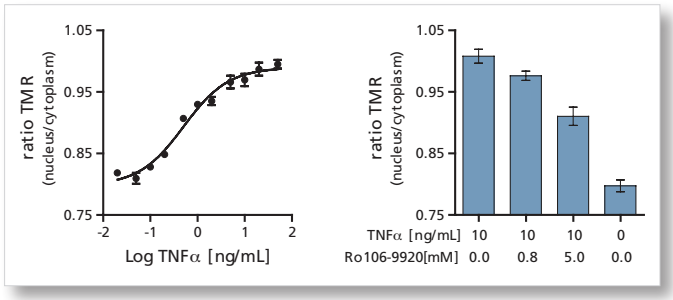

Figure 3. NF-κB translocation at the time point of maximum response (30 min post stimulation) (A) TNFα dose dependent nuclear translocation of p65-HT in HEK293 cells. The ratio between nuclear and cytoplasmic TMR fluorescence increases with increasing TNFα concentrations. N=3 wells, Z'=0.76. (B) Inhibition of NF-κB signaling by Ro106-9920. The fraction of nuclear p65-HT decreases dose-dependently with increasing Ro106-9920 concentrations. Concentrations of Ro106-9920 > 5 µM were cytotoxic in these experiments. N=3 wells.

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