Live Cell GFP Assays - Tools for Screening Key Cell Signalling Events

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Abstract
Monitoring the effect of potential new drug candidates on key intracellular signalling pathways is an important process in the drug development pipeline. Live cell GFP (Green Fluorescent Protein) assays can be used for screening such important events in a high throughput environment. A number of GFP assays have been developed; each performance validated assay is based on a mammalian cell line stably expressing an individual Aequorea victoria GFP tagged GNA construct. These enable dynamic mapping of the intensity, localization or translocation of the expressed GFP fusion protein when inhibited / stimulated by test samples. Images and data from a number of GFP assays generated using the IN Cell Analyzer systems will be presented.

Introduction
The development of each GFP assay for use in the screening environment involves thorough assay optimization, validation experiments, image analytic parameter optimization as well as assay performance and stability testing. The key experiments forming part of the development and optimization process are discussed with example images and data from our GFP assays portfolio.

Effect of assay media.
A cell screening assay monitoring the NFKB1 signaling pathway has been developed. Lymphoma was used as an example agonist to activate the cytoplasmic to nuclear translocation of EGFP-NFKB1 stably translocated in U-2 OS cells. To determine the effect of different assay media on the lymphoma induced EGFP-NFKB1 fusion protein translocation, the static U-2 OS cells were plated in Ham Nutrient Mixture F-12 Ham (Ham F-12) media with a range of additions (10 mM HEPEs, BSA, and FCS). As illustrated in Figure 1. The optimum assay media was found to be Ham F-12 supplemented with 10 mM HEPEs and 10% FCS. As illustrated in Figure 1. The optimum assay media was found to be Ham F-12 supplemented with 10 mM HEPEs and 10% FCS.

Timecourse of translocation event.
A cell screening assay monitoring the response of the Rac1 signaling pathway has been developed. Rac1 was used as an example agonist to activate the cytoplasmic to nuclear translocation of GFP-Rac1 stably translocated in CHO-HIR cells. Establishing the timecourse and therefore the time for maximum response of the Rac1 translocation provided valuable information, as illustrated in Figure 4.

Figure 1. The effect of different assay media on the lymphoma induced translocation of EGFP-NFKB1. Error ± SD, n = 4 replicates per data point.

Effect of cell density.
A cell screening assay monitoring the AKT1 signaling pathway has been developed. IGF-1 was used as an example agonist to activate the cytoplasmic to nuclear translocation of AKT1-EGFP stably translocated in CHO-HIR cells (Figure 2). Establishing the effect of cell density on the IGF-1 induced AKT1-EGFP fusion protein translocation was a key experiment in the development of the assay.

Figure 2. CHO-hIR AKT1-EGFP cells 4 mins after stimulation with control (A) and 2 µg/ml IGF-1 (B). Hoeschst* nuclear stain also present.

Figure 3. shows that significant differences were observed between stimulated (2 µg/ml IGF-1) and control populations at a range of cell densities. Seedings cells at a density of 0.6 x 10^4 cells per well was recommended.

Stability and Performance trials.
Once fully developed and optimized, assays are validated under underlying stability and performance trials. A cell screening assay monitoring the PI3K signaling pathway has been developed. PI3K inhibitors have been used to monitor the intracellular translocation of EGFP-tetracycline responsive fos fusion protein from its initial location bound to P-300 in early endosomes, to the cytoplasm in stably translocated U-2 OS cells. Table 1. shows a summary of the experiments conducted from 15 assays, performed to test assay stability on different occasions, giving an indication of inter assay variation. To assess assay stability the effect of passage number on assay performance was monitored (data not shown).

Figure 4. Timecourse of GFP-Rac1 translocation on stimulation with the reference agonist Insulin. Maximal response is seen after 6–8 min. Error ± SD, n = 6 replicates per data point.

Figure 5. IL-6 dose response curve using the EGFP-STAT3 cell line. Error ± SD, n = 4 replicates per data point. Data were collected 25 min after addition of agonist, and demonstrate an EC50 of 19.4 ng/ml (consistent with literature values), 95% CI = 17.5 - 21.2, R2 = 0.98.

Antagonist dose response data.
A cell screening assay monitoring the MAPKAP-k2 signaling pathway has been developed. Anisomycin was used as an example agonist to activate the cytoplasmic to nuclear translocation of GFP-MAPKAP-k2 fusion protein in stably transfected HIR cells. A key experiment in the development and validation of this assay was to monitor the dose dependent response of the antagonists SB203580 which significantly inhibits the kinase activity of p38 MAPK. In the presence of the agonist Anisomycin at EC50, 300 nM, the results are shown in Figure 7. Solvent sensitivity. A cell screening assay examining the Transforming Growth Factor (TGF-β1 signaling pathway has been developed. TGF-β1 was used as an example agonist to activate the cytoplasmic to nuclear translocation of EGFP-SMAD2 fusion protein in stably transfected CHO-HIR cells.

Figure 6. IL-6 dose response curve using the EGFP-STAT3 cell line. Error ± SD, n = 4 replicates per data point. Data were collected 25 min after addition of agonist, and demonstrate an EC50 of 19.4 ng/ml (consistent with literature values), 95% CI = 17.5 - 21.2, R2 = 0.98.

Table 1. The results of assays performed by different users on different occasions using the fully optimized assay methodology. SD shown in the standard deviation of the assays. 15 assays in total were performed, n=4 replicates per data point in each individual assay.

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Conclusions
A number of key experiments forming part of the GFP assay development, optimization and validation process have been presented.

These GFP assays have been developed for use in the screening environment and will prove to be important tools in the development of new drug candidates.

GFP Assays are compatible with a range of subcellular imaging systems such as the IN Cell Analyzer platforms (only data from IN Cell Analyzer 3000 and associated analysis modules shown).