APPLICATION NOTE

Homogeneous solution for GPCR assays with FLIPR Calcium 5 Assay Kit

Introduction

Cell-based assays have become an indispensable method for screening and compound profiling in the early drug discovery process. To date, such assays have proven to be some of the most reliable and reproducible methods in receptor characterization studies, primary screening campaigns and compound profiling programs.

For G_q-coupled GPCR targets specifically, homogeneous fluorescent calcium flux assays with masking technology are the methodology of choice. These assays, such as the FLIPR® Calcium 5 Assay Kit, do not require washing after dye loading because the masking technology reduces extracellular background as seen in Figure 1. Some commercially available assays are designated as no-wash assays. However, as they do not use masking technology, they tend to have very high background and produce a poor signal window, making it difficult to detect small responses from endogenous or low expression level systems. A larger signal window enabled by a homogenous assay is preferable to enable the reliable determination of EC_{50} and IC_{50} estimates, to calculate EC_{30} concentrations of agonist required to study allosteric modulators or to permit the use of endogenous cell lines with lower receptor expression.

In addition to no-wash assays, many researchers use fluorescent dyes that must be washed out following the dye loading step. Although some targets respond acceptably following this wash step, these assays are labor-intensive, time-consuming and have higher well-to-well and day-to-day variability. Furthermore, there are significant challenges to scale such wash methods to a high throughput screening mode.

FLIPR Calcium 5 Assay Kit

The FLIPR Calcium 5 Assay Kit is a homogeneous, fluorescence reagent kit that solves the challenges of using traditional wash method assays, or other no-wash dyes with high background signals. This assay kit works by using a calcium fluorophore that is taken into the cell cytoplasm during a dye loading step, while the masking technology remains outside the cell and inhibits background fluorescence. Upon receptor-ligand binding, calcium is released into the cell cytoplasm, and then binds to the dye thereby increasing the fluorescence signal (Figure 1).

The masking technology in the FLIPR Calcium 5 Assay Kit offers enhanced dynamic range and superior signal-to-background ratios which contribute to improved assay data fidelity. Because washing is not required, there is reduced

Benefits

- **Superior signal-to-noise ratio over competitive dyes and kits**
- **Z factor > 0.9**
- **Low well-to-well variation, even with frozen cells**
- **True homogenous protocol, mix-and-read**

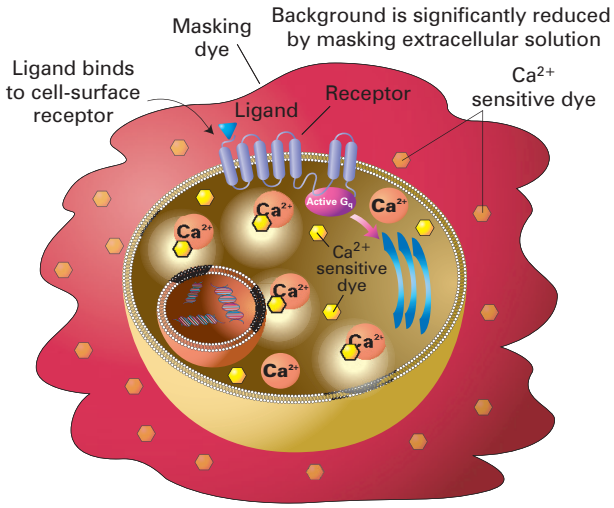

Figure 1. FLIPR Calcium 5 Assay Kit principle. During the response to GPCR activation, the fluorescent signal inside the cell increases as released calcium binds to the novel fluorophore. Background signal is minimized by the extracellular masking technology, improving the signal-to-background ratio.

well-to-well variability which improves the quality (Z factor) and reliability (CV %) of assays. As a result, the FLIPR Calcium 5 Assay Kit is especially beneficial for assays using cell lines with low receptor expression, assays looking at endogenously expressed receptors and other targets that give small calcium responses.

Materials and Methods

Calcium flux assay on the FLIPR Tetra® System

For the experimental data shown here, an assay examining the endogenous muscarinic receptor expressed at low levels in 1321N1 cells (ECACC catalogue #86030402) was developed using the FLIPR Calcium 5 Assay Kit and then compared to other available kits. The "assay ready" frozen cells have become a suitable and frequently used alternative to cells in culture for many biological assays. When combined, the FLIPR Calcium 5 Assay Kit and frozen cells enable an accelerated assay workflow that further increases throughput with fewer experimental steps and less hands-on time.

The 1321N1 cells were thawed rapidly and plated at approximately 13,000 cells per well in black-wall, clear bottom 384-well microplates and incubated at 37°C, 95% humidity and 5% CO_2 overnight. On the following day, cell plates were loaded with the appropriate calcium reagents following manufacturers' recommendations (including water soluble probenecid) and incubated for 60 minutes.

The calcium indicators used for the comparison were:

- FLIPR Calcium 5 Assay Kit (Molecular Devices)
- Fluo-4 AM Calcium Indicator (Life Technologies)
- Fluo-4 NW Calcium Assay Kit (Life Technologies)
- Fluo-4 Direct Calcium Assay Kit (Life Technologies)
- FluoForte Calcium Assay Kit (Enzo Life Sciences)
- Screen Quest Rhod-4 No Wash Calcium Assay Kit (AAT Bioquest)

Parameter	FLIPR Calcium 5 Assay Kit*	Rhod-4 NW Calcium Assay Kit
Excitation wavelength	470–495 nm	510–545 nm
Emission wavelength	515–575 nm	565–625 nm
LED intensity	80 %	30 %
Camera gain	2000	2000
Exposure	0.53 sec.	0.53 sec.
Gate	6.00 %	6.00 %
Interval	1 sec.	1 sec.
Dispense volume	12.5 µL	12.5 µL
Dispense height	35.0 µL	35.0 µL
Dispense speed	30.0 µL/sec.	30.0 µL/sec.
Tip up speed	20 mm/sec.	20 mm/sec.
Tips in well	No	No

* The same parameters also apply to Fluo-4 AM Calcium Indicator, Fluo-4 NW Calcium Assay Kit, Fluo-4 Direct Calcium Assay Kit, and FluoForte Calcium Assay Kit.

Table 1. FLIPR Tetra System parameters for calcium assay comparison.

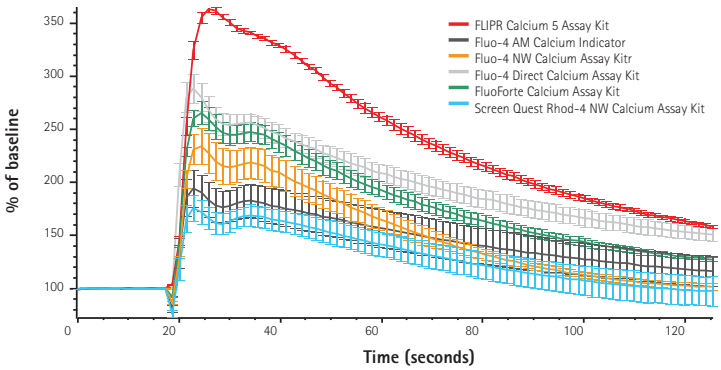

Figure 2. Kinetic traces from the FLIPR Tetra System. Representative signal traces on the FLIPR Tetra System for acetylcholine induced agonism of the endogenous muscarinic M3-receptor in "assay ready" 1321N1 cells compared using different calcium assay kits.

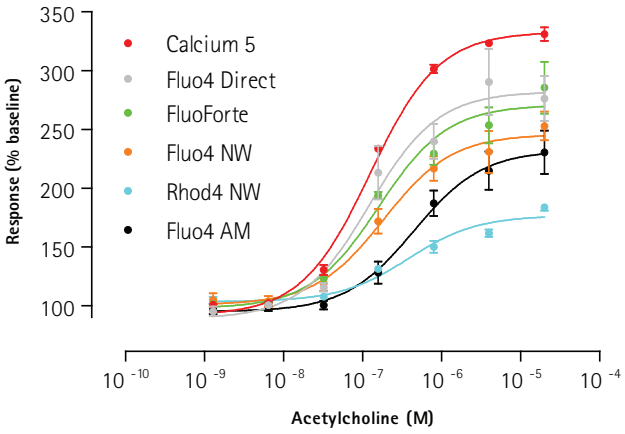

Figure 3. Comparison of calcium assay signal window: agonism. Agonist assay: Comparison of the fluorescent signal in "assay ready" 1321N1 cells during acetylcholine stimulation of the endogenous muscarinic M3-receptor. This illustrates the enhanced signal-to-background ratio obtained by the FLIPR Calcium 5 Assay Kit with proven masking technology.

Cell plates loaded with Fluo-4 AM were washed three times with HBSS/HEPES following the 60 minute incubation, for all other indicators the plates were not washed after dye loading; the initial assay volume was 50 µL per well.

FLIPR Tetra System setup

Cell plates were maintained at 37°C inside the FLIPR Tetra System; compounds were prepared in Corning 384-well polystyrene plates and FLIPR Tetra Pipette Tips (black) were used for compound transfer. The specific parameters for the FLIPR Tetra System are indicated in Table 1.

Results

Larger signal window in less time

The mean kinetic responses of the individual calcium dyes to a single concentration of acetylcholine can be seen in Figure 2. The data shows how the novel fluorophore and inclusion of a masking dye technology offer a number of benefits over similar competitive assay kits, traditional dyes such as Fluo-4 AM, and no-wash reagents that do not utilize masking technology. The signal magnitude was significantly higher for FLIPR Calcium 5 Assay Kit and unlike some of the other reagents, there was no addition artifact observed. Furthermore, the standard error bars were smaller, suggesting a more optimized protocol.

Figure 3 shows acetylcholine concentration-response curves generated using the various calcium assay kits on the FLIPR Tetra System. Broadly consistent EC_{50} values were obtained with all assays kits tested, although a slight rightward-shift was observed when using the Fluo-4 AM Calcium Indicator and Rhod-4 NW Calcium Assay Kit. The EC_{50}, Z and Signal/Baseline (S/B) values are shown in Table 2; the FLIPR Calcium 5 Assay Kit provides a larger signal window, consistent pharmacology, and improved Z factor compared to other reagents.

	Acetylcholine			Atropine	
	EC_{50}	Z at EC_{80}	S/B	IC_{50}	Z
FLIPR Calcium 5	1.2×10^{-7} M	0.93	331	9.8×10^{-9} M	0.94
Fluo-4 AM	4.5×10^{-7} M	0.42	232	N/A	N/A
Fluo-4 NW	1.8×10^{-7} M	0.53	245	6.9×10^{-9} M	0.34
Fluo-4 Direct	1.2×10^{-7} M	0.65	282	8.6×10^{-9} M	0.74
FluoForte	1.6×10^{-7} M	0.63	270	8.3×10^{-9} M	0.69
Rhod-4 NW	3.8×10^{-7} M	0.72	177	N/A	N/A

Table 2. EC_{50} Z and signal/baseline values.

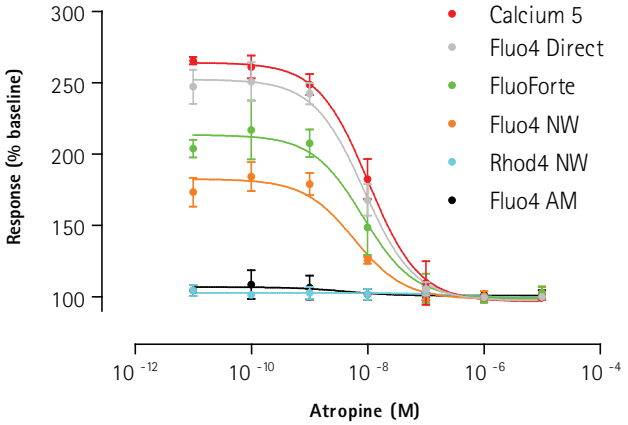

Figure 4. Comparison of calcium assay signal window: antagonism. Antagonist assay: Atropine inhibition of calcium flux in response to an EC_{80} challenge of acetylcholine in "assay ready" 1321N1 cells, evaluated with six different calcium reagents on the FLIPR Tetra System.

A similar situation is also observed when the assay is run in antagonist format using atropine to inhibit the responses to an EC_{80} concentration of acetylcholine (Figure 4). Table 2 shows the calculated IC_{50} and Z values, and consistent with the agonist assay, the FLIPR Calcium 5 Reagent provides pharmacology consistent with previous studies and an improved Z factor compared to competitive products. Under the experimental conditions used in this particular antagonist study, Fluo-4 AM and Rhod-4 NW loaded cells did not respond as expected to the acetylcholine challenge.

Conclusion

Combining a novel fluorophore and proven masking technology, the FLIPR Calcium 5 Assay Kit delivers reliable pharmacology, a larger signal window, and improved assay performance. With the FLIPR Calcium 5 Assay Kit and FLIPR Tetra System, consistent screening of a variety of receptors and targets, especially those with small calcium signal responses, can be obtained in an easy-to-use, homogeneous format. The FLIPR Calcium 5 Assay Kit is amenable to both adherent and non-adherent cells in 96-, 384- and 1536-well formats.

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