Interchangeable Labeling Technology and Its Complimentary Use in High Content Screening

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Abstract

The ability to specifically label proteins is a valuable technique for understanding their function in living cells. There are numerous commercially tools available for imaging live or fixed cells that express transfected protein or protein fusions, enabling researchers to study posttranslational modification of labeled fusion proteins. Activation of NFκB p65 can be measured by its translocation from the cytoplasm to the nuclear region of the cell. In this study, we used the HaloTag® interchangeable labeling technology to fluorescently label the NFκB p65 protein and quantitate its activation within living and fixed HEK293 cells stably expressing an NFκB p65-HaloTag vector. Comparable results between the interchangeable labeling technology and standard antibody-based NFκB High Content Screening (HCS) assays were found, exhibiting similar TNFa-dependent translocation of NFκB p65. To further assess the applicability of the labeling technology, stably expressing NFκB p65 HEK293 cells were transiently transfected with a TNFR siRNA in order to evaluate the knockdown of TNFR pathway on NFκB activation. Samples were fluorescently labeled with the HaloTag ligand, fixed, and analyzed using the Cellomics® ArrayScan® HCS Reader and image analysis software. When evaluating the amount of NFκB nuclear translocation with TNFa stimulation, control cells (transfection reagent only) expressed a 48% increase in NFκB activation, compared to a 12% increase in cells transiently transfected with the TNFR (SF11A) siRNA. Detecting biologically relevant differences in translocation, while maintaining cellular functionality makes the interchangeable labeling technology a viable option as a HCS fluorescent assay reagent.

Introduction

The HaloTag protein is a genetically engineered derivative of a hydrolase gene, designed to form an efficient covalent bond with the variety of HaloTag ligands. HaloTag protein is a monomer and can be fused to a protein of interest at either the N- or C-terminus. HaloTag ligands are chemical tags that comprise the HaloTag reactive linker and functional reporter. Once the protein is tagged with the HaloTag protein, it is expressed in mammalian cells, then differently labeled HaloTag ligands are used to visualize it, illustrated in Figure 1. Three fluorescent ligands (TMR, diAcFAM, and Coumarin), as well as Biotin-containing ligands are available for labeling the HaloTag protein. The HaloTag ligands readily cross the cell membrane, therefore, the HaloTag protein can be labeled and imaged either live or after fixation, shown in Figures 2 and 3. The HaloTag system allows for multiplexing and flexibility which makes it a complimentary system for High Content Screening assays.

NFκB is a transcription factor that is activated in response to ligands, such as IL-1a and tumor necrosis factor, and is associated with the activation of many cellular defense genes. NFκB is normally present in the cytoplasm as a complex with members of the IκB inhibitor family. Upon phosporylation and degradation of IκB, NFκB translocates to the nucleus. NFκB p65 is one of the end point molecules in the NFκB activation pathway. HEK293 cells were stably transfected with the NFκB p65-HaloTag protein and assessed for their ability to translocate NFκB using a traditional immunofluorensant antibody-based labeling assay and HaloTag ligand accordingly when activated with IL-1a or TNFa.

Additional siRNA knockdown experiments were done to assess the multiplexing ability of the HaloTag technology with the popular knockdown system. If the Tumor Necrosis Factor Receptor (TNFR) is sufficiently knocked down by the siRNA transfection, the ability of NFκB to translocate to the nucleus with TNFa stimulation should be suppressed.

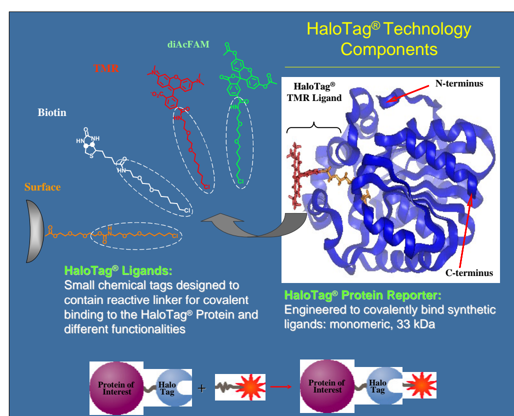

Figure 1: HaloTag Technology components include the protein reporter joined with the protein of choice and the ligand which allows the protein to be visualized and imaged.

Materials and Methods

HEK293 cells were transfected with NFκB p65-HaloTag fusion protein per Promega user documentation. Stably transfected NFκB p65 HEK293 cells were plated at 8,000 cells per well (cpw) in a 96-well collagen coated micro-titer plate. For live cell imaging, stably transfected NFκB p65 HEK293 cells were first labeled with HaloTag 5 μM TMR or 10 μM diAcFAM for 20 minutes, washed 2x with warm 1xPBS, allowed to recover for 3 hrs in complete growth media, then stimulated with IL1a (R & D Systems) at 10 ng/mL or human TNFa (Calbiochem) at 10 ng/mL diluted in complete growth media. The plate was then moved to a Cellomics ArrayScan VTI Live (set at 37°C, 5%CO_2) for image acquision and analysis. For fixed end point experiments, the same sequence was followed as in live cell labeling except after the 20 minute IL1a (10 ng/mL) or 30 minute TNFa (10 ng/mL) stimulation the cells were fixed with 3.7% formaldehyde and processed according to the Cellomics HCS Reagent Kit for NFκB Activation using Alexa Fluor® 488 for those cells pre-labeled with HaloTag TMR ligand or Alexa Fluor 555 for the cells labeled with HaloTag diAcFAM ligand.

For siRNA experiments, cells were plated at 2,000 cpw in 96-well collagen coated plates, 24 hrs later cells were transfected using DharmaFECT™ 1 with ON-TARGETplus™ siRNA TNFR(SF11a) (Dharmacon) or an appropriate control (media only or transfection reagent only) according to manufacturer's protocol. Cells were incubated for 48 hrs at 37°C, 5% CO_2, then labeled and stimulated according to fixed cell protocol above.

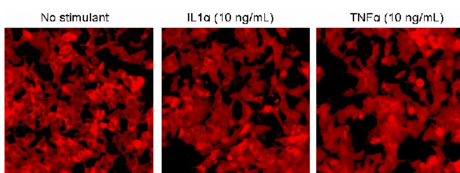

Figure 2: NFkB p65-HaloTag protein in HEK293 cells labeled with the HaloTag TMR ligand, activated with IL1a (10 ng/mL) or TNFa (10 ng/mL), then imaged and analyzed on the Cellomics VTI Live. Images were acquired at 20 minute and 30 minute incubation times respectively. Quantitative measurements of the cytoplasmic to nuclear translocation of the NFκB protein when stimulated IL1a or TNFa are made simultaneously as the images are acquired.

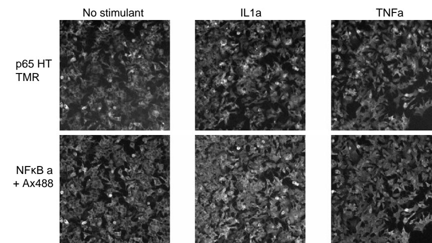

Figure 3: NFkB p65-HaloTag protein in HEK293 cells labeled with the HaloTag TMR ligand, stimulated with IL1a (10 ng/mL) or TNFa (10 ng/mL) for 20 minutes and 30 minutes respectively, then fixed and labeled with the antibody for NFκB and secondary Alexa Fluor 488. Images in the upper panel and lower panel are from the same field of cells, illustrating the translocation of the NFκB protein with the HaloTag TMR (red) and the NFκB + Ax488 antibody method (green) simultaneously.

Fixed-end Point NFκB Assay

The NFκB p65 HEK293 cells were stimulated with IL1a, TNFa or media only after HaloTag ligand labeling followed by fixation and standard immunofluorescent labeling to assess the translocation of the NFκB protein. Figure 3 above shows identical fields of cells labeled with HaloTag TMR ligand in red channel and NFκB antibody with Alexa 488 secondary in the green channel. Nuclear translocation of the NFκB protein with stimulation can be visualized in both. The assay results are shown in Figure 4, quantitative analysis of the NFκB protein translocation was done using the Cellomics Compartmental Analysis BioApplication as the cells were imaged on the HCS instrument. The ratio of NFκB protein intensity in the nucleus compared to the cytoplasm (i.e., CircRingAvgIntenRatio) increased approximately 2-fold with IL1a stimulation and 3-fold with TNFa addition compared to the no stimulant control of media only in both assays.

TNFR knockdown

The NFκB p65 HEK293 cells were transfected with the siRNA for TNFR (S11A) in an effort to knockdown the TNFR pathway, thus negating the TNFa stimulatory effects on the translocation of NFκB. A minimum of six replicate wells for each experimental case was used. As each well was imaged, the cells were tagged as positive responders if the nuclear NFκB intensity minus the cytoplasmic NFκB intensity was greater than zero, demonstrating a translocation of the NFκB from the cytoplasm to the nucleus (i.e., CircRingAvgIntenDiff). The histograms in Figure 6 were derived from the cell level statistics for a representative well with and without siRNA transfection and stimulation. Cytoplasm to nuclear translocation of NFκB occurred as expected in the untreated and transfection reagent only controls. The cells transfected with the TNFR siRNA showed knockdown of the TNFa pathway.

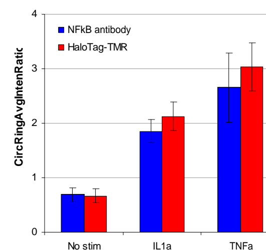

Figure 4: NFkB p65-HaloTag protein in HEK293 cells was comparably stimulated in both the HaloTag labeled assay and the standard immunofluorescent NFκB assay. The ratio of the fluorescent intensity of the NFκB in the nucleus (circ region) compared to the cytoplasm (ring region) is increased approximately 2-fold with IL1a and 3-fold with TNFa compared to the unstimulated control cells. Error bars refer to the standard deviation.

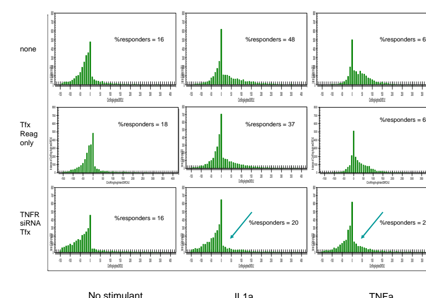

Figure 5: Specific inhibition of NFκB nuclear translocation in HaloTag NFκB-p65 HEK293 cells treated with TNFα and IL1a after TNFR siRNA transfection.

Results

Stably transfected NFκB p65-HEK293 cells were labeled with the HaloTag ligands and imaged live on the Cellomics ArrayScan VTI Live. Nuclear translocation of the NFκB protein in the cells was optimally observed and quantified using IL1a (10 ng/mL) and TNFa (10 ng/mL) at 20 minutes and 30 minutes respectively.

The standard immunofluorescent anti-body based assay to measure NFκB translocation was compared to the HaloTag ligand labeling in the stable cell line and both showed similar results with 2-3 fold increases in nuclear translocation of the NFκB protein in stimulated cells compared to control cells.

Specific inhibition of NFκB nuclear translocation in NFκB p65-HaloTag HEK293 cells treated with TNFα after TNFR siRNA transfection. 48 hours post-transfection, cells were labeled with HaloTag TMR ligand, treated with TNFα (10 ng/mL) or IL1α (10 ng/mL) for 20 min and 30 min respectively, NF-κB was immunolabeled, and the images acquired and analyzed with the Cellomics ArrayScan HCS Reader. %responders are defined as those cells with a difference of the fluorescent intensity of the NFκB in the nucleus compared to the cytoplasm greater than zero, a positive value indicates the translocation of NFκB. As expected, the difference of the fluorescent intensity of the NFκB in the nucleus compared to the cytoplasm increases when the cells are stimulated with IL1a and TNFa and decreased approximately 2-fold in the cells transfected with the TNFR siRNA. IL1a stimulation should not be affected by the knockdown of TNFR as it lies along IL-1R not the TNFR pathway. The positive knockdown effects can most likely be attributed to off-target effects of the siRNA. HaloTag ligand labeling and immofluorescently labeled NFκB assays showed comparable results when TNFR was knocked down with the siRNA.

Conclusion

The HaloTag technology from Promega is a viable quantitative assay solution for live and fixed cell imaging. It provides an alternative to GFP in live cell and fixed end point HCS assays. HEK 293 cells stably transfected with NFκB p65-HaloTag gave comparable results to the standard immunofluorscent labeling to assess the translocation of the NFκB p65 protein. Stable HaloTag cell lines give numerous multiplexing options. The stable NFκB p65-HaloTag HEK 293 cells transfected with a TNFR siRNA showed quantitative knockdown effects could be quantitated within the cells efficiently.

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