Application Notes

GPCR induction followed by a fluorometric Ca^{2+} assay in Thermo Scientific Varioskan LUX

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Key Words

Cellular signal, G-Protein Coupled Receptor, muscarinic acetylcholine receptor, fluorometric Ca^{2+} assay, multimode reader, Varioskan LUX

Goal

This application note describes a method to assess changes in the concentration of free cytoplasmic calcium (Ca^{2+}) in cultured cells in response to activation of G-Protein Coupled Receptors (GPCRs). Our model system consists of cultured Chinese hamster ovary (CHO) cells that over-express the rat M1 muscarinic acetylcholine receptor. The assay was performed with the Thermo Scientific™ Varioskan™ LUX multimode microplate reader. The instrument was controlled and data was analyzed by the Thermo Scientific SkanIt™ software. The SkanIt software provides tools even for challenging microplate assays, such as monitoring of fast kinetic measurements from the very start of the reaction.

Introduction

GPCRs mediate several biochemical signals from outside the cells through activating diverse signal transduction pathways within the cells leading to ultimate cellular responses. One of the two principal signal transduction pathways that involve the G protein–coupled receptors is the phosphatidylinositol signal pathway through which muscarinic receptors are acting. Muscarinic receptors are stimulated by the neurotransmitter acetylcholine in the nervous system. A cascade of resulting intracellular changes includes increase of cytosolic concentration of calcium (1). G-protein-coupled receptors are involved in many diseases and, as a consequence, they are frequent targets of modern drug discovery and pharmacological screening. The selective actions of several drugs through GPCRs can be investigated by measuring the modulation of the cytosolic concentration of calcium.

Fluo calcium indicators are widely used for monitoring changes in the intracellular calcium levels in flow cytometry experiments, fluorescence and confocal microscopy, and microplate screening applications. Their use is based on the large fluorescence intensity increase in response to Ca^{2+} binding (2). The acetoxymethyl (AM) ester derivatives of fluorescent calcium indicators can permeate cell membranes and remain stable within the cell as they convert into a non-leaking compound format. The AM ester used in the assays is colorless and nonfluorescent until hydrolyzed by binding to the target ion, i.e. to calcium.

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Materials and methods

Instrument

- Varioskan LUX multimode microplate reader (Thermo Fisher Scientific)

Plates

- Thermo Scientific™ Nunc™ MicroWell 96-Well Optical-Bottom Plates with Polymer Base, Cell Culture surface coating (Thermo Fisher Scientific 165305)

Reagents

- Chinese hamster ovary cells (M1WT2, ATCC® CRL1984™)
- Ham's F-12K (Kaighn's) Medium (Thermo Fisher Scientific 21127-022), with fetal bovine serum, 10%, penicillin/ streptomycine (HyClone SV30010)
- Fluo-4 Direct™ Calcium Assay Kit (starter pack Thermo Fisher Scientific F10471)
- Fluo-4 NW Calcium Assay Kit (Thermo Fisher Scientific F36205)
- Carbachol (Carbamylcholine chloride, 99%, ACROS Organics™, Fisher Scientific AC108240050)

Experimental setup

Cells were grown and sub-cultured according to standard protocols at 37°C and 5% CO_2 in a cell incubator (Thermo Scientific™ Heracell™ 150). 30000 cells/well in 50 µl media were plated to a 96-well black-walled plate and cultured in a cell incubator for 24 hours. 50 µl of 2X Fluo-4 Direct™ calcium reagent loading solution was added directly to the cells. Plates were incubated for 30 minutes at 37°C and 30 minutes at room temperature. Fluorescence was measured with the Varioskan LUX instrument (Figure 1).

Fluorescence signal (excitation 490 nm, emission 520 nm, bottom reading) was followed in a kinetic measurement: 200 readings as fast as the instrument could measure (i.e. with interval time zero). The receptor was induced by adding different volumes of 2 µM carbachol stock solution to the cells by an onboard dispenser of the Varioskan LUX. The dispensing speed was set to Medium High in order to mix the reagents efficiently. All samples were run in triplicates. The Area Selection step of the SkanIt software was used to define the groups of wells with different carbachol volumes to be added.

The reaction was so fast that it was important to measure all kinetic readings from one well at a time before proceeding to the next well. This was programmed with the Well Loop step of the SkanIt software. Varioskan LUX supports simultaneous dispensing and measurement, which was necessary to follow the signal progress without delay after dispensing carbachol. The kinetic reading at which to dispense the reagent can be freely selected. In this assay, the background fluorescence was measured for 19 kinetic readings, then carbachol was dispensed at the 20th reading. The order of the protocol steps programmed in the SkanIt software is shown in Figure 2.

In the data analysis stage, baseline subtraction was performed by utilizing the Kinetic Baseline Subtraction function of the SkanIt software (Figure 3). The dose-dependent calcium response to the agonist was calculated by defining a Custom Formula in the SkanIt software (Figure 4).

Figure 1. Varioskan LUX multimode microplate reader.

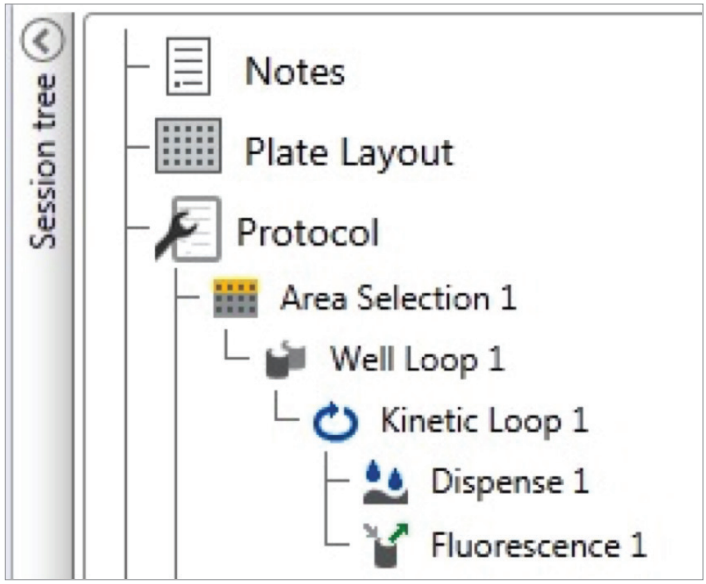

Figure 2. Protocol steps for the fluorometric Ca^{2+} -assay in the SkanIt software controlling Varioskan LUX. The use of Area Selection, Well Loop and Kinetic Loop and the timing of the Dispense step are explained in the text.

Results and discussion

CHO M1 cells were treated with an agonist and assayed for calcium response using Fluo-4 Direct Calcium Assay Kit in Varioskan LUX. Typical responses were in line with published results (Figures 5-7). We have achieved similar data by using the Fluo-4 NW Calcium Assay Kit. Both kits gave similar data on 384-well plates (data not shown).

The Varioskan LUX multimode reader equipped with an onboard dispenser and controlled through the SkanIt software supports simultaneous dispensing and measurement. In an assay like this, it was inevitable to continue the measurement without delay after adding the active reagent, since the reaction was really rapid. So rapid, that the measurement had to be executed by one well at a time to monitor the fast reaction from the very start to the end of the reaction. This was enabled by using the Well Loop step of the SkanIt software. The instrument allows adding reagents in any phase of the kinetic assay. We chose to measure the background fluorescence for the first 19 readings and dispense the activating reagent at the 20th reading. Automatic dispensing also ensures reproducible distribution of reagents from well to well, which is shown by uniform signals from wells of sample replicates (Figure 5).

The in-built calculations of the SkanIt software and the simplicity of defining custom formula provide versatile tools for analyzing kinetic data. In the assay described here, the in-built kinetic baseline subtraction function was used to automate baseline subtraction. The dose-dependent calcium response to muscarinic 1 (M1) receptor agonist can be visualized as averaged kinetic curves of replicated samples (Figure 6). As an alternative, relative change in fluorescent signal during the reaction (ΔRFU) can be used as a quantitative descriptor of the dose response and plotted against the concentration of the studied chemical compound (Figure 7). The custom formula option of the SkanIt software facilitated this calculation (Figure 4).

The assay presented here gives an example how Varioskan LUX can be used to characterize G-protein-coupled receptor pharmacology of compounds. The study is easy to perform, and therefore it can be adapted to high-throughput experiments.

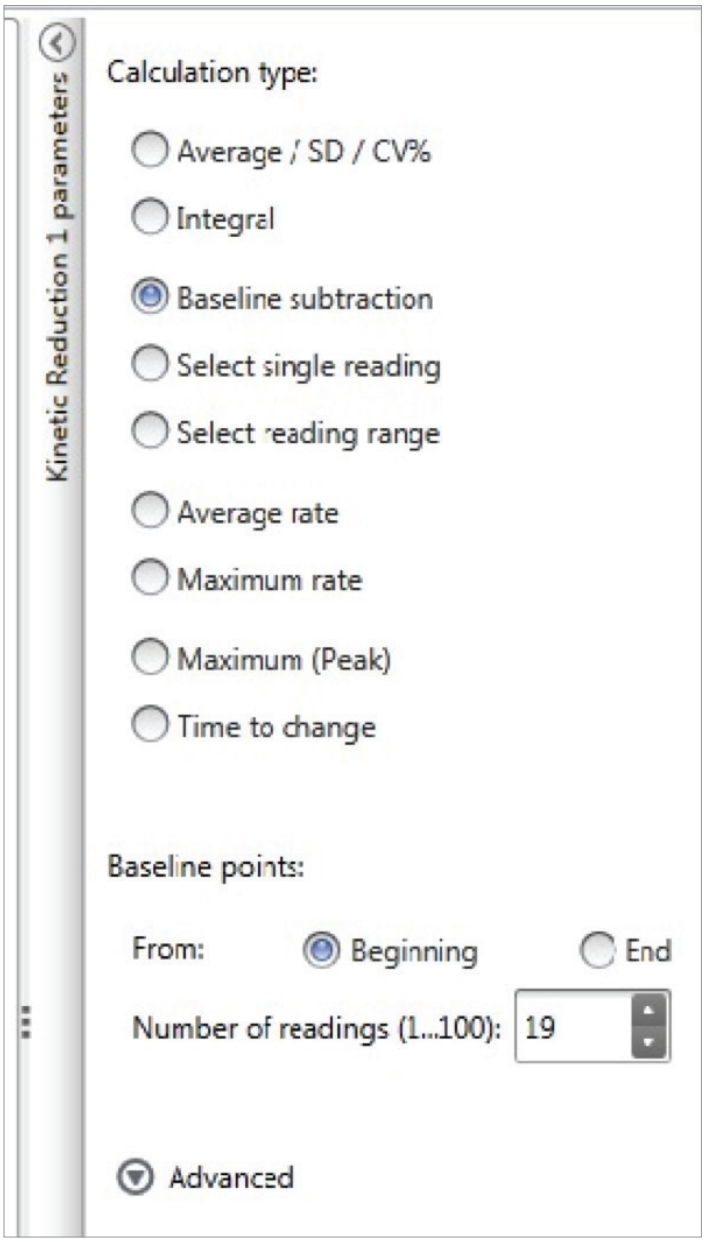

Figure 3. Baseline subtraction was performed by using the in-built Kinetic Baseline Subtraction function of the SkanIt software. The function averages the defined baseline points, here the first 19 readings before dispensing the agonist. This value is then subtracted from each data point to normalize the curve.

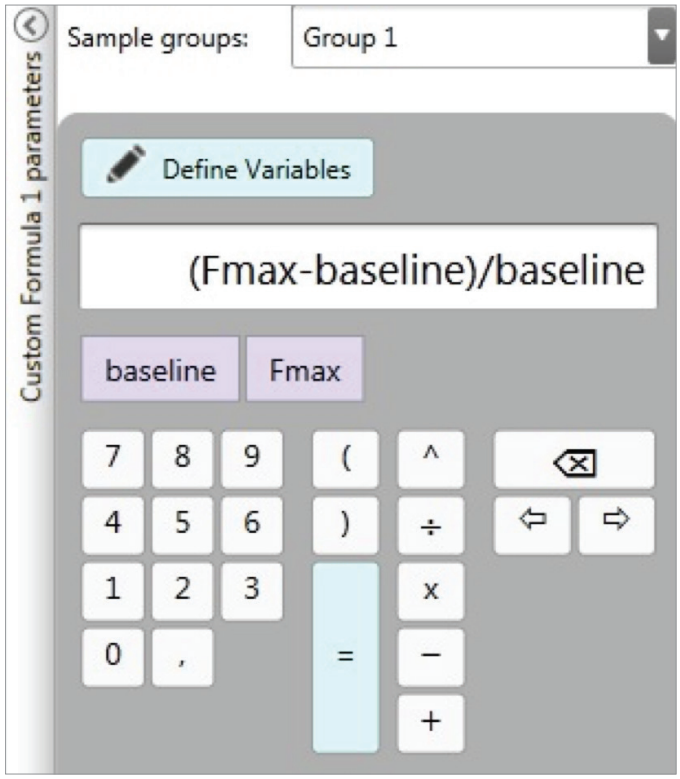

Figure 4. Dose-dependent calcium response (ΔRFU) was calculated as the maximum response minus the minimum response divided by the minimum response. Calculation is straightforward by defining a Custom Formula in the SkanIt software.

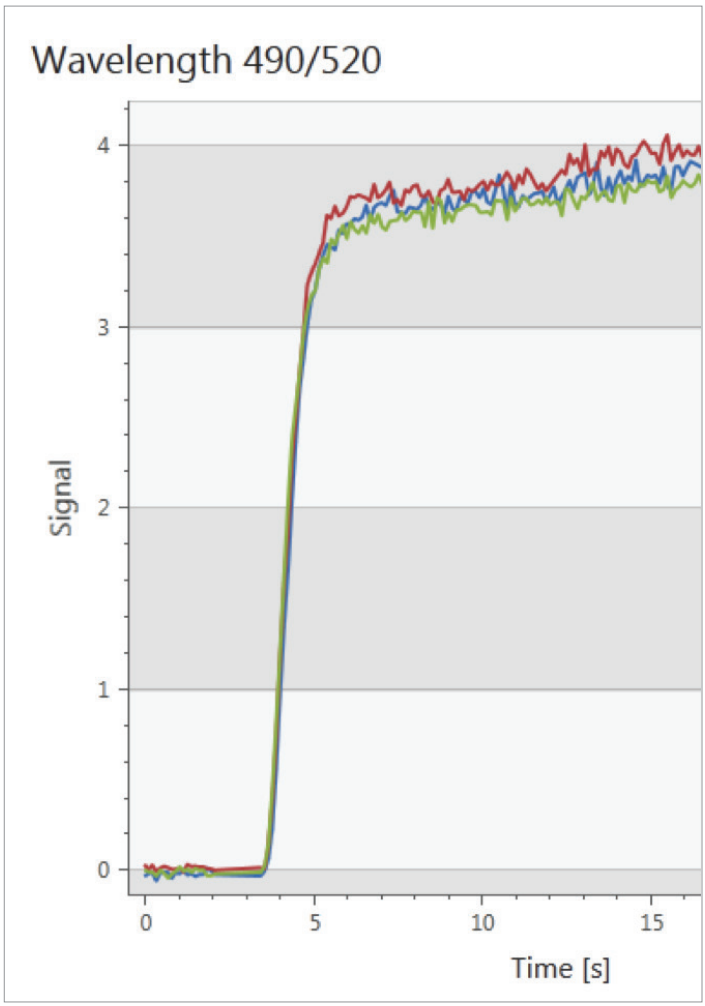

Figure 5. Increase of cytosolic Ca^{2+} levels in response to stimulation of M1 muscarinic receptors in CHO cells was assayed by the Fluo-4 Direct calcium indicator. The agonist stimulating the receptor-transmitted $Ca2^{+}$ -binding was 1 µM Carbachol. Baseline-subtracted fluorescence values

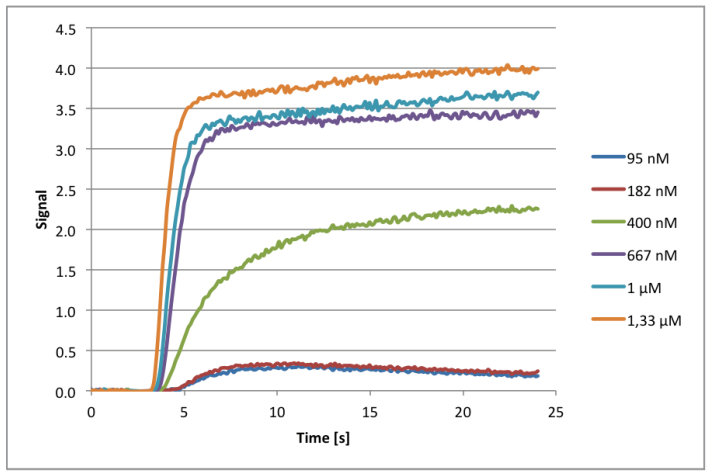

Figure 6. Dose response to carbachol in CHO cells stably expressing the M1 muscarinic receptor is visualized by kinetic curves of baseline-subtracted fluorescence values; average of three replicates.

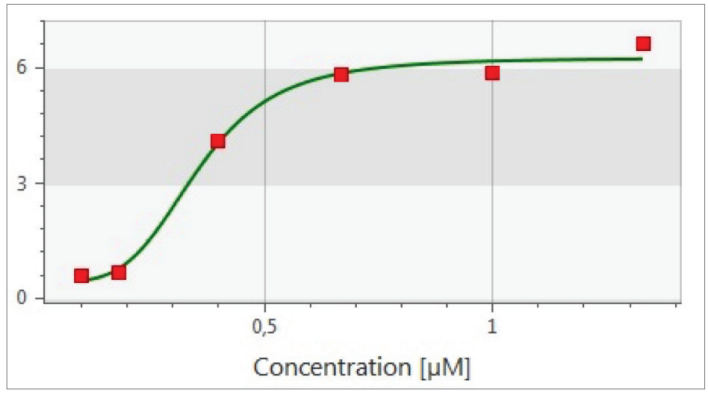

Figure 7. Dose-dependent calcium response (ΔRFU) as a function of carbachol concentration was calculated by defining a Custom Formula (see Figure 4) and fitting a four parameter logistic (4PL) standard curve in the SkanIt software; average of three replicates; R^2=0,993.

Conclusions

By combining the Varioskan LUX multimode reader and the Fluo-4 Direct reagent we were able to assess changes in free cytoplasmic calcium (Ca^{2+}) concentrations in cultured cells induced by activation of muscarinic receptors. The Varioskan LUX multimode reader with the SkanIt software provides a range of benefits for this type of cell-based measurements, including:

- Simple protocol setup aided by the intuitive interface of the SkanIt software
- Well loop step for measuring fast kinetic reactions one well at a time
- Simultaneous dispensing and measurement for signal monitoring from the start of a rapid reaction
- Possibility to execute instrument actions separately for different groups of wells
- Real-time kinetic curves shown during the measurement
- Effortless data analysis using SkanIt software's in-built kinetic calculations
- Calculation of average over replicates of kinetic data
- Diverse custom calculations of SkanIt software

References

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