Screening of signaling events in live cells using novel GFP redistribution Assays.

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INTRODUCTION

The **LEADseeker™ Cell Analysis System** is an integrated screening system combining fast confocal line scanning with optimised software enabling real time data analysis of live cell assays at subcellular resolution. Amersham Biosciences have collaborated with BioImage A/S, in order to develop a range of assays for proteins that undergo intracellular translocation following inhibition/stimulation. We have generated chimeric constructs using a proprietary *Aequorea victoria* Green Fluorescent Protein (GFP). All assays follow a similar format in that stable cell lines which express the protein:GFP chimera are generated and validated. In this poster, we present three stable cell lines expressing NF-κB-GFP, GFP-Rac-1 and GFP-PLCδ-Plekstrin Homology (PH) domain chimeras. Each protein undergoes a functionally relevant translocation following activation. All assays were performed and all images were obtained and analysed on the LEADseeker Cell Analysis System.

RESULTS

In unstimulated Chinese Hamster Ovary CHO-hIR cells NF-κB-GFP (p65 Rel A-GFP) is located in the cytoplasm (green fluorescence). However upon stimulation by an agonist (e.g. cytokine) the protein translocates to the nucleus (red), as shown by the appearance of a yellow signal (fig 1). NF-κB-GFP translocates to the nucleus in a robust manner in response to a range of stimuli including IL-1β and IL-2.

Figure 1. NF-κB translocation

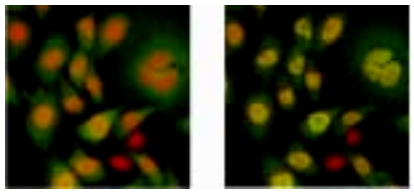

Figure 1. CHO-hIR cells were stably transfected with a p65-GFP (Rel-A-GFP) construct and selected by antibiotic resistance. Cells were plated in 96 well plates (5,000 cells per well). The following day, cells were stimulated with 40 ng/ml IL-1β for varying times. Draq5 nuclear stain (final concentration 5 μM) was added to all wells 15 minutes prior to imaging. The images shown were obtained before stimulation (control) and 40 minutes after agonist addition (stimulated).

Timecourse experiments reveal that translocation of NF-κB-GFP is rapid and observable as early as 5 minutes after agonist addition (fig 2). Maximal translocation is achieved 40 minutes after agonist addition.

Figure 2. Timecourse of NF-κB translocation in response to IL-1β

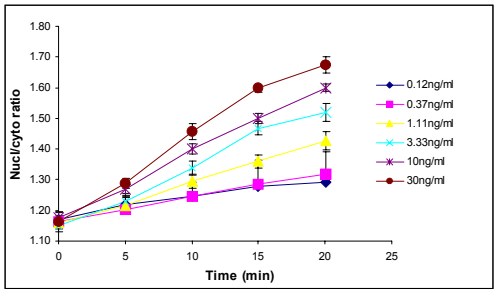

Figure 2. Experiment was performed as in the legend to figure 1. Data are means +/- S.D. from a 96-well plate, > 1000 cells per determination.

In unstimulated cells, the GFP-PLCδ-PH domain protein is principally localised to the plasma membrane, as evidenced by the peripheral green fluorescence seen at membrane edges. Following agonist addition, the GFP-PLCδ-PH protein is seen to move away from the cell periphery and hence the membrane (fig 3). This translocation is both rapid (occuring within seconds of agonist addition) and transient, with GFP-PLCδ-PH returning to the plasma membrane pre-stimulated state several minutes after agonist addition.

Data generated from fig 3 were analysed using a proprietary algorithm and it was found that the relative cytoplasmic intensity had increased in a reproducible fashion (fig 4).

Figure 3. PLC-PH translocation

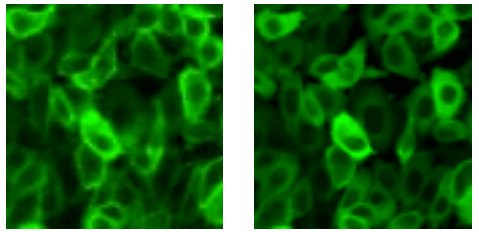

Figure 3. CHO-hIR cells were stably tranfected with a GFP-PLCδ-PH construct and selected by antibiotic resistance. Cells were plated in 96 well plates (5,000 cells per well). The following day, cells were stimulated with ATP (300 μM). The images shown were obtained prior to (control) or 20 seconds after agonist addition (stimulated).

Figure 4. Analysis of PLC-PH translocation

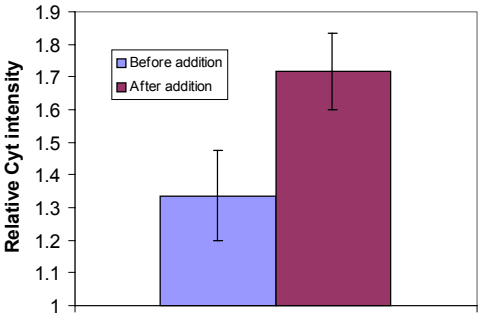

Figure 4. Analysis of PLC-PH translocation from Fig 3 using LEADseeker Cell Analysis System software. Data are means +/- S.D. from a 96-well plate, > 6000 cells per determination.

In unstimulated cells, the GFP-Rac-1 protein is principally localised to the plasma membrane, as evidenced by the peripheral green fluorescence. Following agonist addition, a significant proportion of the GFP-Rac-1 protein is seen to relocate to ruffling plasma membrane (fig 5). This localisation is transient, returning to near basal levels within 20 minutes of agonist addition.

Figure 5. Rac-1 translocation

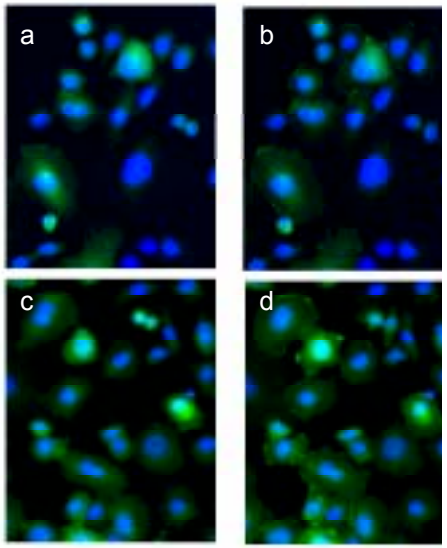

Figure 5. CHO-hIR cells were stably transfected with a GFP-Rac-1 construct and selected by antibiotic resistance. Cells were plated in 96 well plates at 5,000 cells per well. The following day, the cells were serum starved for two hours and then imaged prior to (a, c) or 7 minutes after the addition of agonist (b,d). The agonist used was either 10nM insulin (a,b) or 30nM Insulin like growth factor-I (IGF-1). Hoechst nuclear stain (final concentration 1 μM) was added to all wells 30 minutes prior to imaging.

CONCLUSIONS

The Construction of Protein:GFP chimeras using a proprietary GFP allow us to follow real time translocations of a range of molecules involved in signal transduction in live cells. We can thus bring difficult targets into the drug discovery arena.

Analysis of translocation events occurs in real-time using proprietary algorithms.

As the translocations are observed in live cells in a 96-well plate, this procedure is suitable for rapid screening of compounds that act as agonists or antagonists of the translocations.

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