APPLICATION NOTE

Comparison of Photina luminescent calcium mobilization assays on the FlexStation 3 reader and FLIPR Tetra System

Introduction

G protein-coupled receptors (GPCRs) represent one of the most important therapeutic targets in drug discovery research. GPCRs are membrane-localized proteins that play an important role in cell signaling. When a receptor is activated by a ligand, the conformation of the receptor is modified, activating G-proteins inside the cell. An active G-protein has the potential to induce various cascades of intracellular messengers, including calcium.

Calcium-activated photoproteins are important tools for detecting receptor-mediated signaling events involving calcium mobilization in mammalian cells. One major advantage of photoproteins is the immediate emission of flash luminescence upon calcium binding to the coelenterazine-photoprotein complex. The background signal of aequorin measurements is close to zero, resulting in high signal-to-background ratios. Furthermore, the light emitted by the oxidation of coelenterazine does not depend on optical excitation, eliminating issues with auto-fluorescence.

There are now a number of commercial sources for photoprotein expressing cell-lines, making this technology more widely accessible. These assay methods are also described in the literature1 and in the United States Patent 6,872,538 and European Patent 1,145,002. Users interested in the patented methods may wish to consult legal counsel in evaluating these patents.

Molecular Devices FlexStation® 3 Multi-Mode Microplate Reader has integrated fluidics, permitting the real-time measurement of fluorescent and luminescent cell-based assays. Up to eight (96-well format) or sixteen (384-well format) wells can be monitored simultaneously before, during, and after compound addition. Assays are easily optimized with user-defined dispense height, speed of compound addition, reagent source location, and tip selection for each addition. Disposable pipette tips reduce the opportunity for cross-contamination between additions and experiments, and decrease plate dead volumes (compared to injectors) saving precious reagents.

The FLIPR Tetra® System from Molecular Devices is the market-leading instrument for monitoring GPCR and ion channel activity, and represents a reliable and flexible HTS/uHTS solution for early identification of lead compounds in the drug discovery process. The FLIPR Tetra System is now available with an aequorin option which includes a novel ICCD camera technology optimized for use with both fluorescent and luminescent assays. Furthermore, the cell suspension system makes the system amenable to both adherent and suspension cell-based assays in 96-, 384- and 1536-well formats.

This application note provides a basic protocol for performing an adherent aequorin assay using the FlexStation 3 reader and FLIPR Tetra System with ICCD camera. Both instruments were used to determine the concentration-response of IMETIT in CHO mito-Photina/H3 cells at various cell concentrations.

Benefits

- **Flexible medium- and high-throughput solution for early identification of lead compounds in the drug discovery process**
- **FlexStation 3 reader permits real-time measurement of fluorescent & luminescent assays**
- **FLIPR Tetra System with aequorin option including an ICCD camera is optimized for fluorescent & luminescent assays**

Materials

- CHO mito-Photina/H3 cells (Axxam SpA). The H3 cell line was generated by stably transfecting CHO-K1 cells with Photina, a mitochondrial membrane-targeted chimeric photoprotein, with Ga16, and with the Histamine H3 gene
- Culture media: Dulbecco's MEM/ Nutrient Mix F12 with 15 mM HEPES (Cat. #11039-047), supplemented with 1.35 mM sodium pyruvate (Cat. #11360-070), 10% FBS (Cat. #10082-147), 1% penicillin/streptomycin (Cat. #15070-063), 500 µg/mL Geneticin (Cat. #10131-027), and 1% L-glutamine (Cat. #25030-081), all from Invitrogen.
- PBS without calcium and magnesium (Invitrogen Cat. #14190-144)
- VERSENE 1:5000 (Invitrogen Cat. #15040-066)
- Native coelenterazine (PharmaTech Int. Cat. #55770-48-1) stock solution at 11 mM diluted in DMSO (Sigma Cat.#D5879) and 1mM glutathione (Sigma Cat.#G-6529)
- Assay Buffer: DMEM/HAM's F12 without phenol red (Invitrogen Cat. #11039-021) with 15 mM HEPES (Invitrogen Cat. #11039-047) and 0.1% BSA (Sigma Cat. #A3294)
- Histamine H3 Agonist: IMETIT (Sigma Cat. #I-135)
- 384-well black-wall, clear-bottom microplates (Corning Cat. #3712)
- FLIPR Tetra System with ICCD camera option (Molecular Devices)
- FlexStation 3 Multi-Mode Microplate Reader (Molecular Devices)

Methods

Assay plate preparation

To create assay plates, CHO mito-Photina/H3 cells were harvested using VERSENE, re-suspended in culture media without selection antibiotics and plated at varying cell concentrations (625, 1250, 2500, and 5000 cells/well) in 25 µL in 384-well, black-wall, clear-bottom plates and incubated overnight at 37°C in 5% CO_2.

Assay plates were removed from the incubator and culture media was removed and replaced with 25 µL assay buffer containing 5 µM native coelenterazine. The coelenterazine-loaded plates were incubated at room temperature for five hours in the dark.

Parameter	Setting
Read Type	Flex
Read Mode	Luminescence, Bottom Read
Wavelengths	All
Run Time	90 Sec. Total
Interval	1.14 sec
Autocalibrate	On
Assay Plate Type	384 W Corning Clear/Flatbottom
Compound Source	Greiner Polypropylene V Base (384-Well)
Compound Addition	
Initial Volume	25 µL
Pipette Height	20 µL
Volume	25 µL
Rate	8 (31 µL/sec)
Time Point	21 sec.

Table 1. FlexStation 3 reader: Photina assay setup parameters.

Parameter	Setting
Reading Mode	Luminescence
Excitation/Emission	None/None
Camera Gain	140000
Gate Open	100%
Exposure Time	0.4 sec
Read Interval	1 sec
Dispense Volume	25 µL
Dispense Height	10 µL
Dispense Speed	50 µL/sec
Removal Speed	10 mm/sec
Expel Volume	0 µL

Table 2. FLIPR Tetra System (with ICCD camera option): Photina assay setup parameters.

Cells/Well	EC_{50} IMETIT (nM)	Z factor at EC_{80}
5000	2.18	0.68
2500	2.67	0.63
1250	1.81	0.54
625	2.41	0.43

Table 3. Summary of Photina adherent assay results on the FlexStation 3 reader.

Cells/Well	EC_{50} IMETIT (nM)	Z factor at EC_{80}
5000	1.54	0.86
2500	1.18	0.67
1250	1.59	0.58
625	3.00	0.35

Table 4. Summary of Photina adherent assay results on the FLIPR Tetra System with ICCD camera option.

Compound plate preparation

Polypropylene 384-well plates were used to prepare a concentration-response series of 2X concentrations of IMETIT, a H3-Histamine receptor agonist, in assay buffer.

Instrument setup and data analysis

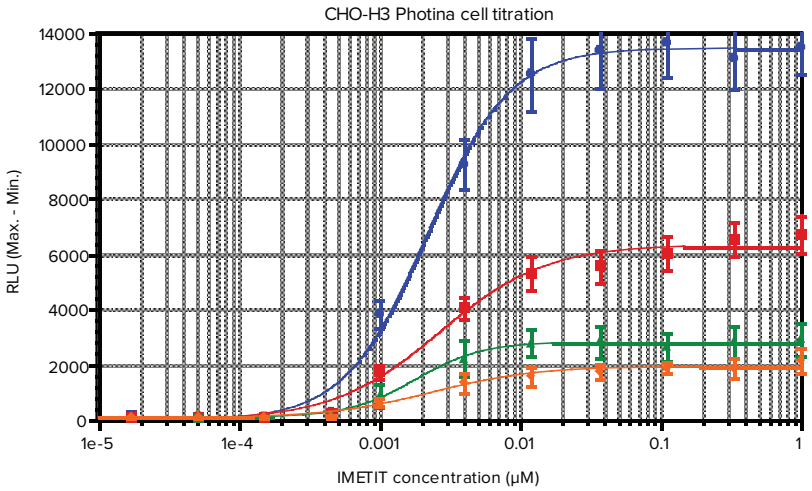
After incubation with coelenterazine, assay plates were placed on the FlexStation 3 reader or FLIPR Tetra System. Protocols were prepared in SoftMax® Pro Software (FlexStation 3) or ScreenWorks® Software (FLIPR Tetra) using the parameters shown in Tables 1 and 2, respectively.

Luminescence readings were taken for ~15 sec. to give a baseline reading and then the instruments' on-board fluidic systems were used to dispense different concentrations of the specific ligand (IMETIT) whilst changes in luminescence were monitored simultaneously. Calculation of average RLU (max-min), standard deviation, EC_{50}, Z factor at EC_{80}, and graphing of concentration response curves were all performed using SoftMax Pro Software. A preconfigured aequorin assay protocol for the FlexStation 3 reader is available in the SoftMax Pro Software.

Results

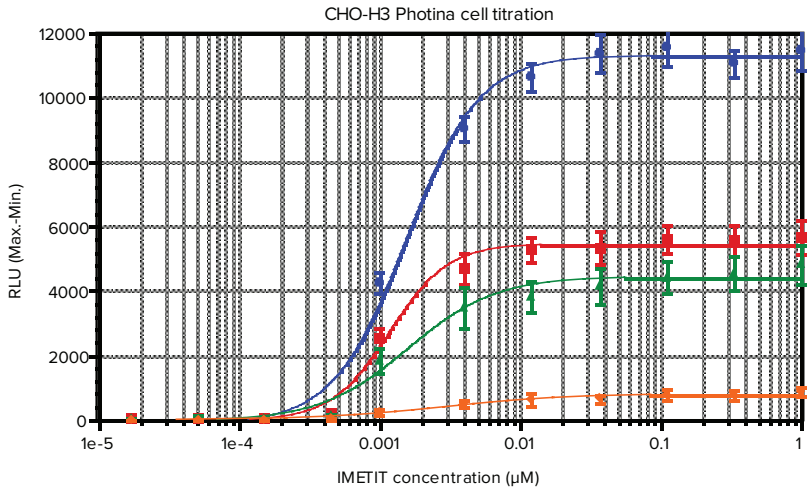
IMETIT-stimulated calcium flux in CHO mito-Photina/H3 transfected cells may be monitored using flash luminescence. Luminescent signal is measured by both the FlexStation 3 reader and the FLIPR Tetra System as Relative Light Units (RLU) vs. time during the assay. The average RLUs (max-min) were plotted against the IMETIT concentration to generate concentration-response curves (Figure 1, FlexStation 3 reader data; Figure 2, FLIPR Tetra System data) and the calculated EC_{50} value.

The IMETIT EC_{50}, and Z-factor at EC_{80} are summarized in Tables 3 and 4. The EC_{50} values for IMETIT using the FlexStation 3 microplate reader range from 1.81 to 2.67 nM , whereas the FLIPR Tetra System with ICCD camera option reported EC_{50} values in the range 1.18 to 3.00 nM. Both sets of results are close to the published EC_{50} value of 3.43 nM. Z-factor values at EC_{80} are also comparable between both instruments.



4-P Fit: y = (A - D)/(1 + (x/C)^B) + D:	A	B	C	D	R^2
5K (5K: Concentration vs MeanValue)	-44.4	1.49	0.00218	1.34e+04	0.996
2.5K (2.5K: Concentration vs MeanValue)	-33.5	1.15	0.00267	6.33e+03	0.991
1.25K (1.25K: Concentration vs MeanValue)	72.4	1.8	0.00181	2.81e+03	0.996
0.625K (0.625K: Concentration vs MeanValue)	53.3	1.16	0.00241	1.93e+03	0.986

Figure 1. Agonist response measured with FlexStation 3 reader. CHO mito-Photina/H3 cells were plated at varying cell concentrations (5000 (●), 2500 (■), 1250 (▲), and 625 (♦) cells/well) in 384-well black-wall, clear-bottom plates without selection antibiotics and incubated overnight at 37°C in 5% CO_2. Culture media was removed and cells were incubated in the dark at room temperature for five hours in 25 µL buffer containing 5 µM native coelenterazine. The FlexStation 3 reader added agonist during real-time luminescent detection as per the settings in Table 1. Results are the average of approximately 16 replicates.



4-P Fit: y = (A - D)/(1 + (x/C)^B) + D:	A	B	C	D	R^2
5K (5K: Concentration vs MeanValue)	-148	1.68	0.00154	1.13e+04	0.994
2.5K (2.5K: Concentration vs MeanValue)	-45.5	2.09	0.00118	5.45e+03	0.993
1.25K (1.25K: Concentration vs MeanValue)	-57.5	1.39	0.00159	4.44e+03	0.985
0.625K (0.625K: Concentration vs MeanValue)	-11.7	0.96	0.003	807	0.982

Figure 2. Agonist response measured with FLIPR Tetra System with ICCD camera option. CHO mito-Photina/H3 cell assay as described in Figure 1 (5000 (●), 2500 (■), 1250 (▲), and 625 (♦) cells/well). FLIPR Tetra System with ICCD camera option added agonist during luminescent read mode as per the settings in Table 2. Results are the average of approximately 32 replicates.

Conclusion

The FlexStation 3 reader and the FLIPR Tetra System with aequorin option (ICCD camera) have been used to measure the Photina adherent luminescent cell assay. The preparation and treatment method of Photina cells for an adherent luminescent assay is the same when using either the FlexStation 3 reader or the FLIPR Tetra System with ICCD camera option. This, along with the correlation of the data produced, supports the use of the FlexStation 3 reader in lower-throughput Photina assays. In addition, the instrument can be used to develop assays which easily transfer to the FLIPR Tetra System for high-throughput screening purposes.

Acknowledgments

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Reference

1. Boie, et al., *Eur J Pharmacol* 340(2-3):227-241 (1997).

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