Calcium Detection Using the NOVOstar and Screen Quest™ Fluo-8 NW Calcium Kit

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Introduction

Analyzing second messengers in cells upon agonist stimulation is an ideal way to measure the activation of G protein coupled receptors (GPCRs). A calcium flux assay, which measures intracellular calcium, is such a method that is commonly used in drug discovery for screening GPCRs.

The Screen Quest™ Fluo-8 NW Calcium Assay kit from ABD Bioquest provides a homogeneous fluorescence-based assay for detecting intracellular calcium mobilization. Cells expressing a GPCR of interest that signals through calcium are pre-loaded with Fluo-8 NW, which crosses the cell membrane. Once inside the cell, the lipophilic blocking groups of Fluo-8 NW are cleaved by esterases resulting in a negatively charged fluorescent dye that stays inside the cell. Subsequent binding to calcium causes the fluorescence of Fluo-8 NW to be greatly enhanced. Its characteristics of a long wavelength, high sensitivity, and ›100 times fluorescence enhancement (when it forms a complex with calcium) make Fluo-8 NW an ideal indicator for measurement of intracellular calcium. The Screen Quest™ Fluo-8 NW Calcium Assay Kits provide an optimized assay method for monitoring G-protein-coupled receptors (GPCRs) and calcium channels. The assay can be performed in a convenient 96-well or 384-well microtiter-plate format and easily adapted to automation.

The NOVOstar from BMG LABTECH is a multifunctional microplate reader with four measurement modes: fluorescence intensity, luminescence, absorbance, and fluorescent polarization. It is an ideal platform to perform cell-based, fast kinetic assays such as intracellular calcium measurement, because it has a unique pipetting system that can add small amounts of agonist (‹1.0 mL) to cells followed by instantaneous reading. The NOVOstar also has two injectors that can be used for reagent and/or substrate addition.

Material and Methods

Detailed protocols for the Screen Quest™ Calcium No Wash Assay, in the presence or absence of medium, are provided with the kits.

- Screen Quest™ Fluo-8 NW Calcium Assay Kit *1% FBS Medium* from ABD Bioquest
- Screen Quest™ Fluo-8 NW Calcium Assay Kit *Medium Removed* from ABD Bioquest
- NOVOstar Microplate Reader from BMG LABTECH
- Other reagents were acquired through normal channels
- Black, clear bottom 96-well Costar® microplate

HEK293 cells were loaded with either Fluo-8 NW, Fluo-4 NW or Fluo-3 AM dyes. The cells were then stimulated with carbachol using the NOVOstar pipette, thereby activating endogenous muscarinic receptors and subsequently causing the release of intracellular calcium.

For all assays, HEK-293 cells were seeded overnight at 40,000 cells per 100 µl per well in a 96-well black wall/clear bottom Costar® plate. Growth medium was removed in figures 1 and 2, but for figure 3 FBS (1%) was kept in the plate.

Cells were incubated with 100 µl of Fluo-8 NW, Fluo-4 NW, or Fluo –3 AM for 1 hr at 37°C. Using the NOVOstar pipettor, either 30 µM of Carbachol (50 µl/well) was added (figue 1) or carbachol (25µl/well) was added to achieve the final indicated concentrations (figures 2 and 3). Two microplates are used with the NOVOstar, a reagent plate with set dilutions of carbachol and an experimental plate with cells.

Carbachol was added at 12 secs and readings were taken up to 50 secs at 1 sec intervals. The fluorescence intensity mode was used along with a 485/10 excitation filter and a 520/10 emission filter.

Results and Discussion

Using the NOVOstar microplate reader with its unique pipetting system, calcium was measured in HEK293 cells after stimulation with carbachol (30 µM). Figure 1 shows that Fluo-8 NW has a 2-fold brighter relative fluorescence than Fluo-4 NW and a 4-fold brighter relative fluorescence than Fluo-3. This shows that Fluo-8 NW is the brightest fluorescent calcium dye currently available.

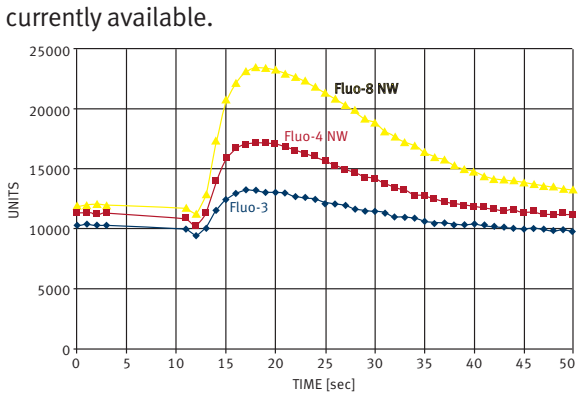

Fig. 1 : Comparisons of Screen Quest™ Fluo-8 NW Assay Kit, Fluo-4 NW, and Fluo-3 AM on HEK-293 cells after carbachol stimulation (30 mM) with the growth media removed.

Further experimentation shows that Fluo-8 NW has a similar binding affinity for calcium as Fluo-4 NW, as determined by their EC_{50}s (Figures 2 and 3). In figure 2, the medium was removed from the cells; whereas in figure 3, the medium (1% FBS) was not removed. Note that the EC_{50}s derived from the two figures are similar, but that the relative fluorescence units (RFU) are more than twice as high when there is no FBS.

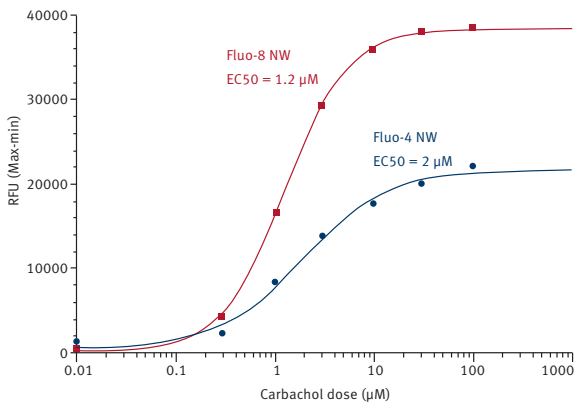

Fig. 2 : Carbachol Dose Response in HEK-293 cells measured with Screen Quest™ Fluo-8 NW Assay kit and Fluo-4 NW Assay Kit with the growth medium removed.

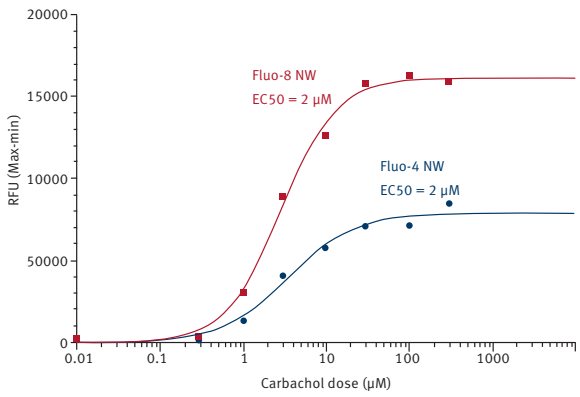

Fig. 3 : Carbachol Dose Response in HEK-293 cells measured with Screen Quest™ Fluo-8 NW Assay kit and Fluo-4 NW Assay Kit with 1% FBS.

Conclusion

Intracellular calcium measurements can be used to detect the activation of a receptor (i.e. GPCR), which in turn can be used to screen specific agonists or antagonists.
There are two keys aspects to a good intracellular calcium assay:

- The ability to deliver an agonist/antagonist to cells followed by instantaneous detection of calcium.
- A membrane permeable dye that greatly increases in intensity upon binding to calcium.

The NOVOstar from BMG LABTECH fulfils criteria 1 by providing the ideal system to perform all of your fast kinetic, cell-based assays. With its unique pipetting system, the NOVOstar can deliver minute amounts (‹1 mL) of agonist or antagonist from the reagent plate to the experimental plate. Detection, using one of its four measurement modes (fluorescence intensity, luminescence, absorbance, or fluorescence polarization), can be initiated prior to, during, and after injection.

Fluo-8 NW is a membrane permeable dye that increases in fluorescence by 100 fold upon binding to calcium. It is more than twice as bright as Fluo-4 NW and it has a similar affinity for calcium as Fluo-4 NW. Thus the NOVOstar and Fluo-8 NW, when combined, create the perfect platform for which to measure intracellular calcium.

Fig. 4: BMG LABTECH's NOVOstar with unique pipetting system allows for instantaneous reading upon agonist stimulation.

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