Cal-520®, Cal-590™, and Cal-630™ Calcium Detection Reagents

Introduction

Cal-520®, Cal-590™ and Cal-630™ provide the most robust homogeneous fluorescence-based assay tools for detecting intracellular calcium mobilization. They are fluorogenic calcium-sensitive dyes with a significantly improved signal to noise ratio and intracellular retention compared to the existing calcium indicators (such as Fluo-3 AM, Fluo-4 AM and Rhod-2 AM). Cells expressing a GPCR or calcium channel of interest that signals through calcium can be preloaded with Cal-520® AM, Cal-590™ AM or Cal-630™ AM which can cross cell membrane. Once inside the cell, the lipophilic blocking groups of Cal 520® AM, Cal-590™ AM or Cal-630™ AM are cleaved by intracellular esterases, resulting in a negatively charged fluorescent dye that stays inside cells. Their fluorescence is greatly enhanced upon binding to calcium. When cells stimulated with agonists, the receptor signals the release of intracellular calcium, which significantly increase the fluorescence of Cal-520®, Cal-590™ or Cal-630™. The characteristics of high sensitivity and >100 times fluorescence enhancement make Cal-520® AM, Cal-590™ AM or Cal-630™ AM ideal indicators for the measurement of cellular calcium. The high S/N ratio and better intracellular retention make the Cal-520®, Cal-590™ or Cal-630™ calcium assay a robust tool for evaluating GPCR and calcium channel targets as well as for screening their agonists and antagonists.

Besides their convenient excitation wavelengths and large fluorescence enhancement by calcium, Cal-520®, Cal-590™, and Cal-630™ are predominantly localized in cytosols unlike Rhod-2 that is mainly localized in mitochondria. In addition, the long Ex/Em wavelengths of Cal-590™ and Cal-630™ make these dyes perfect calcium indicators compatible for multicolor detection with green fluorescent protein (GFP) cell lines. In addition, Cal-520®, Cal-590™ or Cal-630™ calcium assays are optimized to be compatible with most of the existing fluorescence instruments. Cal-520 can be well excited at 488 nm, and used with FITC filter set. Cal-590 is optimized to be excited at 555 nm, and used with TRITC/Cy3 filter set. Cal-590 is optimized to be excited at 594 nm, and used with Texas Red® filter set. The spectral and calcium binding properties are summarized below (see Table 1).

Table 1. Spectral and Ca^{2+}–Binding Properties of Cal-520®, Cal-590™ or Cal-630™ Ca^{2+}Detection Reagents

Ca^{2+} Indicator	**Excitation (nm)**	**Emission (nm)**	**K_d (nM)**
Cal-520®	492 nm	514	320
Cal-590™	558	584	561
Cal-630™	607	623	792

Use of Cal-520® AM, Cal-590™ AM, or Cal-630™ AM Esters

1. Load Cells with Cal-520®, Cal-590™ or Cal-630™ AM Esters:

AM esters are the non-polar esters that readily cross live cell membranes, and rapidly hydrolyzed by cellular esterases inside live cells. AM esters are widely used for loading a variety of polar fluorescent probes into live cell non-invasively. However, cautions must be excised when AM esters are used since they are susceptible to hydrolysis, particularly in solution. They should be reconstituted just before use in high-quality, anhydrous dimethylsulfoxide (DMSO). DMSO stock solutions may be stored desiccated at –20 °C and protected from light. Under these conditions, AM esters should be stable for several months. Following is our recommended protocol for loading Cal-520® AM,Cal-590™ AM or Cal-630™ AM esters into live cells. This protocol only provides a guideline, and should be modified according to your specific needs.

a) Prepare a 2 to 5 mM stock solution of Cal-520® AM, Cal-590™ AM or Cal-630™ AM esters in high-quality, anhydrous DMSO.

b) On the day of the experiment, either dissolve Cal-520® AM, Cal-590™ AM or Cal-630™ AM in DMSO or thaw an aliquot of the indicator stock solution to room temperature. Prepare a dye working solution of 10 to 20 µM in Hanks and Hepes buffer (HHBS) or the buffer of your choice with 0.04% *Pluronic® F-127*. The exact concentration of the indicator required for cell loading must be determined empirically.
Note: The nonionic detergent Pluronic® F-127 is sometimes used to increase the aqueous solubility of Cal-520® AM, Cal-590™ AM or Cal-630™ AM esters. A variety of Pluronic® F-127 solutions can be purchased from AAT Bioquest.

c) If your cells (such as CHO cells) containing the organic anion-transports, probenecid (1-2 mM) may be added to the dye working solution (final in well concentration will be 0.5-1 mM) to reduce leakage of the de-esterified indicators.
Note: A variety of ReadiUse™ probenecid including water soluble sodium salt and stabilized solution can be purchased from AAT Bioquest

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Technical Support: support@aatbio.com; Tel: 408-733-1055

d) Add equal volume of the dye working solution (from Step b or c) into your cell plate.
e) Incubate the dye-loading plate at a cell incubator for 60 to 90 minutes, and then incubate the plate at room temperature for another 30 minutes.
Note: Incubate the dye longer than 2 hours gives better signal intensity for some cell lines.
f) Replace the dye working solution with HHBS or buffer of your choice (containing an anion transporter inhibitor, such as 1 mM probenecid, if applicable) to remove excess probes.
g) Run the experiments at Ex/Em = 490/525 nm (for Cal-520® AM), 540/590 nm (for Cal-590™ AM) or 600/640 nm (for Cal-630™ AM).

2. Measure Intracellular Calcium Responses:

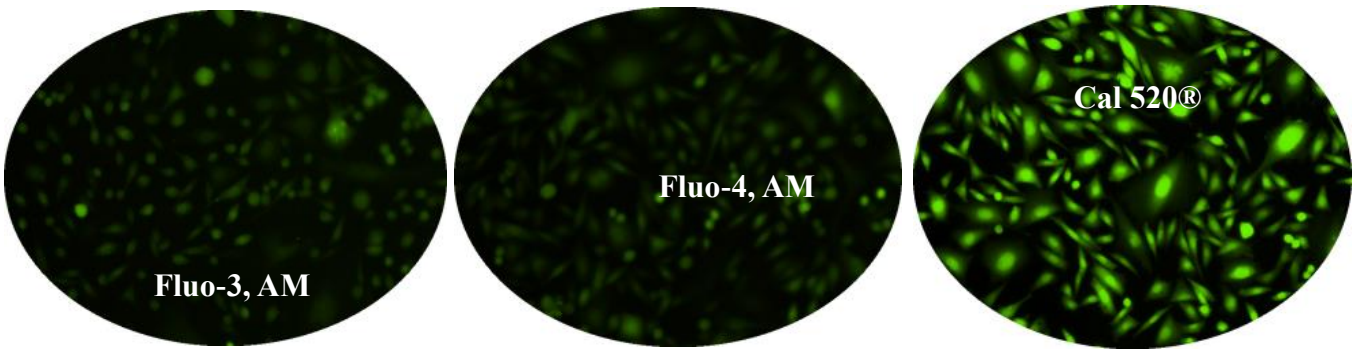

Figure 1. **Response of endogenous P2Y receptor to ATP in CHO-M1 cells without probenecid.** CHO-M1 cells were seeded overnight at 40,000 cells per 100 µL per well in a 96-well black wall/clear bottom costar plate. 100 µl of 4 µM Fluo-3 AM, Fluo-4 AM or Cal 520® AM in HHBS were added into the wells, and the cells were incubated at 37 °C for 2 hour. The dye loading medium were replaced with 100 µl HHBS, 50 µl of 300 µM ATP were added, and then imaged with a fluorescence microscope (Olympus IX71) using FITC channel.

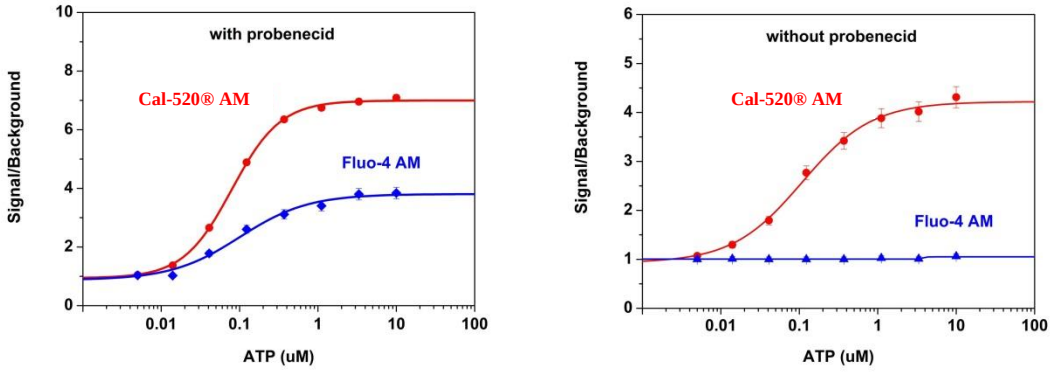

Figure 2. **ATP-stimulated calcium response of endogenous P2Y receptor in CHO-K1 cells measured with Cal-520® or Fluo-4 AM.** CHO-K1cells were seeded overnight in 50,000 cells per 100 µL per well in a 96-well black wall/clear bottom costar plate. 100 µL of 5 µM Fluo-4 AM or the Cal-520® AM with (A) or without (B) 2.5 mM probenecid was added into the cells, and the cells were incubated at 37°C for 2 hours. ATP (50µL/well) was added by FlexStation (Molecular Devices) to achieve the final indicated concentrations.

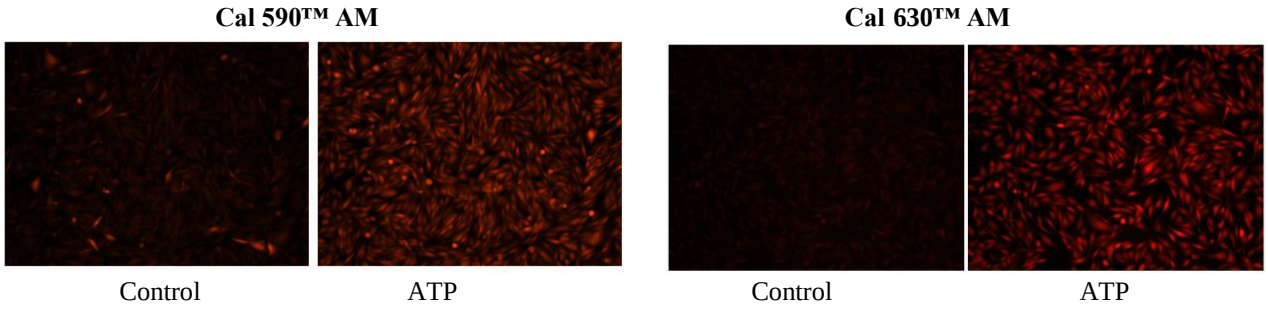

Figure 3. **Response of endogenous P2Y receptor to ATP in CHO-K cells.** CHO-K cells were seeded overnight at 40,000 cells per 100 µL per well in a 96-well black wall/clear bottom costar plate. 100 µl of 4 µM Cal 590™ AM or Cal 630™ AM in HHBS with 1 mM probenecid were added into the wells, and the cells were incubated at 37 °C for 2 hour. The dye loading mediums were replaced with 100 µl HHBS and 1 mM probenecid , then imaged with a fluorescence microscope (Olympus IX71) using TRITC channel before and after adding 50 µl of 300 µM ATP .

Use of Cal-520®, Cal-590™, or Cal-630™ Salts

Calcium calibration can be carried out by measuring the fluorescence intensity of the salt form (25 to 50 µM in fluorescence microplate readers) of the indicators in solutions with precisely known free Ca^{2+} concentrations. Calibration solutions can be used based on 30 mM MOPS EGTA Ca^{2+} buffer. In general, water contains trace amount of calcium ion. It is highly recommended to use 30 mM MOPS + 100 mM KCl, pH 7.2 as buffer system. One can simply make a 0 and 39 µM calcium stock solutions as listed below, and these 2 solutions are used to make a serial solution of different Ca^{2+} concentrations

A. 0 µM calcium: 30 mM MOPS + 100 mM KCl, pH 7.2 buffer + 10 mM EGTA

B. 39 µM calcium: 30 mM MOPS + 100 mM KCl, pH 7.2 buffer + 10 mM EGTA + 10 mM $CaCl_2$

To determine either the free calcium concentration of a solution or the K_d of a single-wavelength calcium indicator, the following equation is used:

$$[Ca]_{free} = K_d[F - F_{min}]/F_{max} - F]$$

Where F is the fluorescence intensity of the indicator at a specific experimental calcium level, F_{min} is the fluorescence intensity in the absence of calcium and F_{max} is the fluorescence intensity of the calcium-saturated probe.

The dissociation constant (K_d) is a measure of the affinity of the probe for calcium. The calcium-binding and spectroscopic properties of fluorescent indicators vary quite significantly in cellular environments compared to calibration solutions. *In situ* response calibrations of intracellular indicators typically yield K_d values significantly higher than *in vitro* determinations. *In situ* calibrations are performed by exposing loaded cells to controlled Ca^{2+} buffers in the presence of ionophores such as A-23187, 4-bromo A-23187 and ionomycin. Alternatively, cell permeabilization agents such as digitonin or Triton® X-100 can be used to expose the indicator to the controlled Ca^{2+} levels of the extracellular medium.

Use of Cal-520®, Cal-590™, or Cal-630™ Dextran Conjugate

Compared to the free ion indicator, dextran conjugates of these same indicators exhibit both reduced compartmentalization and much lower rates of dye leakage. Since the molecular weight of the dextran, net charge, degree of labeling, and nature of the dye may affect the experiment, researchers are advised to consult the primary literature for information specific to the application of interest.

Conclusions

Because of the importance of Ca^{2+} in biology, numerous techniques/methods for analyzing the mechanisms of cellular and/or subcellular Ca^{2+} activity have been established. Although each method for analyzing Ca^{2+}activity has certain advantages over the others, each also suffers from drawbacks. With the outstanding properties described above, we believe

that Cal-520®, Cal-590™ and Cal-630™ calcium detection reagents provide a new powerful tool for intracellular calcium analysis and monitor in a variety of biological systems.

As might have been predicted, the interests of many researchers in Ca^{2+} analysis shifted from the cellular level to the subcellular level. It has been found that Ca^{2+} is not even distributed throughout the whole cell and that intracellular heterogeneity of Ca^{2+} (such as Ca^{2+} waves and Ca^{2+} sparks) is observed in a variety of cells (e.g., oocyte, heart muscle cell, hepatocyte, and exocrine cell). With the advent of the confocal laser scanning microscope (CLSM) in the 1980s and advanced microplate readers in 2000s (such as FLIPR, FDSS and NOVOStar dedicated for intracellular Ca^{2+} detections), the measurement of intracellular Ca^{2+} has accelerated significantly. Confocal laser scanning microscopy and more recently multiphoton microscopy allow the precise spatial and temporal analysis of intracellular Ca^{2+} signaling at the subcellular level in addition to the measurement of its concentration.

Product Ordering Information

Cat. #	Product Name	Unit Size
20605	Cal-520® -Biotin Conjugate	5x50 µg
20606	Cal-520® -Biocytin Conjugate	5x50 µg
20609	Cal-520® NHS Ester	100 µg
20610	Cal-520® Maleimide	100 µg
21130	Cal-520®, AM *Cell-permeable*	10x50 µg
21131	Cal-520®, AM *Cell-permeable*	1 mg
21135	Cal-520®, sodium salt	10x50 µg
21136	Cal-520®, sodium salt	1 mg
21140	Cal-520®, potassium salt	10x50 µg
21141	Cal-520®, potassium salt	1 mg
21142	Cal-520FF™, AM *Cell-permeable*	1 mg
21143	Cal-520FF™, AM *Cell-permeable*	10x50 µg
21144	Cal-520FF™, potassium salt	10x50 µg
20600	Cal-520®-Dextran Conjugate *MW 3,000*	1 mg
20601	Cal-520®-Dextran Conjugate *MW 10,000*	5 mg
20508	Cal-590™-Dextran Conjugate *MW 3,000*	1 mg
20509	Cal-590™-Dextran Conjugate *MW 10,000*	1 mg
20510	Cal-590™, AM *Cell-permeable*	5x50 µg
20511	Cal-590™, AM *Cell-permeable*	10x50 µg
20512	Cal-590™, AM *Cell-permeable*	1 mg
20530	Cal-630™, AM *Cell-permeable*	5x50 µg
20515	Cal-590™, sodium salt	5x50 µg
20518	Cal-590™, potassium salt	5x50 µg
20531	Cal-630™, AM *Cell-permeable*	10x50 µg
20532	Cal-630™, AM *Cell-permeable*	1 mg
20535	Cal-630™, sodium salt	5x50 µg
20538	Cal-630™, potassium salt	5x50 µg
20545	Cal-630-Dextran Conjugate *MW 3,000*	1 mg
20546	Cal-630-Dextran Conjugate *MW 10,000*	1 mg
20588	Cal Red™ R525/650 potassium salt	5x50 µg
20590	Cal Red™ R525/650 AM	1 mg
20591	Cal Red™ R525/650 AM	10x50 ug

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