



Combining a Fluo-4 Calcium Mobilization Assay and the Beta-Lactamase Reporter System: Two GPCR Screens with One Cell Line

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Abstract



G-protein coupled receptors (GPCRs) represent the largest and most frequently screened class of receptors. Activation of certain sub-classes of GPCRs results in intracellular calcium mobilization. Calcium mobilization assays that make use of dyes that become highly fluorescent in the presence of calcium are commonly used GPCR screening tools. Here we take one such calcium mobilization assay, a Fluo-4 Assay, and multiplex it with the highly sensitive beta-lactamase reporter system. We show that a Fluo-4 and a beta-lactamase assay can both be run on a stable cell line expressing a Gi/o coupled GPCR, a promiscuous G protein, and beta-lactamase linked to an NFAT reporter in a homogeneous assay which allows data to be acquired with two independent detection methods. Results from a screen of the 5HT1a cell line with the LOPAC™ library of small molecules are shown. In the future, it should also be possible to extend this technology to screening two different GPCR targets responding through different signaling pathways within the same well thus providing selectivity information.

Introduction

GPCRs represent the largest and most frequently screened class of receptors. Activation of one sub-class of GPCRs, the G_q coupled receptors, results in stimulation of phospholipase C and intracellular calcium mobilization. Two of the most popular methods of high throughput screening for GPCRs are Ca^{2+} flux assays and reporter gene assays. In reporter gene assays, GPCR signaling can be monitored by activation of specific transcriptional response elements placed 5' to a reporter gene whereas calcium flux assays make use of dyes that become highly fluorescent in the presence of calcium. Here we take one such calcium mobilization assay, a Fluo-4 assay, and combine it with the highly sensitive beta-lactamase reporter system. The assay was conducted with CHO-K1 cells stably expressing the 5HT1a serotonin receptor and the promiscuous $G_{\alpha 15}$ protein which redirects the $G_{i/o}$ coupled 5HT1a receptor pathway to the G_q coupled, calcium signaling pathway. This cell line also stably expresses the beta-lactamase reporter gene under the control of an NFAT response element. We show that a Fluo-4 and a beta-lactamase assay can both be run on this cell line in a homogeneous assay which allows data to be acquired with two independent detection methods. Results from a screen of the 5HT1a cell line with the LOPAC™ library of small molecules are shown. In the future, it may be possible to extend this technology to screening two different GPCR targets responding through different signaling pathways within the same well thus providing selectivity information.

Figure 2—GPCR signaling monitored by Beta-Lactamase

G-protein coupled receptors (GPCRs), regardless of G_{α} subunit coupling (G_s , G_q , G_i/G_o) can be monitored using beta-lactamase. Stable cell lines expressing the NFAT response element (for monitoring Ca^{2+} flux) or the cAMP response element (CRE) linked to the beta-lactamase gene have been developed. These Master cell lines can be used as building blocks to develop target-specific GPCR assays. G_i -coupled receptors can be transfected into the Ca^{2+} -responsive NFAT-bla cell line and G_s -coupled receptors can be transfected into the cAMP-responsive CRE-bla cell line to follow agonist or antagonist binding events. To track $G_{i/o}$ -coupled GPCRs, the promiscuous G-protein, $G_{\alpha_{15}}$, can be co-transfected with G_i -coupled receptors into the NFAT responsive cell line to re-direct $G_{i/o}$ -coupled signaling to the G_q pathway. Upon stimulation (agonist binding) these cell lines respond with an increase in β -lactamase expression. The β -lactamase response can be quantified using the FRET-based substrate, CCF4-AM, and a standard fluorescence plate reader.

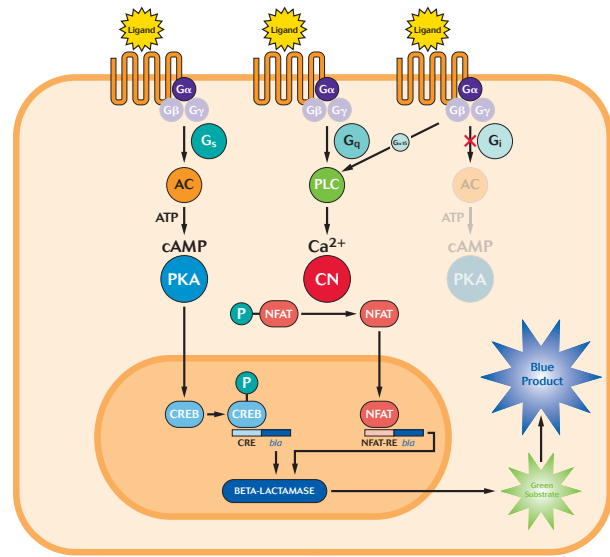
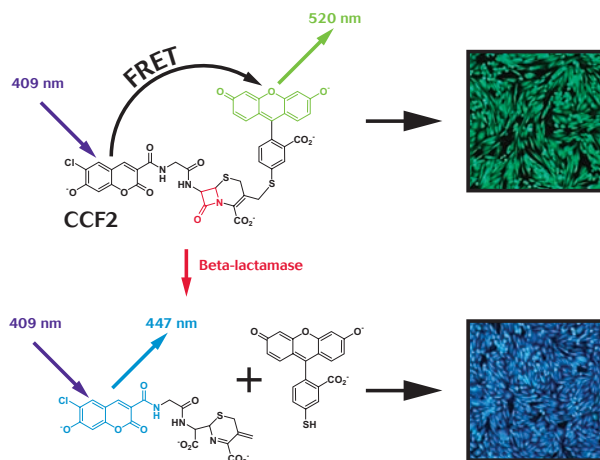
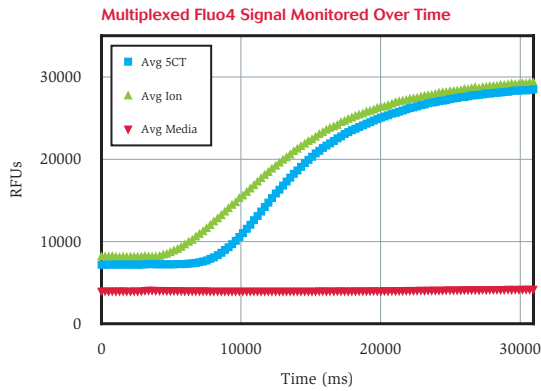


Figure 1—The GeneBLazer® Beta-Lactamase Reporter System



CCF4-AM is a fluorescence resonance energy transfer (FRET) based substrate for beta-lactamase. Once CCF4-AM enters a cell, it is converted to the negatively charged CCF4 by endogenous esterases. Excitation of this substrate at 409 nm leads to efficient FRET between the coumarin and fluorescein moieties, resulting in green fluorescence detectable at 530 nm. Expression of β -lactamase leads to cleavage of CCF4 and results in a loss of FRET, resulting in a robust blue fluorescent signal detectable at 460 nm.

Figure 4—GPCR Signaling Monitored by Fluo-4



The 5HT1a-Gα15-NFAT-*bla* CHO-k1 stable cell line was loaded with 2 μM Fluo-4-AM in a solution containing probenecid, to inhibit export by drug pumps, and Solution C (a background suppression dye used with the beta-lactamase reporter system) for 45 minutes at room temperature. A GENios Pro Multifunctional reader was used in kinetic mode to inject either media alone, a 5HT1a serotonin receptor agonist (5-Carboxamidotryptamine maleate), or the Ca²⁺ ionophore (ionomycin). An increase in fluorescence signal is detected with both the 5HT1a agonist and with the Ca²⁺ ionophore but is not seen when media alone is added.

Figure 3—The Fluo-4 Ca²⁺ Mobilization Assay

The Ca²⁺ indicator Fluo-4 has been used extensively in high-throughput screening assays for drug discovery. Fluo-4 is essentially non-fluorescent in the absence of Ca²⁺ and exhibits a calcium dependant increase in fluorescence emission upon binding Ca²⁺. Shown is the fluorescence emission spectra for the closely related Ca²⁺ indicator, Fluo-3, at various Ca²⁺ concentrations.

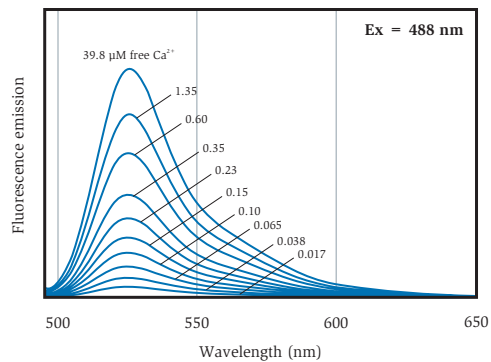


Figure 5—Multiplexing Fluo-4 with the Beta-Lactamase Reporter System

The 5HT1a-Gα15-NFAT-*bla* CHO-K1 cell line was loaded with Fluo-4 as above. Following the Fluo-4 reading, the plate was incubated at 37°C for 5 hours then loaded at room temperature for 2 hours with the fluorescent, ratiometric beta-lactamase substrate, CCF4-AM. Beta-lactamase response ratios are calculated by taking the blue:green ratio of stimulated cells and dividing by the blue:green ratio of unstimulated cells. The Z'-factor and Response Ratio were calculated from replicates of 10 wells. The Z'-factor and the Response Ratios from the beta-lactamase assay were unaffected by multi-plexing with Fluo-4.

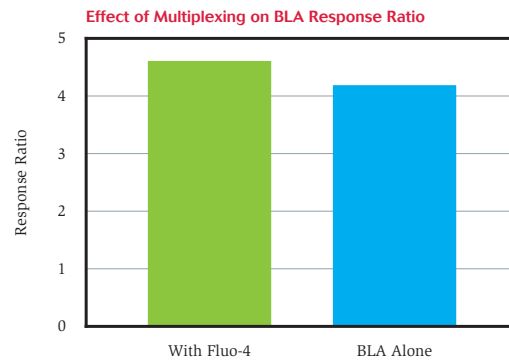
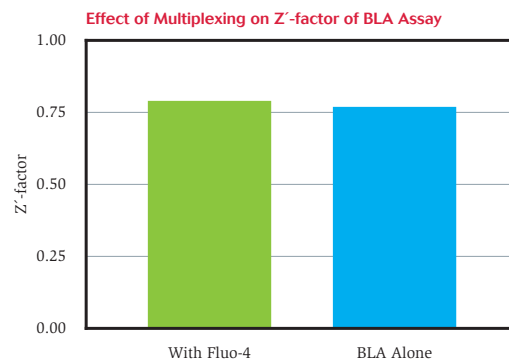
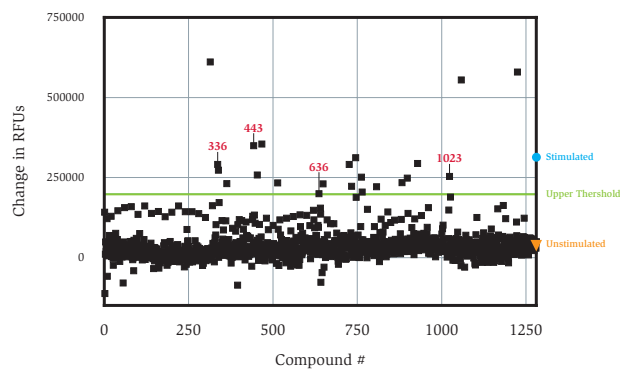


Figure 6—Results from LOPAC¹²⁸⁰™ Screen of 5HT1A Fluo-4 Assay

The 5HT1a-NFAT-*bla* CHO-K1 cells were loaded with Fluo-4 as above, and the assay was run in homogeneous format with the LOPAC¹²⁸⁰™ library of small molecules to search for compounds that trigger the Ca²⁺ flux pathway upon binding to the serotonin receptor. The 5HT1a cell line was able to detect serotonin ligands present in the library with a high degree of subtype selectivity. Several 5HT1a serotonin receptor subtype selective compounds were detected including: #336, N, N-Dipropyl-5-carboxamidotryptamine maleate; #443, 5-Carboxamidotryptamine maleate; #636, R-(+)-8-Hydroxy-DPAT hydrobromide; #1023, PAPP.

**Figure 7—Results from LOPAC¹²⁸⁰™ Screen of 5HT1A Beta-Lactamase Assay**

The 5HT1a-Gα15-NFAT-*bla* CHO-k1 assay was also run in agonist mode with the LOPAC¹²⁸⁰™ library of small molecules using beta-lactamase as the detection method. Once again the 5HT1a assay was able to detect serotonin receptor ligands in the library with a high degree of subtype selectivity. The beta-lactamase assay was able to detect an additional 5HT1a subtype selective compound (#746, Oxymetazoline hydrochloride) missed in the Fluo-4 assay.

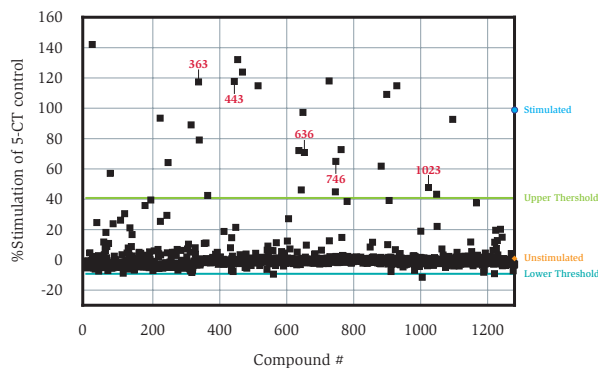


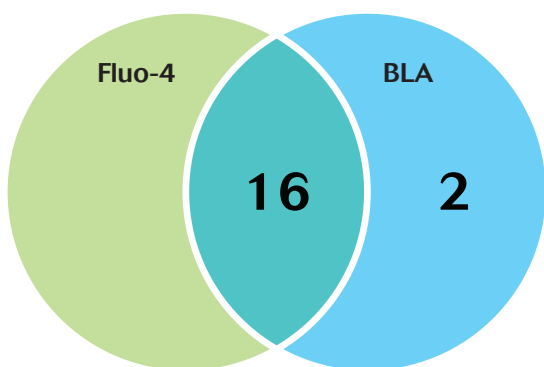
Figure 8—Statistical Data Determined from Screen

Parameter	Fluo-4 Assay		BLA Assay	
	# of Compounds	%	# of Compounds	%
Hit Rate	22	1.7	22	1.7
Known 5HT _{1A} Agonist detected	16	1.2	18	1.4
Other agonists detected	6	0.5	4	0.3
Non-5HT_{1A} serotonin receptor selective compounds in LOPAC^{1280TM}	15	1.1	15	1.1
Non 5HT _{1A} subtype selective compounds detected in screen	0	0	0	0
5HT_{1A} serotonin agonist not detected	6	0.5	5	0.4
Partial agonist	3	0.2	3	0.2
Racemic compound (active isomer was detected)	1	0.1	1	0.1
Other Agonist	2	0.2	1	0.1

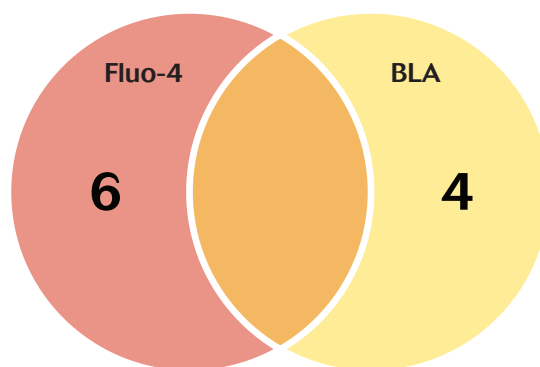
Figure 9—Venn Diagram Showing Overlap of Hits Between Fluo-4 and BLA Assay

Out of 18 known serotonin receptor agonists detected by the Beta-lactamase assay, 16 were also detected by the Fluo-4 assay. In contrast, the 4 other compounds which were detected by the Beta-lactamase assay were not detected by the Fluo-4 assay, and the 6 additional compounds detected by the Fluo-4 assay were not detected by the beta-lactamase assay. Therefore, using both assays can serve as a validation of hits to determine which ones are true agonists.

Known Serotonin Receptor Agonists Detected



Other Compounds Detected



Conclusions

- A Fluo-4 assay and a beta-lactamase assay can be multiplexed into one homogeneous assay without affecting the Z'-factor or response ratio of the beta-lactamase assay.
- Data from a screen with the LOPAC^{12807™} library of small molecules indicates that the Fluo-4 assay and the beta-lactamase assay give very similar results.
- The beta-lactamase assay was able to detect two known serotonin receptor agonists (including one 5HT1a subtype selective compound) that were not detected with the Fluo-4 assay.
- The GeneBLAzer® GPCR assays which signal through the Ca²⁺ pathway can also be assayed with traditional Ca²⁺ indicators such as Fluo-4.
- Using both a beta-lactamase assay and a Fluo-4 assay allows for two independent modes of detection for a screen to verify hits.
- In the future, it may be possible to extend this technology to screening two different GPCR targets responding through different signaling pathways within the same well thus providing selectivity information.



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