Detecting the FRET Response of the GeneBLAzer® Cell Line D1 CRE-*bla* CHO-K1 to Agonists and Antagonists using Microplate Cytometry

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Abstract

The GeneBLAzer® CHO.K1-D1 cell line (Invitrogen) stably expresses both the β-lactamase gene downstream of the cAMP response element (CRE) and the dopamine D1 receptor. Stimulation of the cells with dopamine D1 receptor agonists, results in transcriptional activation of the β-lactamase gene through CRE. A FRET-enabled substrate (CCF4-AM) fluoresces green, in the absence of β-lactamase reporter activity, and blue when cleaved. This technology has been measured by bulk fluorescence readers, which report data on a whole well basis. In this study, the violet laser in the Acumen Explorer 405 microplate cytometer was used to excite CCF4-AM substrate and the resulting fluorescent emissions simultaneously detected in the blue and green channels. High content data for dopamine D1 receptor activation were calculated from ratios of blue to green fluorescence in cell populations. We have shown that the Acumen Explorer 405 has the ability to accurately measure β-lactamase activity, and generates good fold activations above baseline. We have also shown that we can obtain toxicological data from the same plate, thereby providing additional, valuable information during the screening process. When coupled with short read times of less than 10 minutes per plate, this technology provides an excellent opportunity for functional genomic applications.

Introduction

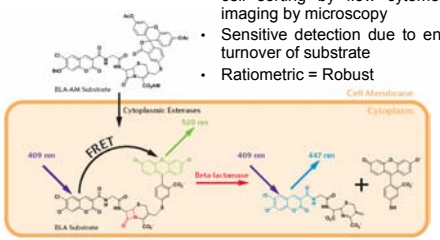
G-protein coupled receptors (GPCRs) represent the largest and most frequently screened class of receptors. GPCRs, regardless of Gα subunit coupling (Gs, Gq, Gi/Go), can be monitored using β-lactamase. Stable cell lines expressing the NFAT response element for monitoring Ca^{2+} flux) or the cAMP response element (CRE) linked to the β-lactamase gene have been developed as a functional tool to identify GPCR activation[1].

β-lactamase activity has conventionally been analysed by bulk fluorescence readers[2] or flow cytometers[3], both of which require large numbers of cells (>10^5) for accurate analysis. Flow cytometry, although highly sensitive, has the added disadvantages of low throughput, and an inability to analyse adherent cell lines *in situ*. Microplate cytometers, such as the Acumen Explorer, provide an alternative method for analysis and reporting of data, requiring as few as 100 cells per well in a 96 well plate. The Acumen Explorer rapidly analyses the whole well simultaneously collecting up to 4 colours. Removing the need for repeated collection of fluorescent emissions, thus decreasing the overall read time in multiplex assays[4], and allowing a high screening potential comparable with current throughputs of many primary screening campaigns[5]

Here we present an alternative method of β-lactamase analysis using the Acumen Explorer fluorescence microplate cytometer. This instrument is capable of scanning the whole well within a 96 well microplate, reporting ratiometric data for β-lactamase activation on a cell-by-cell as opposed to well-by-well basis, resulting in an increased assay window. We have used the Acumen Explorer equipped with a 405nm solid-state laser to analyse D1 receptor activation. Scanning the whole well allows the total cell number to be determined, which can be used as a toxicological indicator. This partnership enables an efficient method of detection and analysis of cell lines expressing the β-lactamase reporter gene system.

1 GeneBLAzer® : β-lactamase (β-lac) Technology

- No mammalian background, bacterial/ fungal origin enzyme
- Fluorescent readout, allows for live cell sorting by flow cytometry & imaging by microscopy
- Sensitive detection due to enzyme turnover of substrate
- Ratiometric = Robust

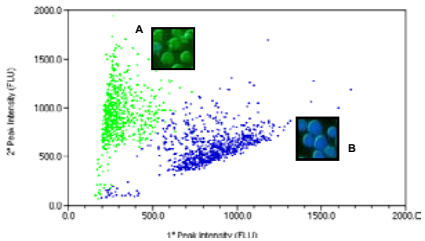


Ratiometric, FRET-Based Reporter Gene Technology

2 Assay Protocol

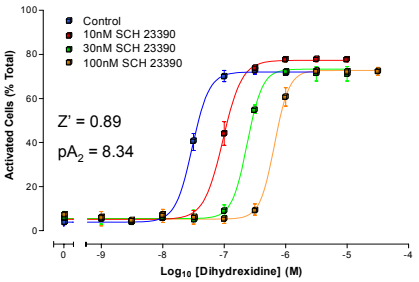
- D1 CRE-*bla* CHO-K1 cells were cultured to 50% confluency. Cells were harvested and resuspended in DMEM + 10% dialysed FBS.
- Cells were seeded to a 96 well plate at 4,000 cells/well and allowed to adhere overnight (37°C/5% CO_2). The assay protocol was followed as described by Invitrogen method 839-040859 - kit # K1086.
- For antagonist experiments, cells were pre-incubated in the presence of antagonist concentrations for 30 minutes prior to the addition of dihydrexidine.
- The toxin saponin was used as a cytotoxic control at a concentration of 0.01% w/v in serum free DMEM.

3 Scatter Plot of Inactive and Active D1 CRE-*bla* CHO-K1 cells



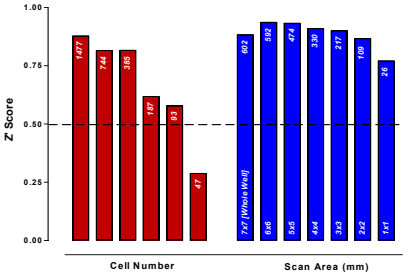
Plot showing Inactive (A, green) and Active (B, blue) cells expressing β-lactamase reporter gene. Note the heterogeneity in green and blue fluorescence for each population.

4 Antagonism of Dihydrexidine Activation by SCH-23390



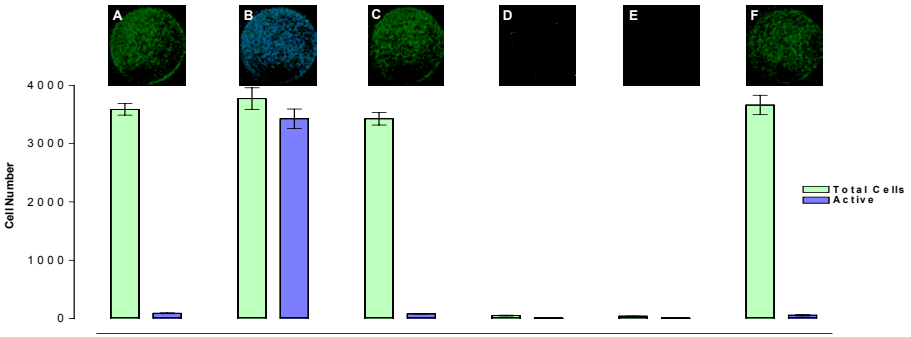
SCH-23390-concentration dependent shift of dihydrexidine curves (data represent means ± S.D. of 4 replicates and are representative of results obtained from 3 separate experiments).

5 Effects of Cell Number and Scan Area on Experimental Z' Score



Cell number was varied from 50 – 1,500 cells per well – Z' > 0.5 was achieved down to 100 cells. Reducing scan area had little effect on Z' but reduced plate read time (whole well – 9 min; 1x1 mm – 3.5 min)

6 Use of Absolute Cell Number as a Toxicity Indicator



Whole well scans: A: Control, B: Dihydrexidine 1μM, C: Dihydrexidine 1μM with SCH23390 100nM, D: Saponin 0.01% w/v, E: Saponin 0.01% w/v with Dihydrexidine 1μM, F: SCH23390 only. Saponin (0.01%) was used to achieve 100% cell death which was detected as an absence of viable cells using the Acumen Explorer 405. Figure F showing SCH23390 antagonist alone, displays control levels of viable cells demonstrating that antagonism is distinguishable from cytotoxicity (saponin, D).

Conclusions

- High content analysis of β-lactamase reporter gene assays reports data on a per-cell basis suitable for antagonist profiling.
- Acceptable Z' scores were obtained using cell numbers as low as 100 cells per well.
- Cell number per well can distinguish D1 antagonism from cytotoxicity effects permitting a multiplex readout with a standard GeneBLAzer® assay.
- The combination of Acumen Explorer 405 and GeneBLAzer® reporter gene technology equips the drug discovery community with a powerful new partnership for use in drug discovery.

References

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