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Quantification of NF-κB signaling in living cells: A high-content workflow for the generation of time-resolved single-cell data

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1 Introduction

The transcription factors of the NF-κB family play a pivotal role in all aspects of immunity and it is well established that aberrant activation of NF-κB signaling is associated with inflammatory diseases like rheumatoid arthritis or asthma. Thus, it is evident that NF-κB signaling remains an attractive target in drug development.

Here, we present a high content analysis (HCA) based assay to study NF-κB signaling in living cells using the Operetta® High Content Imaging System and the Promega HaloTag® technology. By analyzing the acquired Operetta images with our Acapella® High Content Imaging and Analysis Software, we were able to track individual cells over time. This approach can be used to study highly dynamic processes like oscillations in NF-κB signaling that have been implicated in differential gene regulation [Ashall *et al.*, 2009].

In summary, the approach we present here demonstrates an automated workflow for the generation of time-resolved single-cell data applicable to screening campaigns.

2 Assay principle

NF-κB translocation was studied in stable HEK293 cells expressing the NF-κB family member p65 c-terminally fused to a HaloTag® (p65-HT). Cells were seeded at densities of 10,000-30,000 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 Hoechst33342 and HaloTag® TMR Ligand. Subsequently, cells were serum-starved for 5 hours to render the cells highly susceptible for TNFα stimulation. Imaging was performed using an Operetta High Content Imaging System equipped with the live cell chamber (set to 37°C and 5% CO_2). Images were acquired using a LWD 40X objective in widefield fluorescence mode.

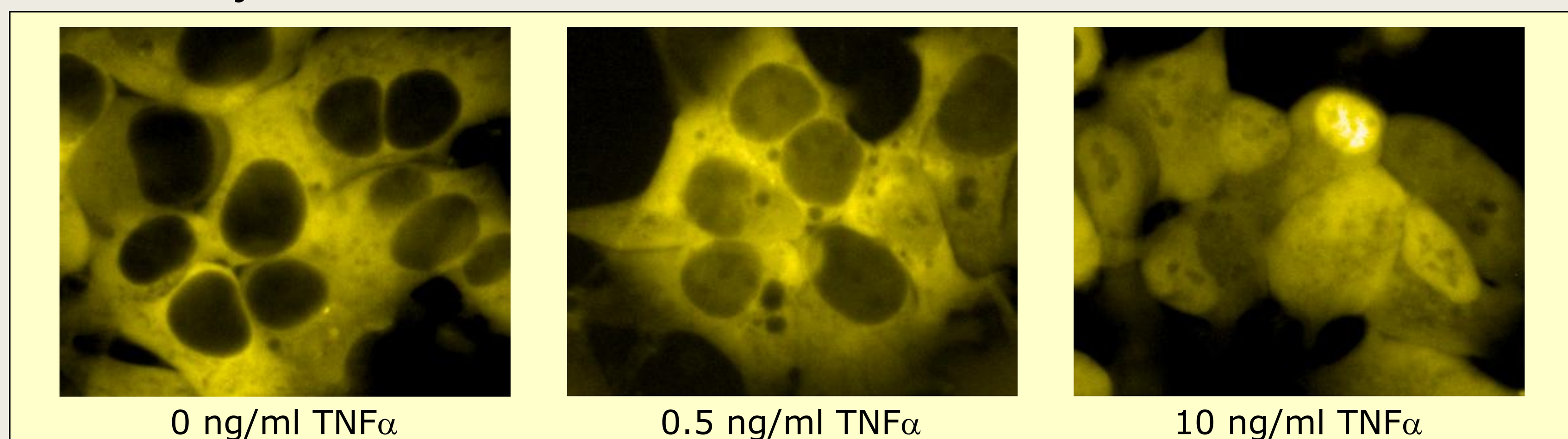

Figure 1. TNFα induces the cytoplasm-to-nucleus translocation of p65. HEK293 p65-HT cells were stained with Hoechst33342 and HaloTag® TMR Ligand and were treated with the indicated TNFα concentrations for 30 minutes. The fraction of nuclear fluorescence increases dose-dependently with TNFα concentration.

3 Population-based analysis of NF-κB translocation

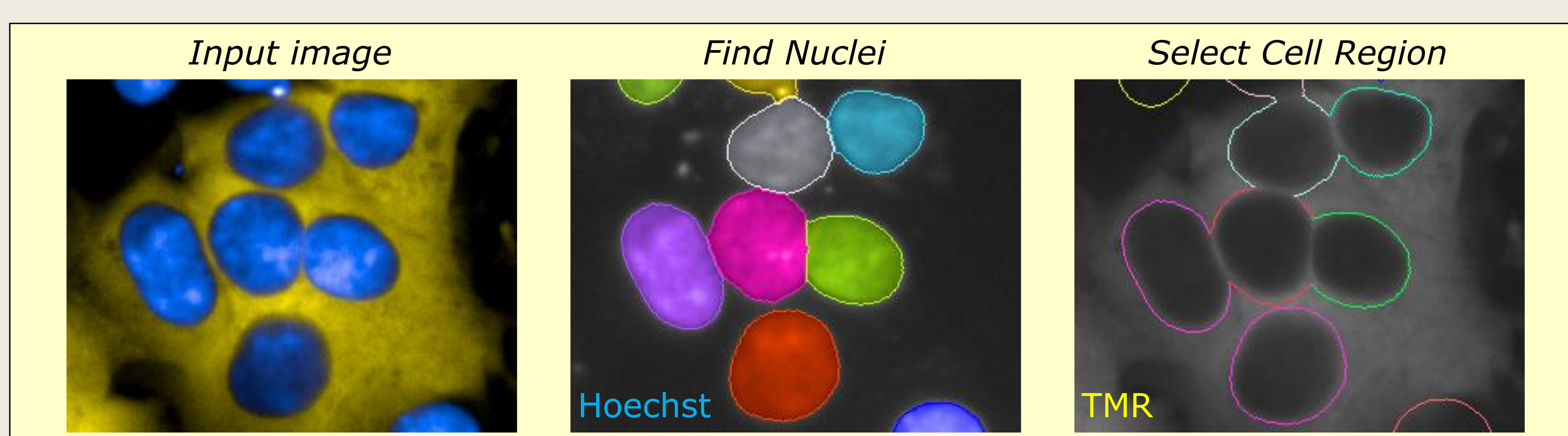

Figure 2. Image analysis strategy with our Harmony® High Content Imaging and Analysis Software. Individual cells were segmented based on the Hoechst channel image via the *Find Nuclei* building block of the Harmony Software. Subsequently, a ring region of 1 px width was generated around the nuclei. The ratio of TMR fluorescence in the nucleus and the cytoplasmic ring region was calculated as the final readout parameter.

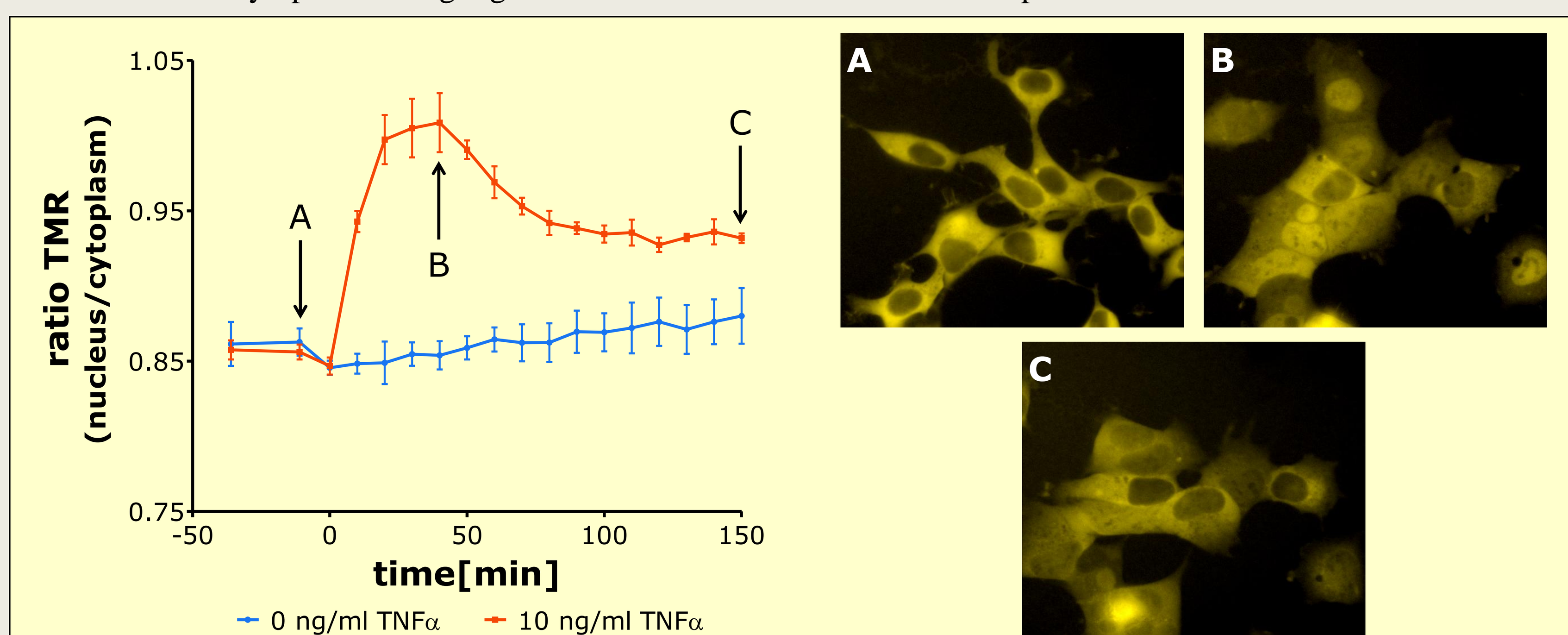

Figure 3. Time course of the nuclear translocation of p65. The fraction of nuclear p65 rapidly increases to a maximum after 30-40 minutes before it slowly decreases to a steady-state between nuclear import and export. Representative images for 3 time points are shown. (A) before stimulation, (B) maximum response, (C) steady-state. N=3 wells, Z'=0.7 at 30 minutes.

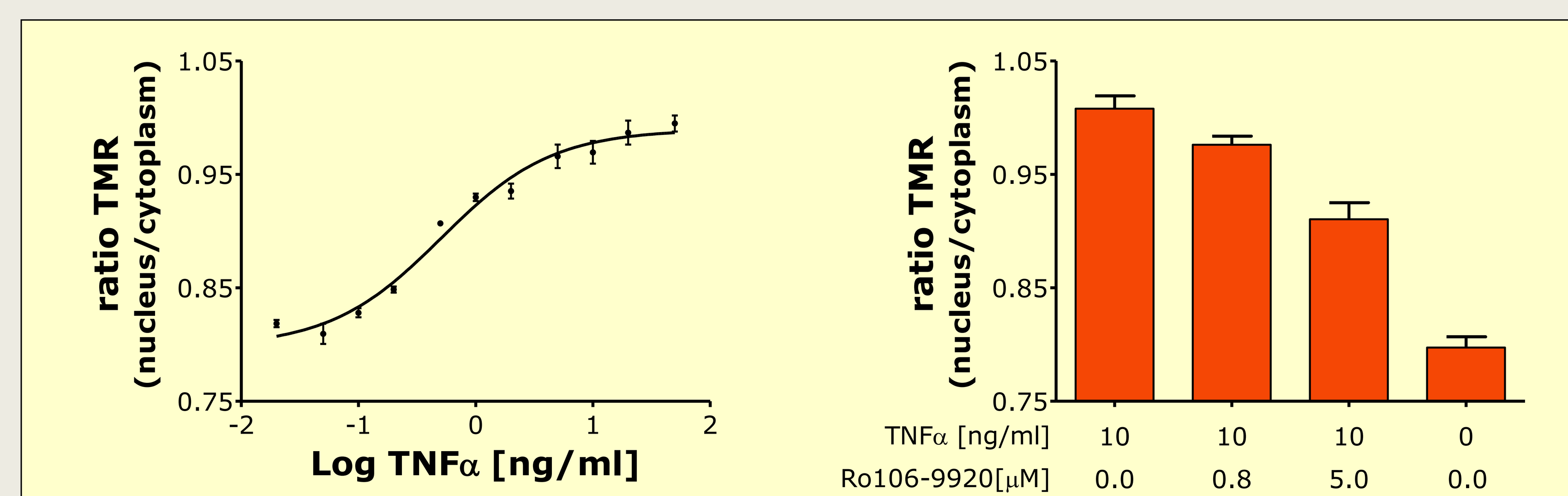

Figure 4. Population based analysis of NF-κB translocation. *Left*: Dose-response curve for TNFα on HEK293 p65-HT cells after 30 minutes of incubation. The calculated EC_{50} is 0.5 ng/ml. *Right*: Inhibition of NF-κB signaling by Ro106-9920 at 30 minutes post TNFα stimulation. The fraction of nuclear p65-HT decreases dose-dependently with increasing Ro106-9920 concentration. At concentrations higher than 5 μM Ro106-9920 was cytotoxic in our experiments. For both experiments N=3 wells, Z'≥0.7 .

4 Single cell-based analysis of NF-κB translocation

The induction of NF-κB signaling results in the expression of proinflammatory genes as well as the IκB gene. IκB expression provides a negative feedback mechanism to the cell. In combination with persistent TNFα stimulation this can result in oscillations in the signaling. Such oscillations in NF-κB signaling have been shown *in silico* and *in vitro* [Sung *et al.*, 2009] and their biological relevance has been demonstrated [Ashall *et al.*, 2009]. By performing automated single cell tracking we tested our model for the occurrence of oscillations in the nuclear translocation of p65-HT.

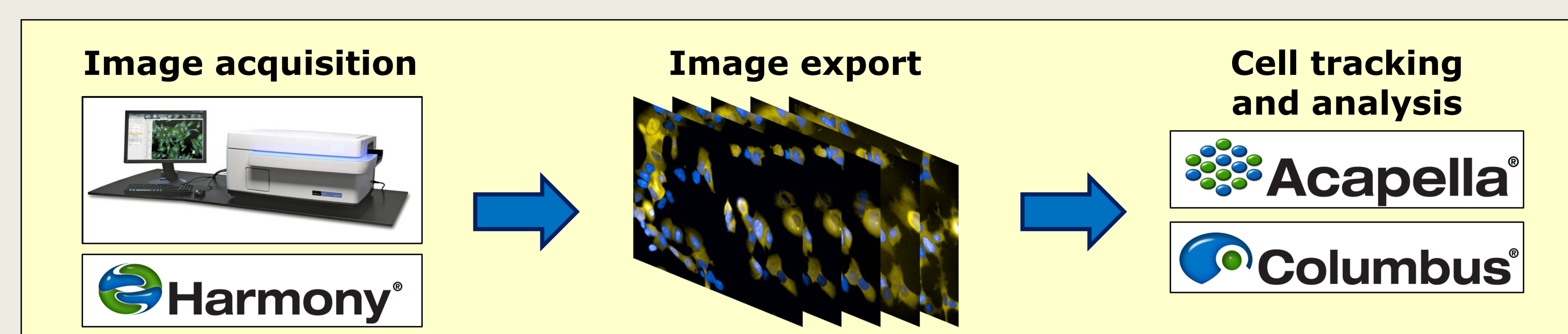

Figure 5. Workflow for automated single cell tracking. Images were acquired with the Operetta system controlled by the Harmony software. Images were exported from the Harmony software database and were analyzed with the Acapella software. The Acapella script used provides numerical properties of the tracked cells and an optional video of the cell tracking. The quality of cell tracking was controlled by visual inspection of a few tracking videos. Alternatively, the Columbus™ Image Data Storage and Analysis System allows for Acapella script-based image analysis, but the generation of tracking videos is not possible.

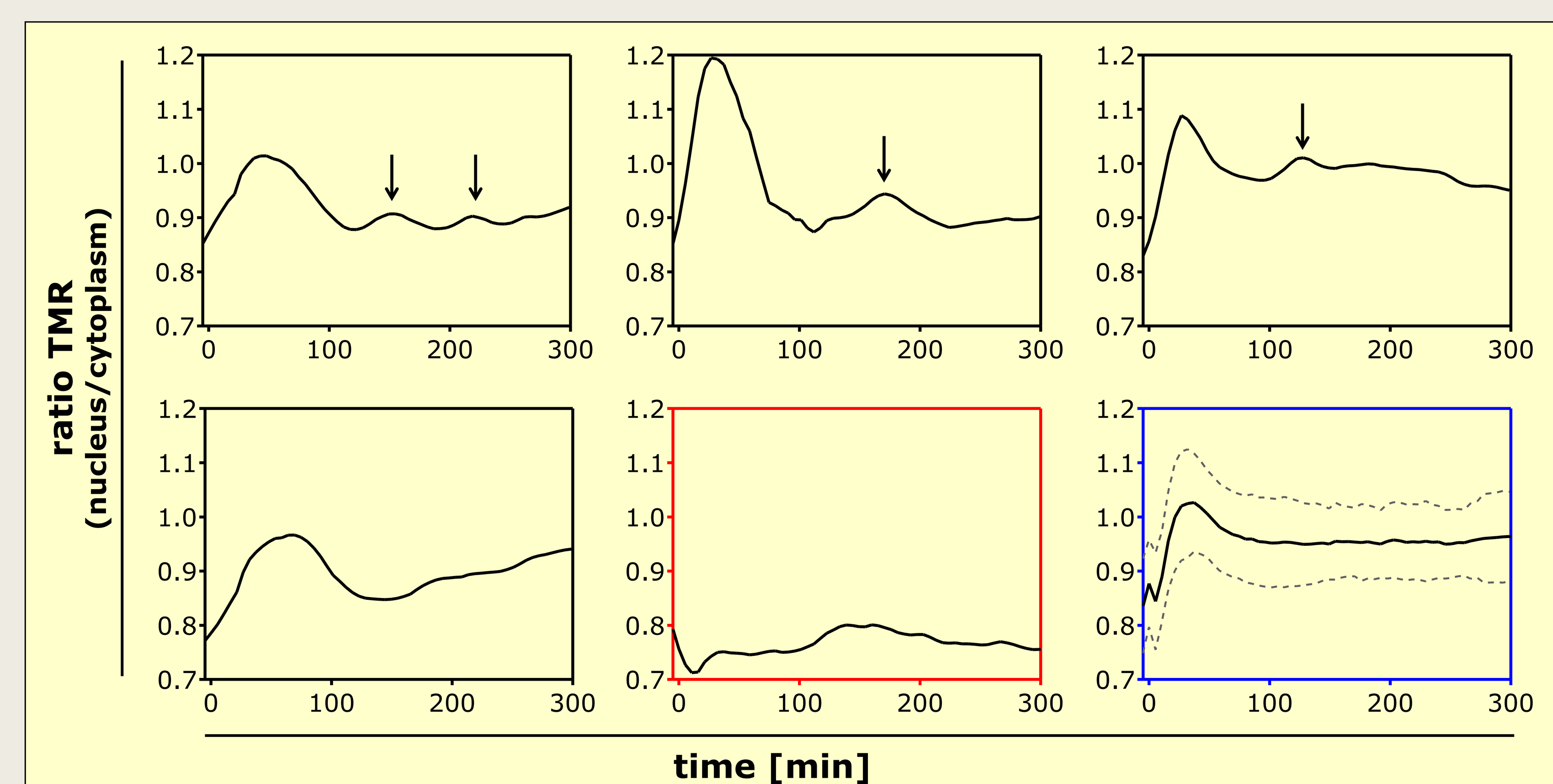

Figure 6. Selected single cell traces of HEK293 p65-HT cells treated with 10 ng/ml TNFα. *Black frames*: Most cells respond to TNFα treatment with a synchronous peak in nuclear NF-κB after 30 minutes. Some cells show a dampened oscillation in NF-κB localization (black arrows) caused by the cellular negative feedback mechanism and the persistent stimulation with TNFα. *Red frame*: A few cells do not respond to TNFα at all. *Blue frame*: Mean trace of 100 tracked cells. The dashed lines represent ± SD demonstrating a high cell-to-cell variability. More than one peak cannot be detected, since the oscillatory behavior of the cells is asynchronous.

5 Summary

Here, we investigate different aspects of the NF-κB signaling kinetics in living cells using the HaloTag® technology and the Operetta High Content Imaging System.
The low background and the interchangeable labeling possibilities render the HaloTag® an ideal tool for multiplexing in live-cell high content assays. Complementary, the Operetta system provides optimal conditions for long-term live-cell imaging without any detrimental effects on cell viability.
Furthermore, by generating time-resolved single-cell data using our sophisticated Acapella software, we revealed oscillations in NF-κB signaling that are masked in population-based data.

Ashall, *et al.* (2009): Pulsatile stimulation determines timing and specificity of NF-kappaB-dependent transcription. *Science*, 324 (5924), 242-246.
Sung, M.-H., *et al.*(2009): Sustained oscillations of NF-kappaB produce distinct genome scanning and gene expression profiles. *PLoS One*, 4 (9), e7163.

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