Cytometry
Journal of the International Society for Advancement of Cytometry

Development of a Robust GABA$_B$ Calcium Signaling Cell Line Using β-Lactamase Technology and Sorting

Mei Cui,[1] Fu-Zon Chung,[2] Christopher J. Donahue[3]*

[1]Cancer Biology, Pfizer Global Research & Development, 10724 Science Center Drive, San Diego, California 92121

[2]Department of Pathology, University of Michigan, Ann Arbor, Michigan 48109

[3]Genetically Engineered Mouse Models COE, Pfizer Global Research & Development, Eastern Point Road, Groton, Connecticut 06340

*Correspondence to: Christopher Donahue, Genetically Engineered Mouse Models COE, Pfizer Global Research and Development, Eastern Point Road, Groton, CT 06340, USA

Email: christopher.donahue@pfizer.com

- **Abstract**
The GABA$_B$ receptor is a member of the "family 3" G protein coupled receptors. The GABA$_B$ receptors modulate activity inwardly rectifying potassium channels and high voltage activated calcium channels. The GABA$_B$ receptors require heterodimerization between two subunits, GABA$_{B1}$ and GABA$_{B2}$, for functional expression. A robust functional calcium cell line was developed that contained both the human truncated GABA$_{B(1b)}$ and human truncated GABA$_{B(2)}$ receptors. The cell line was analyzed and sorted using β-lactamase as a reporter. Single cell clones were sorted and isolated using flow cytometry based on high β-lactamase expression. The single cell clones were further tested in a 384-well calcium mobilization assay using the Fluo-4 AM calcium indicator on the fluorescent imaging plate reader system (FLIPR). Twenty-seven clones were grown up from single cell collections and 10 clones demonstrated a high response to GABA stimulation. The 10 clones were re-evaluated based on agonist dose response and EC$_{50}$. Clone-16 was identified and utilized in high throughput screening (HTS) assay development. Using sorting and β-lactamase as a reporter, we were able to develop a robust, functional cell-based, GABA$_B$, calcium mobilization assay. The cell line described here can be used for high throughput FLIPR screening and also to compare and rank the potency and selectivity of agonists, antagonists and potentiators of the GABA$_B$ receptor. © 2008 International Society for Advancement of Cytometry

- **Key terms**
β-lactamase; calcium assay; CCF4; FLIPR; flow cytometry; Fluo-4; GABA$_B$; sorting

GABA$_B$ receptors are metabotropic transmembrane receptors for gamma-aminobutyric acid (GABA) that are linked via G-proteins to potassium channels (1). These receptors are found in the central and peripheral autonomic nervous system (2). There are two subtypes of the receptor, GABA$_{B1}$ and GABA$_{B2}$, which need to assemble as a heterodimer to be functional (3,4). The GABA$_{B1}$ subunit consists of the extracellular binding site, whereas the GABA$_{B2}$ subunit mediates G-protein coupled signaling (5,6). The two subunits assemble and interact allosterically, such that GABA$_{B2}$ increases the affinity of GABA$_{B1}$ for agonists and GABA$_{B1}$ facilitates the coupling of GABA$_{B2}$ to G-proteins (6,7). The activation of GABA$_B$ causes an inhibition of adenylate cyclase activity together with a decrease in calcium or inwardly rectifying potassium channels (3). This activation prevents sodium channels from opening, and thus inhibits neurotransmitter release.

The G protein-coupled GABA$_B$ receptor was described over 20 years ago yet only recently has been cloned (2). With this, has come the identification of its unique heterodimeric structure. It is crucial to determine if GABA$_B$ receptor subtypes exist, and whether or not they can be exploited pharmacologically to determine if other proteins or small molecules can bind with GABA$_{B1}$ to form a functional receptor, to establish whether the receptor has other cellular functions, and to assess the clinical value of GABA$_B$ agonists, antagonists, and positive allosteric modulators.

The ability to screen millions of compounds in a chemical library to identify potential drug candidates is a valuable asset for any pharmaceutical drug research

ISAC
International Society for Advancement of Cytometry

and development program. The calcium flux assay is the most popular high throughput screening approach because it is a rapid and direct signal for biological activity of GPCRs. The measurement of intracellular calcium response in cells is a popular functional assay for GPCRs.

The FLIPR system allows for the rapid and kinetic determination of the rise in cytosolic calcium levels using cell permeable calcium sensing fluorescent indicators such as Fluo-4 AM (8). The FLIPR was developed as a high throughput screening instrument for cell-based, fluorescent screening (9). The FLIPR system is unique because it can read all the wells of a 96, 384, or 1,536-well microplate simultaneously, with kinetic updates in under a second (9,10). The FLIPR assay has become one of the primary assays for measuring GPCR function because it is amenable to robotic automation and miniaturization to 1,536-well microplates (10).

To develop a sensitive and robust high throughput screening cell line the reporter must be compatible with flow cytometry and sorting. The success of developing a cell-based assay depends entirely on the quality of the reporter gene. β-lactamase is an ideal reporter system for monitoring gene expression in live cells (11). β-lactamase is a reporter system that can be used to monitor gene expression and is also amendable for flow cytometry and sorting (12,13). Neither the β-lactamase enzyme nor the CCF4 AM substrate is toxic to live cells and thus can be used to rapidly develop stably transfected clonal cell lines (12,13). β-lactamase provides a reliable marker for gene expression in a variety of cell types, and can be used with flow cytometry to isolate low and high expressing clonal populations (13).

The coexpression of G-proteins with Gq chimeras allows for the measurement of the calcium response through the Gq pathway (14–16). A cell line was developed that contains both GABA$_{B1}$ and GABA$_{B2}$ receptors, which were cloned and coexpressed in Gqi3 (5), containing CHO cells (Fig. 1). The cell line was analyzed and sorted using β-lactamase as a reporter. GABA$_B$ receptor activation triggers calcium signaling through the binding and activation of G-proteins and β-lactamase expression is activated from the NFat response element upon stimulation of Gq-coupled receptors. Single cell clones were sorted and isolated using flow cytometry based on high β-lactamase expression. High responders expressing GABA$_B$ receptor clones were further tested and evaluated on the FLIPR. Using an isolated single-cell clone, a sensitive FLIPR, calcium-based functional assay has been established and validated.

MATERIALS AND METHODS

Materials and Reagents

The β-lactamase substrate CCF4-AM, the cell culture and molecular cloning reagents were purchased from Invitrogen (Carlsbad, CA). Propidium iodide (PI) and the calcium indicator Fluo-4 AM were purchased from Molecular Probes (Eugene, OR). GABA was purchased from Sigma (St. Louis, MO), and the GABA$_B$ antagonist CGP54626 was purchased form Tocris Bioscience (Ellisville, MO). The 384-well clear
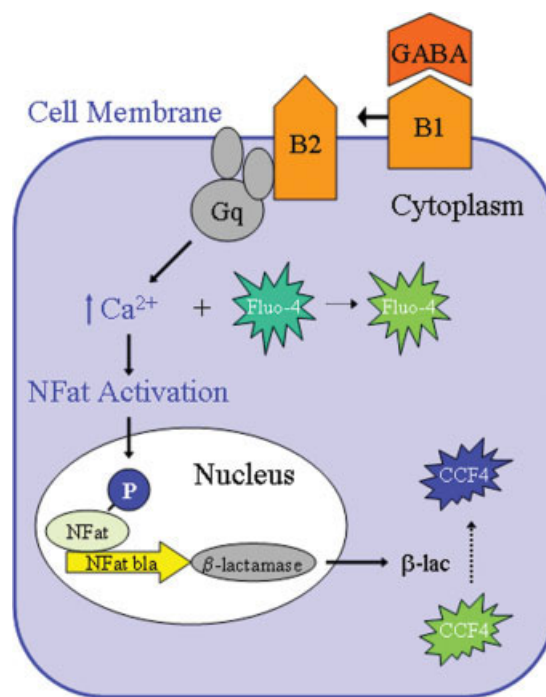

Figure 1. Schematic illustration of the GABA$_B$ signaling pathway and the use of CCF4 AM or Fluo-4 AM to monitor calcium mobilization or β-lactamase expression. The GABA$_{B1}$ subunit consists of the extracellular binding site, whereas the GABA$_{B2}$ subunit mediates G-protein coupled signaling. The two subunits assemble and interact allosterically, such that GABA$_{B2}$ increases the affinity of GABA$_{B1}$ for agonists and GABA$_{B1}$ facilitates the coupling of GABA$_{B2}$ to G-proteins. GABA$_B$ receptor activation triggers calcium signaling through the binding and activation of G-proteins. β-lactamase expression is activated from the calcium sensitive NFat response element (NFat bla). Fluo-4 AM is a green fluorescent indicator, which exhibits a calcium dependent increase in fluorescence emission upon calcium binding. CCF4 AM is a fluorescent resonance energy transfer based substrate for β-lactamase. β-lactamase cleaves CCF4 AM disrupting the FRET resulting in a change of fluorescence from green to blue.

bottom black plates were purchased from VWR (West Chester, PA).

Plasmid Construction

GABA$_B$ receptors without the intracellular coiled-coil domains were designed and used. Truncated human GABA$_{B1b}$ and truncated human GABA$_{B2}$ were cloned from the human whole brain cDNA library from BD Bioscience, (San Jose, CA) using the following primers (synthesized by Invitrogen): GABA$_{B1b}$ primers, 5′-ACCACCATGGGGCCCGGGGCCCCT TTTGCC-3′ and 5′-TTACCCTCGGGTGATCAGCCTGCGC AT-3′; GABA$_{B2}$ primers, 5′-ACCACCATGGCTTCCCCGCGA AGCTCCGGG-3′ and 5′-TTAGACCGAGGTGGACGTTTTAG AATC-3′. The PCR products were then cloned into the pcDNA3.1 vector using the TOPO cloning kit from Invitrogen (Carlsbad, CA). The resulting plasmid DNAs were checked by restriction enzyme digestions and the correct plasmids were confirmed by sequencing (see supplementary materials for full length sequences). While the C-terminal domains of GABA$_B$

subunits mediate intracellular trafficking, they are not essential for heterodimeric assembly and receptor signaling. In this study, we used truncated forms of both $GABA_{B1}$ and $GABA_{B2}$ receptors to promote cell surface expression and formation of a functional heterodimer at the cell surface.

Transfections

Plasmid DNA encoding truncated $GABA_{B(1b)}$ and that encoding truncated $GABA_{B(2)}$ were cotransfected into CHO-Gqi3 (5)-NFat-β-lactamase cells (Aurora Biosciences, San Diego, CA) using electroporation. Cells were grown in T75 flask to confluence in DMEM medium supplemented with 25 mM HEPES, 1× NEAA, 10% dFBS, 100 μg/ml Zeocin, and 2.5 μg/ml blasticindin. The cells were then detached, and 20 μg of each DNA were added to 10 E 7 cells in 0.8 ml of serum free medium in an electroporation chamber. After electroporation, cells were diluted and grown in the above medium with geneticin at 1 mg/ml. After ∼10 days, most cells died and the remaining resistant cells were sorted using flow cytometry.

Flow Cytometry Analysis and Sorting

Flow cytometric analysis was performed using a FACS-Vantage SE flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA) equipped with an Enterprise II laser and an Innova 302C Krypton Ion Laser (Coherent, Santa Clara, CA). The 488-nm line was positioned as the primary laser beam, and the 407-nm line was positioned in the secondary laser position. Propidium iodide was excited at 488 nm, and the emission of propidium iodide was measured using a 675 nm bandpass filter, and a 650 dichroic short pass filter. CCF4 AM was excited at 407 nm, and the emission of CCF4 AM was measured using a 525 nm bandpass filter (green channel) and a 460 nm bandpass filter (blue channel). A 490 nm dichroic long pass filter was used to separate the signals. Viable cells were gated based on the absence of detectable incorporation of propidium iodide. Singlets were selected using forward scatter peak versus forward scatter width signals. High β-lactamase expressing cells were sorted based on bright blue fluorescence and low green fluorescence. Twenty-five thousand (25k) gated events were counted for each sample.

FLIPR Analysis

The fluorescence imaging plate reader (FLIPR) analysis was performed on the FLIPR 384 system (Molecular Device, Sunnyvale, CA). The $GABA_B$ stable cell line was maintained in DMEM medium, with 25 mM HEPES, 5% dFBS, 1× NEAA, 100 μg/ml zeocin, 2.5 μg/ml blastocidin, and 500 μg/ml geneticin. Twenty-four hours prior to the experiment, the cells were plated in the 384-well clear-bottom black-well plate at the density of 15k cells/well in the above medium without antibiotics. On the day of the experiment, cells were washed with assay buffer (HBSS, with 20 mM HEPES and 2.5 mM probenecid), and then loaded with Fluo-4 AM for 60 min at 37° C. The cells were washed again with the assay buffer and 30 μl volume was left in each well. All compounds were diluted to the desired concentration in the assay buffer. The cell plates were then analyzed on the FLIPR. Normal FLIPR-384 instru-

ment settings were used with the exception of laser power which was set to 600 MW power to reduce the signal of the calcium response to get the fluorescence signal on-scale.

Data Analysis

In the FLIPR software, spatial uniformity correction, baseline subtraction, and maximum fluorescence change were used.

RESULTS

In the $GABA_B$ cell line, we can measure calcium mobilization using Fluo-4 AM and β-lactamase expression using CCF4 AM. The $GABA_B$ receptor activation triggers calcium signaling through the binding and activation of G-proteins. β-lactamase is expressed through the activation of the NFat response element through the Gq-coupled receptors mobilization of calcium. Fluo-4 AM is a green fluorescent indicator that exhibits a calcium dependent increase in fluorescence emission upon calcium binding. CCF4 AM is a fluorescent resonance energy transfer based substrate for β-lactamase. β-lactamase cleaves CCF4 AM, disrupting the FRET, which causes a change of fluorescence from green to blue (Fig. 1).

Following transfection, the cells were analyzed and sorted based on the expression of β-lactamase. The purpose of the first sort was to identify a pool of cells that responded to GABA. The cells were treated with either 200 μM GABA, 200 nM thapsigargin, or vehicle for 4 h and loaded with CCF4 AM. In comparison with the vehicle population, the treated group demonstrated a low change in β-lactamase expression. This small fraction of cells, which consisted of a bright-blue and low-green fluorescence population (upper left quandrant), was identified and sorted (Fig. 2). Thapsigargin can increase the cytosolic concentration of calcium by opening IP3-gated channels in the ER and by activating plasma membrane calcium channels, allowing an influx of calcium into the cytosol without signaling through the $GABA_B$ receptor. The thapsigargin control showed a robust response at 200 nM.

The sorted pool of cells were expanded and reanalyzed for β-lactamase expression following stimulation of GABA. The sorted pool of cells demonstrated a four-fold increase in the percentage of cells of the highest β-lactamase expressing cells over the vehicle group. This high β-lactamase expressing population was identified and sorted for single cell clones to isolate a clonal $GABA_B$ cell line (Fig. 3). The sorted pool of cells showed a robust response when treated with 200 nM thapsigargin.

The clonal cell lines were expanded and seeded into a 384 well plate, loaded with Fluo-4 AM, and analyzed on the FLIPR. Twenty-seven clones were grown from single cell collections and 10 clones demonstrated a high response to GABA stimulation, compared with the sorted pool of cells. The 10 clones were re-evaluated based on GABA dose response and EC_{50}. Clone-16 was identified and utilized in high throughput assay development (Fig. 4). We found that many clones had a robust signal in the FLIPR assay. Several of the high response clones were further evaluated, and clone 16 consistently showed a robust assay window.
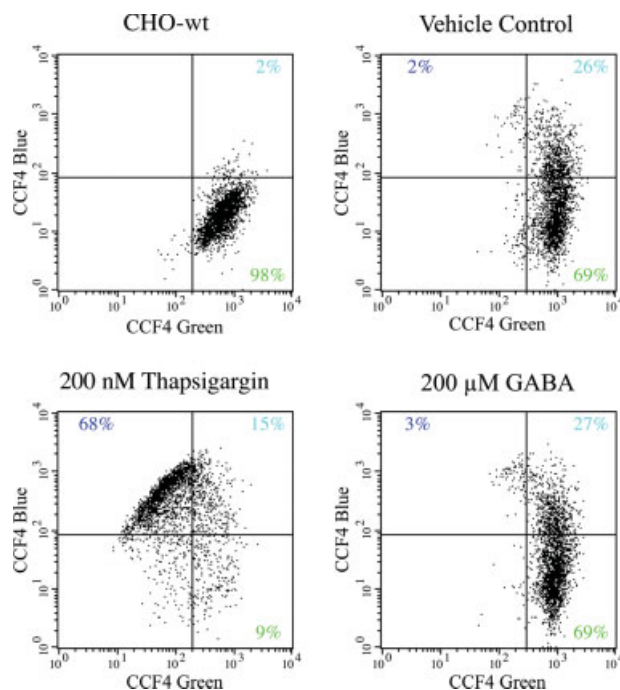
Figure 2. Blue Pool Sort. Dual parameter flow cytometric analysis of cells loaded with CCF4 AM. CCF4 green fluorescence was measured on the x-axis and CCF4 blue fluorescence on the y-axis. Upper left-histogram: Wild-type CHOs representing no β-lactamase activity. Upper right-histogram: The CHO $GABA_B$ cell line treated with vehicle (negative control) demonstrating β-lactamase background activity. Lower left-histogram: The CHO $GABA_B$ cell line treated with 200 nM thapsigargin (positive control). Lower right-histogram: The CHO $GABA_B$ cell line treated with 200 μM GABA.
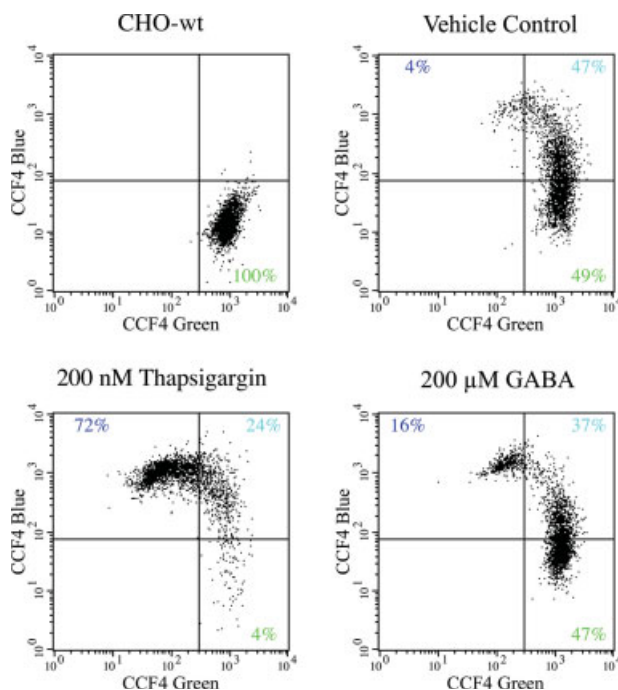
Figure 3. Single Cell Clone Sort. Dual parameter flow cytometric analysis of cells loaded with CCF4 AM. CCF4 green fluorescence was measured on the x-axis and CCF4 blue fluorescence on the y-axis. Upper left-histogram: Wild-type CHO's representing no β-lactamase activity. Upper right-histogram: The CHO $GABA_B$ cell line treated with vehicle (negative control) demonstrating β-lactamase background activity. Lower left-histogram: The CHO $GABA_B$ cell line treated with 200 nM thapsigargin (positive control). Lower right-histogram: The CHO $GABA_B$ cell line treated with 200 μM GABA.

Twenty-four hours prior to the experiment, clone-16 cells were seeded into a 384 well plate at 15k cells/well. Cells were loaded with Fluo-4 AM and analyzed on the FLIPR. GABA was added to the well 6 s after the start of data acquisition. The average FLIPR well response (16 wells/trace) to a GABA serial dilution is shown in Figure 5. The EC_{50} for the GABA serial dilution is 45.3 nM (Fig. 6). CGP54626 (GABA antagonist) was added to the well 5 min prior to the addition of 100 nM GABA. The average FLIPR well response (16 wells/trace) to a CGP54626 serial dilution was calculated. The IC_{50} for the CGP54626 serial dilution is 3.57 nM (Fig. 7). There was no response of the parental cell line (no GABA-receptor) to GABA (data not shown).

Discussion

Here we described the development of a human $GABA_B$, CHO-Gqi3 (5)-NFat, β-lactamase cell line for use in a high throughput, calcium mobilization assay. The clonal cell line displayed a sensitive dose dependent response to GABA and demonstrated a robust signal-to-noise window excessive for high throughput FLIPR screening. This assay will help identify and characterize selective agonists, modulators, and antagonists of the $GABA_B$ receptor.

We used flow cytometry sorting along with the β-lactamase technology to develop a high responding $GABA_B$, calcium signaling, clonal cell line. By linking β-lactamase to the
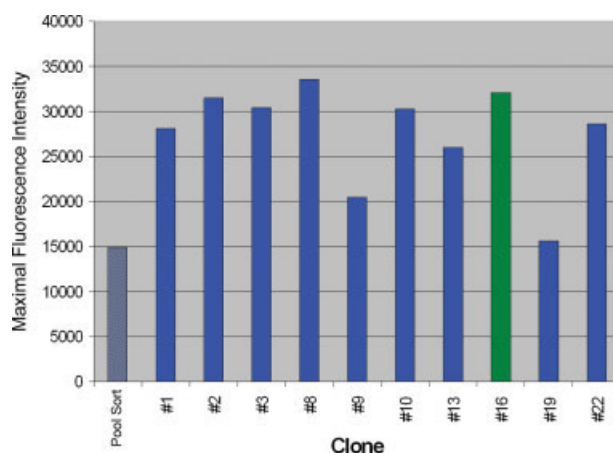

Figure 4. FLIPR Analysis of the Sorted $GABA_B$ Clones. 10 expanded single cell clones and the blue-sorted pool were loaded with Fluo-4 AM in a 384-well plate and analyzed on the FLIPR. The clones were evaluated with the blue-sorted pool (x-axis) based on agonist dose response using maximal fluorescence intensity (y-axis). Clone-16 was identified and utilized in later studies and for HTS assay development.
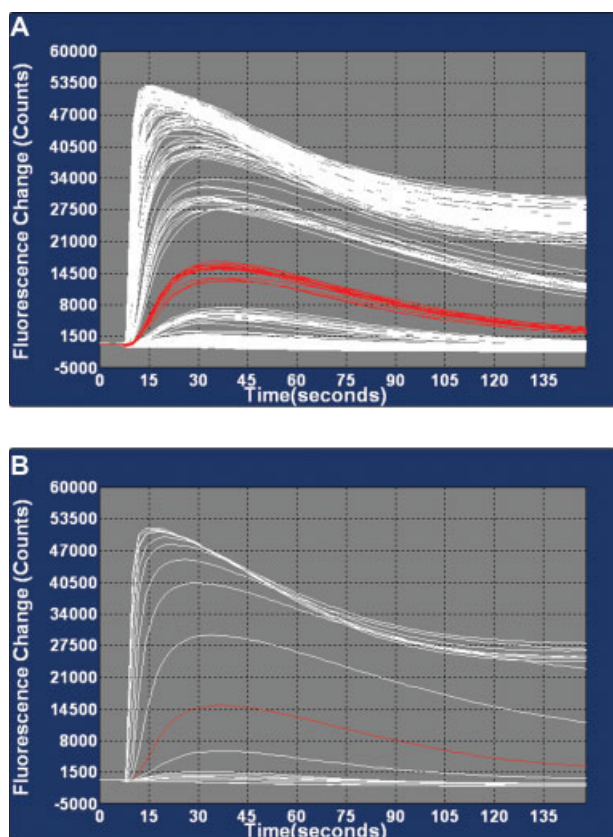
Figure 5. FLIPR Analysis of Clone-16: GABA Serial Dilution. Clone-16 cells were loaded with Fluo-4 AM in a 384-well plate and analyzed using the FLIPR. Fifteen thousand cells were seeded into each well one day prior to the experiment and the response to GABA is shown. Time is on the *x*-axis and fluorescence change (counts) is on the *y*-axis. 25 nM GABA is shown in red. (**A**) 384 Multiwell Overlay. (**B**) Multiwell Average. 16 wells per concentration of GABA were measured, and means ± standard deviation are shown. GABA was added to the well 6 s after the initial read.
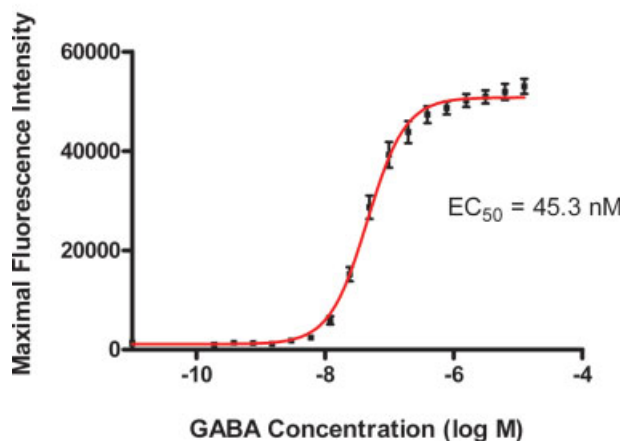

Figure 6. GABA EC_{50} calculation of Clone-16. Clone-16 cells were loaded with Fluo-4 AM in a 384-well plate and analyzed using the FLIPR. Fifteen thousand cells were seeded into each well one day prior to the experiment and the average FLIPR well response to GABA is shown. GABA concentration is on the *x*-axis and maximal fluorescence intensity is on the *y*-axis. GABA was added to the well 6 s after the initial read. The GABA EC_{50} determination for Clone-16 was 45.3 nM. Sixteen wells per concentration of GABA were measured, and means ± standard deviation are shown.

calcium signaling pathway provided us with a direct method to isolate a high responding calcium signaling clones. Sorting high β-lactamase expressing clones correlated to high responding calcium signaling clones on the FLIPR. Flow cytometry sorting increases productivity of cell line development efforts through the efficiencies of automated single-cell cloning. Using CCF4 AM fluorescence as a marker for β-lactamase activity, we were able to identify, sort, and isolate thousands of cells per second based on a preferred phenotype or response.

Assays of transcriptional reporter genes are an important tool for HTS. Novel cell lines that express a recombinant receptor, transcriptional response element, and a reporter gene can be used to isolate clones used in a variety of assay types. Flow cytometry sorting can be used to reduce high constitutive activity, thus creating cell lines with superior signal-to-noise ratios. Sorting can be used to isolate high responding clones using calcium fluorescent indicators such as Indo-1 AM or Fluo-4 AM, although this is a much harder approach than using the β-lactamase technology as CCF4 AM is an end-

point measurement which does not require additional instrumentation or manipulation of the cells on your flow cytometry system.

We describe the creation of a truncated heterodimerized GPCR stable cell line that can be used in both β-lactamase and
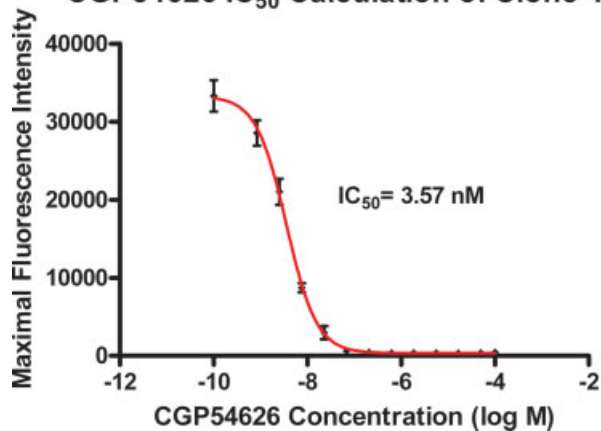

Figure 7. CGP54626 IC_{50} calculation of Clone-16. Clone-16 cells were loaded with Fluo-4 AM in a 384-well plate and analyzed using the FLIPR. Fifteen thousand cells were seeded into each well one day prior to the experiment and the average FLIPR well response to 100 nM GABA is shown. CGP54626 concentration is on the *x*-axis and maximal fluorescence intensity is on the *y*-axis. CGP54626 was added to the well 5 min prior to the GABA addition to the well. The CGP54626 IC_{50} determination for Clone-16 was 3.57 nM. Sixteen wells per concentration of inhibitor were measured, and means ± standard deviation are shown.

FLIPR assays. This multiplex approach allows the researcher to test compounds with either short term or long term exposures. The cell line allows for the robust and consistent measure of calcium signaling using the FLIPR. The cell line contains the truncated heterodimerized receptor, which allows for the measurement of different types of ligands that can inhibit, activate, or potentiate the receptor. The cell line contains the NFat β-lactamase reporter gene which can measure downstream effects of the signaling pathway using CCF4 AM. The calcium mobilization assay can be miniaturized to 1,536 on the FLIPR and the β-lactamase assay can be miniaturized to 3,456 on a fluorescence plate reader.

The FLIPR calcium mobilization assay is the preferred high throughput assay for GPCR drug screening. The FLIPR can acquire data rapidly and can obtain consistent dose-response curves from compound screening. When trying to identify antagonists or modulators, the cells only interact with compound for a few minutes. In the case of screening for agonists, the cells only interact with compound for a few seconds. This is a very important criterion not to be overlooked in drug screening as some compounds will degrade or precipitate in an aqueous environment. Generally, FLIPR data has tighter CVs, robust signal-to-noise ratios and lower EC_{50}s than other assay types. The FLIPR calcium flux assay is the most popular high throughput screening approach because it is a rapid and a direct signal for biological activity of GPCRs.

LITERATURE CITED

1. Chen K, Li H, Ye N, Zhang J, Wang J. Role of GABA$_B$ receptors in GABA and baclofen-induced inhibition of adult rat cerebellar interpositus nucleus neurons in vitro. Brain Res Bull 2005;67:310–318.

2. Bowery NG, Doble A, Hill DR, Hudson AL, Shaw JS, Turnbull MJ, Warringon R. Bicuculline-insensitive gaba receptors on peripheral autonomic nerve terminals. Eur J Pharm 1981;71:53–70.

3. Bowery NG, Enna SJ. Aminobutyric acid$_B$ receptors: First of the functional metabotropic heterodimers. J Pharmacol Exp Ther 2000;292:2–7.

4. Jones KA, Borowsky B, Tamm JA, Craig DA, Durkin MM, Dai M, Yao WJ, Johnson M, Gunwaldsen C, Huang LY, Tang C, Shen Q, Salon JA, Morse K, Laz T, Smith KE, Nagarathnam D, Noble SA, Branchek TA, Gerald C. GABA(B) receptors function as a heteromeric assembly of the subunits GABA(B)R1 and GABA(B)R2. Nature 1988;396:674–679.

5. Bowery NG, Bettler B, Froestl W, Gallagher JP, Marshall F, Raiteri M, Bonner TI, Enna SJ. International Union of Pharmacology XXXIII. Mammalian γ aminobutyric acidB receptors: structure and function. Pharmacol Rev 2002;54:247–264.

6. Pin JP, Kniazeff J, Binet V, Liu J, Maurel D, Galvez T, Duthey B, Havlickova M, Blahos J, Prezeau L, Rondard P. Activation mechanism of the heterodimeric GABA$_B$ receptor. Biochem Pharmacol 2004;68:1565–1572.

7. Liu J, Maurel D, Etzol S, Brabet I, Ansanay H, Pin JP, Rondard P. Molecular determinants involved in the allosteric control of agonist affinity in the GABA$_B$ receptor by the GABA$_{B2}$ subunit. J Biol Chem 2004;279:15824–15830.

8. Gee KR, Brown A, Chen W-N U, Bishop-Stewart J, Gray D, Johnson I. Chemical and physiological characterization of fluo-4 Ca^{2+}-indicator dyes. Cell Calcium 2000; 27:97–106.

9. Schroeder KS, Neagle BD. FLIPR: A new instrument for accurate, high throughput opitcal screening. J Biomol Screen 1996;1:75–80.

10. Hodder P, Mull R, Cassaday J, Berry K, Strulovici B. Miniaturization of intracellular calcium functional assays to 1536-well plate format using a fluorometric imaging plate reader. J Biomol Screen 2004;9:417–426.

11. Qureshi SA. β-lactamase: An ideal reporter system for monitoring gene expression in live eukayotic cells. Biotech 2007;42:91–96.

12. Zlokarnik G, Negulescu PA, Knapp TE, Mere L, Burres N, Feng L, Whitney M, Roemer K, Tsien RY. Quantitation of transcription and clonal selection of single living cells with β-lactamase as reporter. Science 1998;279:84–88.

13. Knapp T, Hare E, Feng L, Zlokarnik G, Negulescu P. Detection of β-lactamase reporter gene expression by flow cytometry. Cytometry 2003;51:68–78.

14. Coward P, Chan SD, Wada HG, Humphries GM, Conklin BR. Chimeric G-proteins allow a high-throughput signaling assay of Gi-coupled receptors. Anal Biochem 1999;270:242–248.

15. Yokoyama T, Kato N, Yamada N. Development of a high-throughput bioassay to screen melatonin receptor agonists using human melatonin receptor expressing CHO cells. Neurosci Lett 2003;344:45–48.

16. Kowal D, Nawoschik S, Ochalski R, Dunlop J. Functional calcium coupling with the human metabotropic glutamate receptor subtypes 2 and 4 by stable co-expression with a calcium pathway facilitating G-protein chimera in Chinese hamster ovary cells. Biochem Pharm 2003;66:785–790.