APPLICATION NOTE

Monitoring G_q protein-coupled receptor activation on the SpectraMax i3x reader with injector module

Introduction

G_q protein-coupled receptor activation is commonly monitored in live cells in real time using calcium-sensitive dyes on a fluorescence plate reader. Automated liquid handling within the plate reader is generally required to deliver agonist compounds to the cells in the microplate while the detection system takes real-time readings of compound-induced changes in fluorescence intensity values. Analysis of the resulting kinetic readings yields information about the compound response profiles, including EC_{50} and IC_{50} values for agonists and antagonists.

In this application note, we describe how the SpectraMax® i3x Multi-Mode Microplate Reader with SpectraMax Injector Module is used to perform calcium assays on two different cell lines. CHO M1WT3 cells are Chinese hamster ovary cells stably transfected with the M1 muscarinic G_q-coupled receptor. 1321N1, a human astrocytoma cell line, expresses endogenous muscarinic cholinergic receptors. Data analysis performed using SoftMax® Pro Software yielded accurate EC_{50} and IC_{50} values for both cell lines, comparable to previous results obtained using high-throughput screening systems. The Acquisition Editor, a graphical user interface in the software, facilitated setup of the assay's injector and detection parameters (Figure 1).

Materials

- CHO M1WT3 cells (ATCC cat. #CRL-1985)
- 1321N1 cells (ECACC cat. #86030402)
- Agonist compounds
  - Carbamoylcholine chloride (carbachol; Sigma cat. #C4382)
  - Acetylcholine chloride (Sigma cat. #A6625)
- Antagonist compound
  - Atropine (Sigma cat. #A0132)
- FLIPR® Calcium 6 Assay Kit (Molecular Devices cat. #R8190)
- 1 M HEPES (Thermo cat. #15630-080)
- ReadiUse™ water-soluble probenecid (AAT Bioquest cat. #20061)
- SpectraMax® i3x Multi-Mode Microplate Reader
  - SpectraMax Injector Module
- FlexStation® 3 Multi-Mode Microplate Reader

Benefits

- **EC_{50} and IC_{50} values agree closely with previously published values on high-throughput screening systems**
- **Minimal dead volume saves costly compounds**
- **Real-time cell monitoring is easily configured using SoftMax Pro Software**
- **Compatible with FLIPR Calcium Assays**

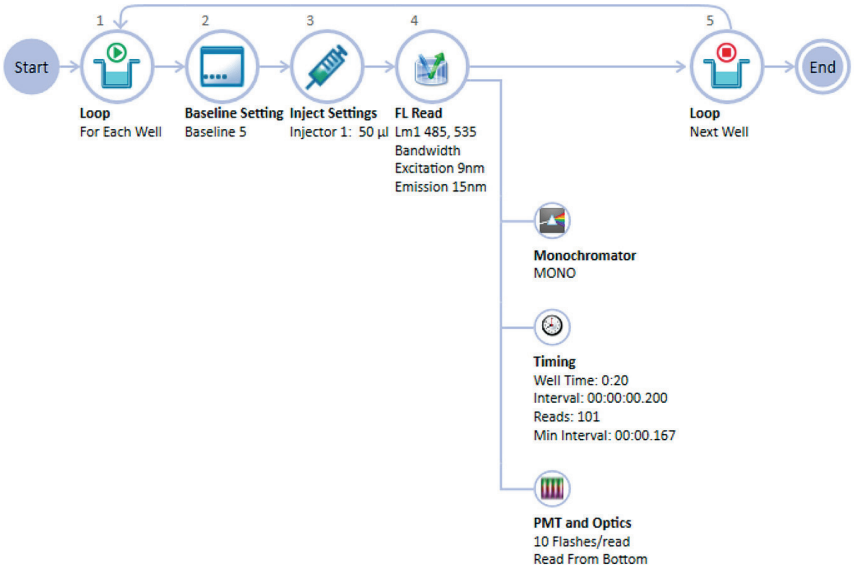

Figure 1. Acquisition Plan for calcium assay on the SpectraMax i3x reader. The Acquisition Editor in SoftMax Pro Software provides a graphical drag-and-drop interface for easy assay setup.

Methods

Cell preparation

CHO M1WT3 cells were seeded into a 96-well black-wall, clear-bottom plate at 50,000 cells per well in 100 µL per well. They were incubated overnight at 37°C, 5% CO_2.

1321N1 cells were used as an 'assay-ready' reagent. Briefly, one vial of frozen cells (~ 10 million cells per mL) was thawed rapidly in a 37°C water bath and re-suspended in 10 mL of DMEM medium. The cells were centrifuged for five minutes at 1,000 RPM, the medium was discarded, and then the cells were suspended at 300,000 cells per mL in fresh DMEM. Cells were then seeded into a 96-well black-wall, clear-bottom plate at 30,000 cells per well in 100 µL per well. They were incubated overnight at 37°C, 5% CO_2.

For both cell lines, calcium assays were performed on the day after cells were seeded in microplates.

Reagent preparation

30 mL of assay buffer were made by adding 0.6 mL of 1 M HEPES to 29.4 mL of 1X HBSS. This assay buffer was used in the preparation of other assay reagents. A 25 mM probenecid stock solution was made by dissolving one vial of probenecid in 10 mL of assay buffer. A solution consisting of 1 mL of 25 mM probenecid and 10 mL of assay buffer was used to reconstitute a vial of Dye Loading Buffer (provided in the Calcium 6 assay kit), and the vial was vortexed for 30 seconds to mix.

Dye loading

To each well of the microplate containing cells, 100 µL of Dye Loading Buffer was added. The plate was then incubated for two hours at 37°C, 5% CO_2. Cells were not washed prior to assay.

Compound preparation

For the agonist assays, 5X working stocks of carbachol (CHO M1WT3 cells) or acetylcholine (1321N1 cells) were prepared in assay buffer. 1:3 dilution series of the working stocks were made in 4-mL polypropylene tubes. A full concentration-response curve, consisting of eight concentrations, was prepared for each compound.

Parameter	Setting
Read type	Flex
Read mode	Fluorescence Bottom read
Wavelengths	Ex 485 nm Em 525 nm Cutoff 515 nm
Sensitivity	Readings: 3 PMT: Medium
Timing	Run time: 90 sec Interval: 2.1 sec
Compound transfer	Initial volume: 150 µL Transfers: 1 Pipette height: 150 µL Volume: 50 µL Rate: 2 Time Point: 19 sec
Triturate	Not used

Table 1. FlexStation 3 reader settings for calcium assay.

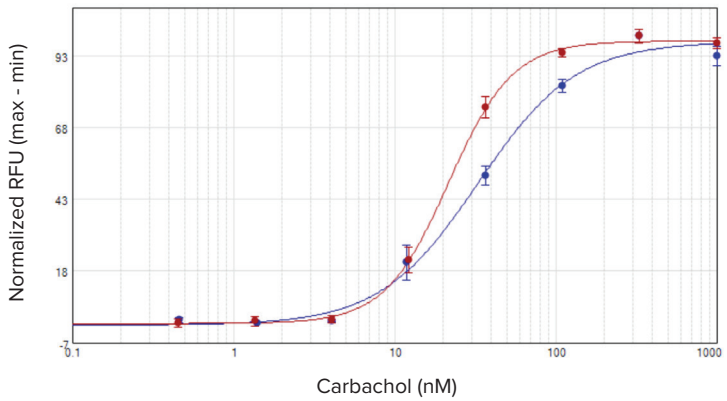

Figure 2. CHO M1WT3 agonist concentration response curves. Cells were treated with carbachol and assayed for calcium response on the SpectraMax i3x reader with injector module (blue) or the FlexStation 3 reader (red).

The antagonist atropine was prepared as a 1:3 dilution series. Working stocks were added to the wells of the assay plates to achieve the appropriate final concentrations, and the plates were equilibrated for 30 minutes. The injector was then used to deliver agonist at EC_{80} concentration to the wells.

Cell-based assay setup

Agonist concentration response curves were run on the SpectraMax i3x reader with a SpectraMax Injector Module, one compound concentration at a time. For each curve, the assay was run from low to high concentration to minimize carryover. Injector 1 was primed with compound, then the dye-loaded cell plate was placed in the instrument and the assay was run on a set of three replicates. For each well, 50 µL of 5X compound working stock was injected into the 200 µL of cells, medium, and Dye Loading Buffer already in the well, for a final 1X concentration of compound.

The injector was then reversed to remove compound from the line and primed with the next highest concentration of compound. A new plate section was created, and the assay was run on the next set of replicate wells. This process was repeated for each compound concentration, with a new plate section being created for each set of replicates, until the concentration-response curve was completed.

The Acquisition Plan in SoftMax Pro Software was used to define the instrument and injector settings (Figure 1).

Antagonist studies were done by manually pipetting antagonist dilution series to the wells of the assay plate containing dye-loaded cells, equilibrating for 30 minutes, and then running the kinetic assay on the SpectraMax i3x reader with injection of a single EC_{80} concentration of agonist into all the assay wells.

As a control, additional wells in the same dye-loaded cell plate were assayed on the FlexStation 3 Multi-Mode Microplate Reader, which uses an integrated multi-channel pipettor to deliver compound to the wells. Instrument settings for the FlexStation 3 reader are shown in Table 1.

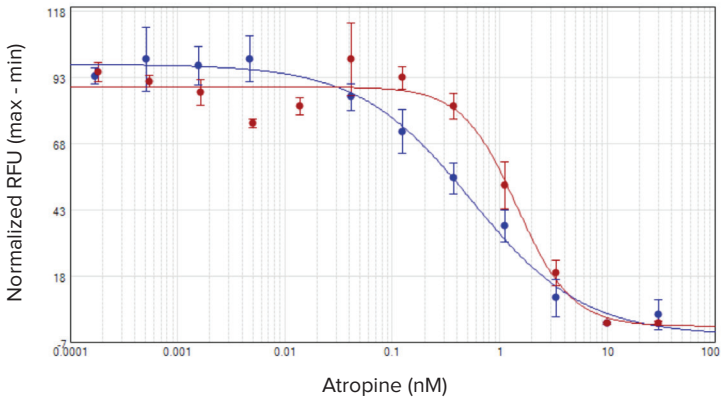

Figure 3. CHO M1WT3 antagonist IC_{50} curves. Cells were treated with increasing concentrations of the antagonist atropine and assayed for response to addition of an EC_{80} concentration of the agonist carbachol. Results are shown for the SpectraMax i3x reader with injector module (blue) and FlexStation 3 reader (red).

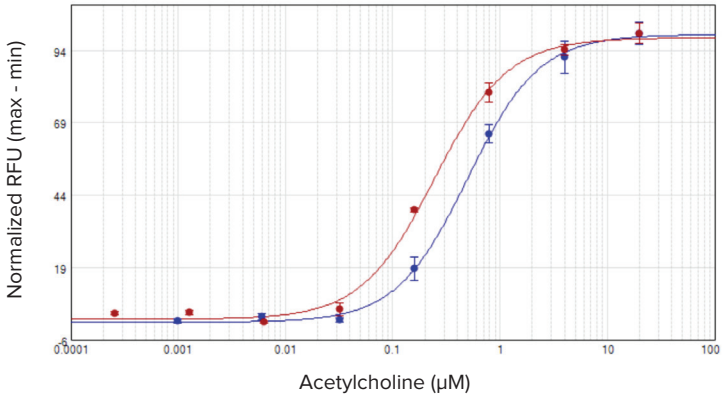

Figure 4. 1321N1 agonist concentration response curves. Cells were treated with acetylcholine and assayed for calcium response on the SpectraMax i3x reader with injector module (blue) or the FlexStation 3 reader (red).

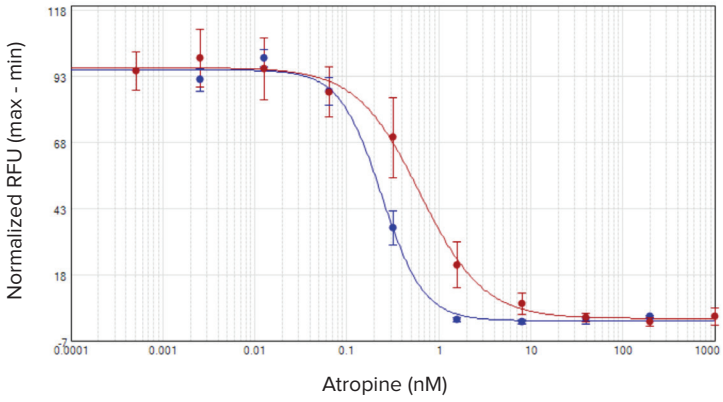

Figure 5. 1321N1 antagonist IC_{50} curves. Cells were treated with increasing concentrations of the antagonist atropine and assayed for response to addition of an EC_{80} concentration of the agonist acetylcholine. Results are shown for the SpectraMax i3x reader with injector module (blue) and the FlexStation 3 reader (red).

Results

Consistent agonist concentration response curves were achieved on the SpectraMax i3x reader by using the injector module to deliver each compound dilution in a separate plate read step. The compounds were run from low to high concentration to avoid carryover issues. Antagonist assays, which involve the delivery of a single EC_{80} concentration of agonist to wells containing cells and antagonist compound, were done as a single plate read.

The agonist carbachol activated the M1 muscarinic receptor expressed in CHO M1WT3 cells with an EC_{50} value of 32.8 nM on the SpectraMax i3x reader and 21.7 nM on the FlexStation 3 reader (Figure 2). Atropine antagonized this activation with IC_{50} values of 0.54 nM and 1.4 nM, respectively (Figure 3). The muscarinic acetylcholine receptor (mAChR) expressed in 1321N1 cells was activated by acetylcholine with EC_{50} values of 0.50 µM and 0.24 µM (Figure 4), and antagonized by atropine with IC_{50} values of 0.24 nM and 0.62 nM (Figure 5). Table 2 summarizes the concentration response values obtained for both cell lines.

Conclusion

Using the SpectraMax Injector Module, we obtained concentration response curves with EC_{50} and IC_{50} values agreeing closely with previously published values on high-throughput screening systems. Compound delivery parameters and real-time cell monitoring were easily configured using the Acquisition View in SoftMax Pro Software. Results using the injector-based system agreed closely with those previously acquired using the FlexStation 3 reader with onboard pipettor, and with data from the FLIPR Tetra® High-Throughput Cellular Screening System.

		SpectraMax i3x reader	FlexStation 3 reader
Agonist EC_{50}	CHO M1WT3 carbachol	32.8 nM	21.7 nM
	1321N1 acetylcholine	0.54 nM	1.4 nM
Antagonist IC_{50}	CHO M1WT3 atropine	0.50 nM	0.24 nM
	1321N1 atropine	0.24 nM	0.62 nM

Table 2. Summary of EC_{50} and IC_{50} values obtained on the SpectraMax i3x reader with injector module and on the FlexStation 3 reader.

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