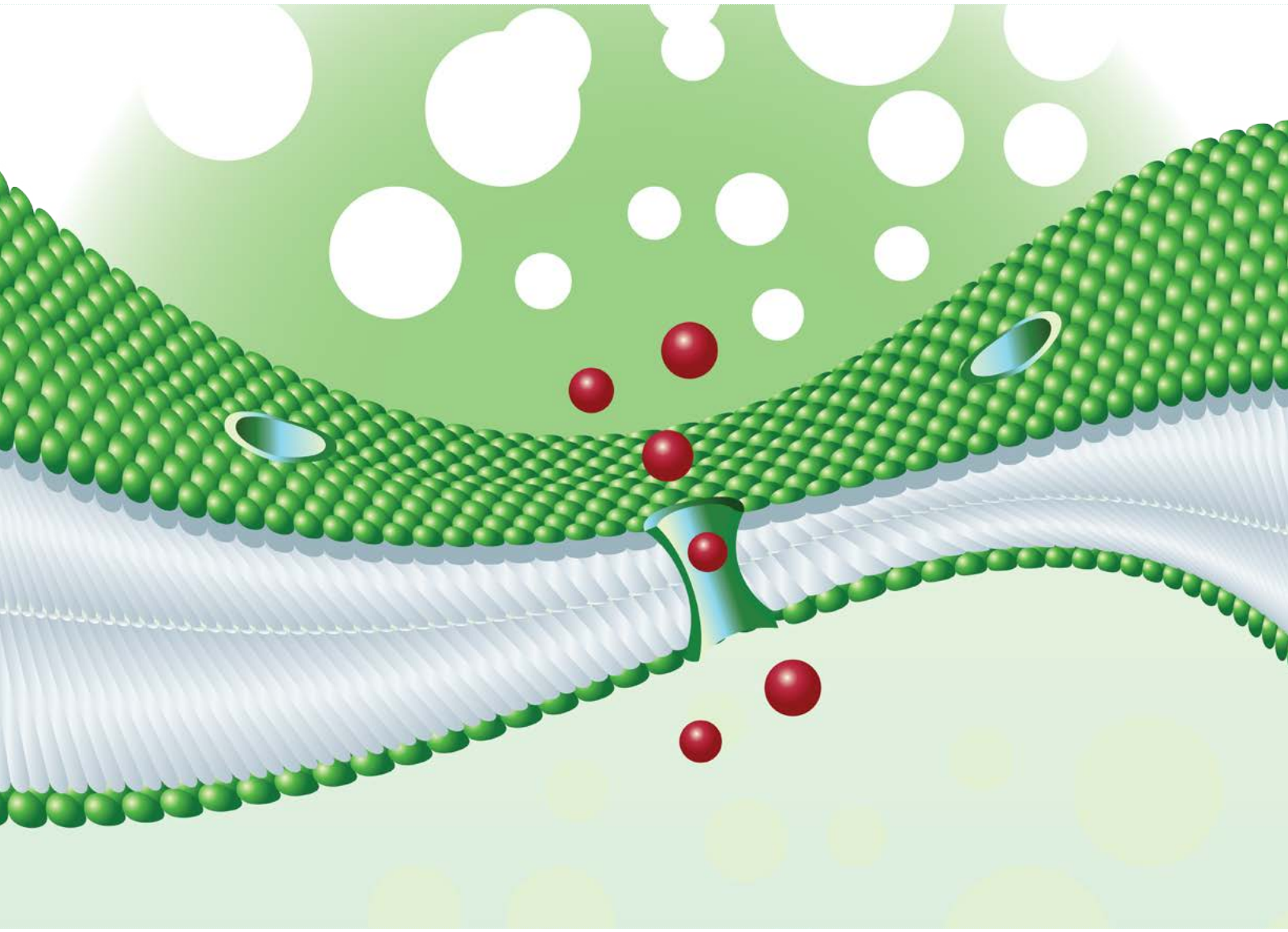
Calcium Detection Probes & Assay Kits



Fluo-8® | **Cal-520®** | **Calbryte™ 520**

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AAT Bioquest®

Fluorescent Single Wavelength Calcium Indicators

Calcium acts as a universal second messenger in a variety of cells. Numerous functions of all types of cells are regulated by Ca^{2+}, thus calcium measurement is critical for various biological investigations. Since the 1920s, scientists have attempted to measure Ca^{2+}, but few were successful due to the limited availability of Ca^{2+} probes. The first reliable measurement of Ca^{2+} was performed by Ridgway and Ashley by injecting the photoprotein aequorin into the giant muscle fiber of the barnacle. Subsequently, in the 1980s, Tsien and colleagues produced a variety of fluorescent indicators. Among them Indo-1, Fura-2, Fluo-3 and Rhod-2 have been the most valuable dyes for measuring Ca^{2+} with a fluorescence instrument. In recent years, AAT Bioquest has introduced the most robust calcium probes: Fluo-8®, Cal-520® & Calbryte™ 520, all of which enable the high throughput screening of GPCR and calcium channel drug discovery targets through the convenient calcium detection. FLIPR® and FlexStation® instruments of Molecular Devices, FDSS®/µCELL of Hamamatsu and NOVOstar of BMG Technologies have further accelerated the high throughput measurement of calcium for GPCR and ion channel research.

Fluorescent probes that show spectral responses upon binding Ca^{2+} have enabled researchers to investigate changes in intracellular free Ca^{2+} concentrations by using fluorescence microscopy, flow cytometry, fluorescence spectroscopy and fluorescence microplate readers. Most of these fluorescent indicators are derivatives of BAPTA chelators that incorporate a PET system responsive to calcium. There are quite a few factors that need be considered when selecting a fluorescent Ca^{2+} indicator. These include:

• ***Spectral Properties:*** For UV excitation, Indo-1 and Fura-2 are widely used. Fura-8™ is a newly developed excitation-ratioable calcium dye. Its AM is superior to Fura-2 AM with higher signal/background ratio in cells. Fluo-8®, Cal-520® & Calbryte™ 520 are preferred for 488 nm excitation while Cal-590™, Calbryte™ 590, Cal-630™, Calbryte™ 630, Rhod-2 and Rhod-4™ are used for red emissions.

• ***Measurement Mode:*** Ion indicators that exhibit spectral shifts upon ion binding can be used for ratiometric measurements of Ca^{2+} concentration, which are essentially independent of uneven dye loading, cell thickness, photobleaching effects and dye leakage. Excitation and emission wavelength preferences depend on the type of instrumentation being used, as well as on sample autofluorescence and on the presence of other fluorescent or photoactivatable probes in the experiment. Indo-1, Fura-2 and our newly developed Fura-8™ are primary choices for ratiometric measurements while Fluo-3, Fluo-4, Fluo-8®, Cal-520®, Calbryte™ 520, Cal-590™, Calbryte™ 590, Cal-630™, Calbryte™ 630, Rhod-2 and Rhod-4™ are predominantly used for single wavelength measurements.

• ***Permeability of Ca^{2+} Indicators (salt or AM ester):*** The salt forms are typically loaded into cells by microinjection, microprojectile bombardment or electroporation, or used for extracellular assays. In contrast, the cell-permeant acetoxymethyl (AM) esters can be passively loaded into cells, where they are cleaved to cell-impermeant products by intracellular esterases.

• ***Dissociation Constant (K_d):*** The desired indicators must have a proper K_d compatible with the Ca^{2+} concentration range of interest. The K_d values of Ca^{2+} indicators are dependent on many factors, including pH, temperature, ionic strength, viscosity, protein binding, the presence of Mg^{2+} and other ions. Consequently, K_d values for intracellular indicators are usually significantly higher than the corresponding values measured in cell-free solutions.

Among the visible light-excitable calcium indicators, Fluo-8®, Fluo-4, Fluo-3, Rhod-2 and Rhod-4™ are most commonly used. Fluo-8® indicators are widely used in flow cytometry and confocal laser-scanning microscopy. More recently, Fluo-8® AM has been extensively used for high throughput screening GPCR targets. Fluo-8® is essentially nonfluorescent unless bound to Ca^{2+} and exhibits a quantum yield of ~0.15 in the presence of saturating Ca^{2+} and a K_d of 390 nM for Ca^{2+}. Cal-520® is a robust green fluorescent calcium indicator with a greatly improved signal/background ratio and intracellular retention. Calbryte™ 520 is by far the best 488 nm-excitable green fluorescent calcium indicator with a exceptionally improved signal/background ratio, intracellular retention as well as easy cell dye loading property.

Table 2.1 Classic Single Wavelength Fluorescent Calcium Indicators

Cat #	Product Name	Size	Ex (nm)	Em (nm)	K_d
20500	Cal Green™-1 [equivalent to Calcium Green™-1]	10x50 µg	506	531	190 nM
20501	Cal Green™-1 AM [equivalent to Calcium Green™-1 AM]	10x50 µg	506	531	190 nM
21011	Fluo-3 AM *UltraPure grade*	1 mg	506	526	390 nM
21018	Fluo-3, pentaammonium salt	1 mg	506	526	390 nM
21017	Fluo-3, pentapotassium salt	1 mg	506	526	390 nM
21016	Fluo-3, pentasodium salt	1 mg	506	526	390 nM
20507	OG488 BAPTA-1, AM [equivalent to Oregon Green® 488 BAPTA-1, AM]	500 µg	494	523	170 nM
20506	OG488 BAPTA-1, hexapotassiu salt [equivalent to Oregon Green® 488 BAPTA-1, hexapotassium salt]	500 µg	494	523	170 nM
21064	Rhod-2 AM *UltraPure grade*	20x50 µg	549	578	570 nM
21067	Rhod-2, tripotassium salt	1 mg	549	578	570 nM
21068	Rhod-2, trisodium salt	1 mg	549	578	570 nM
21070	Rhod-5N AM	1 mg	551	577	0.3 mM
21072	Rhod-5N, tripotassium salt	1 mg	551	577	0.3 mM

The long-wavelength Rhod-4™ is a valuable alternative Ca^{2+} indicator to the green fluorescent Fluo-8®, Fluo-4 and Fluo-3 for experiments in cells and tissues that have high levels of autofluorescence. Rhod-5N has a lower binding affinity for Ca^{2+} than any other BAPTA-based indicator (K_d = ~320 µM) and is suitable for Ca^{2+} measurements from 10 µM to 1 mM. Like the parent Rhod-2 indicator, Rhod-5N is essentially nonfluorescent in the absence of divalent cations and exhibits strong fluorescence enhancement with no spectral shift upon binding Ca^{2+}. Both Fluo and Rhod indicators are available as cell-impermeant potassium salts or as cell-permeant AM esters.

Blue-Green Fluorescent Calcium Indicators

Cal-500™

Cal-500™ is a unique violet laser-excitable fluorescent calcium indicator with excitation at 390 nm and emission at 500 nm. Its excitation wavelength matches the violet laser line of flow cytometer, which makes it convenient for measuring calcium response using flow cytometry. It can also be used to detect calcium response using fluorescence microscopes and microplate readers. Upon binding to calcium, Cal-500™ enhances its fluorescence by 64 folds. Cal-500™ AM (Cat# 20410) has an increased cellular calcium response around 4 folds.

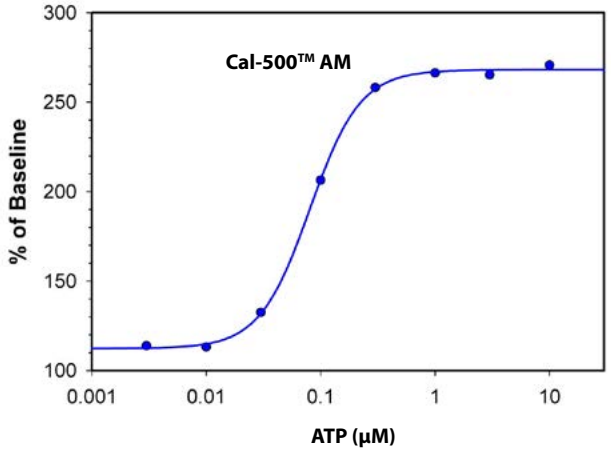

Figure 2.1 The ATP dose dependent intracellular calcium release was measured by Cal-500™ AM (Cat# 20410) in CHO-K1 cells. Cells were incubated with Cal-500™ AM dye for 60 minutes at 37 °C before different concentration of ATP was added into the cells. The response was measured over time on FlexStation®.

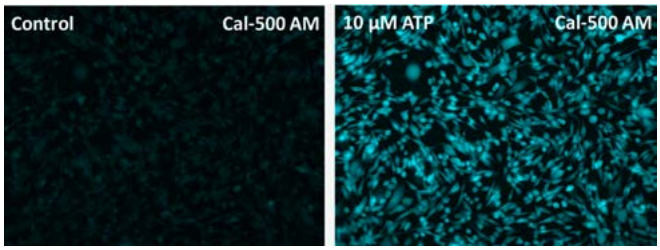

Figure 2.2 Response of endogenous P2Y receptor to ATP in CHO-K1 cells. CHO-K1 cells were seeded overnight at 40,000 cells per 100 µL per well in a 96-well black wall/clear bottom Costar plate. 100 µL of Cal-500™ AM in HHBS with probenecid were added into the wells, and the cells were incubated at 37 °C for 60 minutes. The dye loading medium were replaced with 200 µL HHBS. Images were taken before and after the addition of 50 µL of 10 µM ATP using a fluorescence microscope (Keyence) using 405 nm and 465 nm long pass filters.

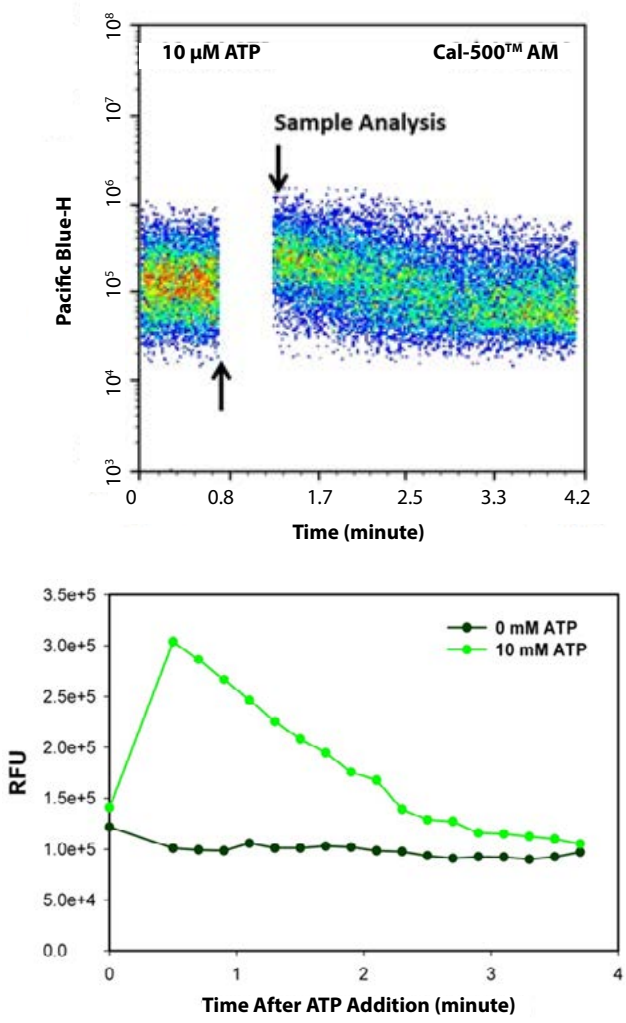

Figure 2.3 The ATP dependent intracellular calcium release was measured by Cal-500™ AM (Cat# 20410) in CHO-K1 cells. Cells were incubated with Cal-500™ AM dye for 60 minutes at 37 °C before 10 µM ATP was added into the cells. **Top:** The baseline was acquired and the rest of the cells were analyzed after the addition of ATP. The response was measured over time. The analysis was done with a NovoCyte™ 3000 flow cytometer. The arrows on the graph indicate the time between addition of ATP and the actual analysis. **Bottom:** Time dependent change of fluorescence. Time is relative to ATP stimulation, time 0 is the stimulation time, and the initial detection point was ~30 seconds relative to stimulation.

Table 2.2 Cal-500™ Calcium Indicators

Cat #	Product Name	Size	Ex (nm)	Em (nm)	K_d
20410	Cal-500™, AM	10x50 µg	390	500	303 nM
20412	Cal-500™, potassium salt	10x50 µg	390	500	303 nM

Green Fluorescent Calcium Indicators

Traditional Green Fluorescent Calcium Indicators

Fluo-2 is the parent compound of Fluo-3 and Fluo-4. These fluorescent calcium indicators have calcium-dependent fluorescence. Fluo-3 and Fluo-4 were the most commonly used visible light-excitable calcium indicators.

The cell-permeant Mag-Fluo-4 AM (Cat# 20401) is an analog of Fluo-4 AM with a K_d of 4.7 mM for Mg ion and a K_d of 22 µM for Ca^{2+} ion, making it useful as an intracellular Mg ion indicator as well as a low-affinity Ca^{2+} ion indicator. This low-affinity fluorescent Ca^{2+} ion indicator has been used to accurately track the kinetics of the spatially averaged free Ca^{2+} ion transient in skeletal muscle. Mag-fluo-4 yields reliable kinetic information about the spatially averaged free Ca^{2+} ion transient in skeletal muscle.

Table 2.3 Traditional Green Fluorescent Calcium Indicators

Cat #	Product Name	Size	Ex (nm)	Em (nm)	K_d
20494	Fluo-2, AM	10x50 µg	494	517	232 nM
20493	Fluo-2, potassium salt	10x50 µg	494	517	232 nM
21011	Fluo-3, AM *ultraPure grade*	1 mg	506	526	390 nM
21018	Fluo-3, pentaammonium salt	1 mg	506	526	390 nM
21017	Fluo-3, pentapotassium salt	1 mg	506	526	390 nM
21016	Fluo-3, pentasodium salt	1 mg	506	526	390 nM
21014	Fluo-3FF, AM	10x50 µg	506	526	~10 µM
21019	Fluo-3FF, pentapotassium salt	10x50 µg	506	526	~10 µM
20551	Fluo-4, AM *UltraPure grade*	10x50 µg	494	516	345 nM
20556	Fluo-4, pentapotassium salt	10x50 µg	494	516	345 nM
20560	Fluo-5F, AM	10x50 µg	494	516	~2.3 µM
20562	Fluo-5F, pentapotassium salt	10x50 µg	494	516	~2.3 µM
20566	Fluo-5N, AM	10x50 µg	494	516	~90 µM
20567	Fluo-5N, pentapotassium salt	10x50 µg	494	516	~90 µM
20401	Mag-Fluo-4, AM	10x50 µg	494	516	22 µM
20400	Mag-Fluo-4, potassium salt	10x50 µg	494	516	22 µM

Fluo-8® Calcium Indicators

Fluo-8® dyes have been developed to improve cell loading and calcium response while maintaining the convenient Fluo-3 and Fluo-4 spectral wavelengths of maximum excitation @ ~490 nm and maximum emission @ ~520 nm. For cell loading, Fluo-8® AM only requires incubation at room temperature while Fluo-3 AM and Fluo-4 AM require incubation at 37 °C. In addition, Fluo-8® AM is 2 times brighter than Fluo-4 AM, and 4 times brighter than Fluo-3 AM in cells. AAT Bioquest offers a set of outstanding Fluo-8® reagents with different calcium binding affinities.

Key Features of Fluo-8® AM

- ***Faster,*** more readily loaded into cells than Fluo-3 AM and Fluo-4 AM. Only room temperature is required.
- ***Brighter,*** much brighter than Fluo-3 AM and Fluo-4 AM in cells.
- ***Convenient,*** almost identical spectra to those of Fluo-4 AM.

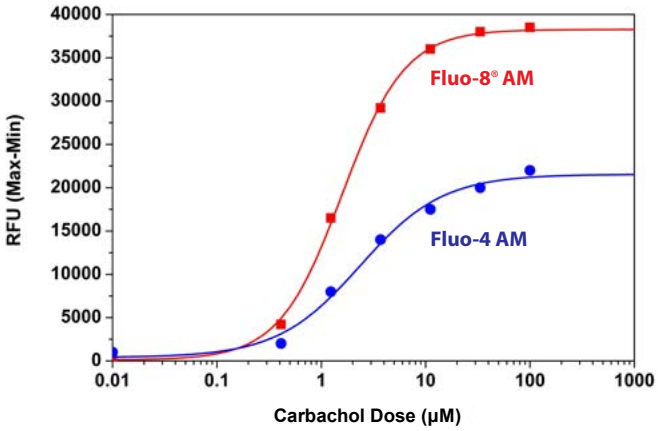

Figure 2.4 Carbachol dose responses were measured in HEK-293 cells with Fluo-8® AM (Cat# 21082) and Fluo-4 AM (Cat# 20551). HEK-293 cells were seeded overnight at 40,000 cells/100 µL/well in a 96-well black wall/clear bottom Costar plate. The growth medium was removed, and the cells were incubated with 100 µL of dye-loading solution containing Fluo-8® AM or Fluo-4 AM for 1 hour at room temperature. Carbachol (25 µL/well) was added by NOVOstar to achieve the final indicated concentrations. The fluorescence signals were measured at Ex/Em = 490/525 nm. The EC_{50} of Fluo-8® AM is about 1.2 µM.

Table 2.4 Fluo-8® Calcium Indicators

Cat #	Product Name	Size	Ex (nm)	Em (nm)	K_d (nM)
21082	Fluo-8® AM	10x50 µg	494	517	389
21088	Fluo-8®, sodium salt	10x50 µg	494	517	389
21089	Fluo-8®, potassium salt	10x50 µg	494	517	389
21104	Fluo-8FF™ AM	10x50 µg	494	517	10,000
21102	Fluo-8FF™, potassium salt	10x50 µg	494	517	10,000
21090	Fluo-8H™ AM	1 mg	494	517	232
21095	Fluo-8H™, sodium salt	10x50 µg	494	517	232
21096	Fluo-8L™, AM	1 mg	494	517	1,860
21098	Fluo-8L™, sodium salt	10x50 µg	494	517	1,860
21100	Fluo-8L™, potassium salt	10x50 µg	494	517	1,860

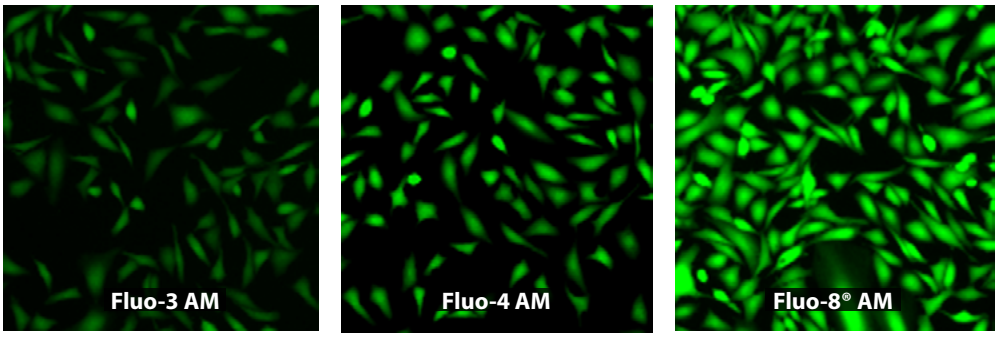

Figure 2.5 U2OS cells were seeded overnight at 40,000 cells per 100 µL per well in a Costar black wall/clear bottom 96-well plate. The growth medium was removed, and the cells were incubated with 100 µL of 4 µM Fluo-3 AM, Fluo-4 AM and Fluo-8® AM in HHBS at 37 °C for 1 hour. The cells were washed twice with 200 µL HHBS, and imaged with Olympus IX71 using FITC channel.

Cal-520® Calcium Indicators

Cal-520® provides a robust homogeneous fluorescence-based assay tool for detecting intracellular calcium mobilization. Cal-520® AM is a new fluorogenic calcium-sensitive dye with a significantly improved signal to background ratio and intracellular retention compared to the existing green calcium indicators (such as Fluo-3 AM and Fluo-4 AM). The higher signal/background ratio and better intracellular retention make the Cal-520® calcium assay a robust tool for evaluating GPCR and calcium channel targets as well as for screening their agonists and antagonists.

Our preliminary in-house research indicated that Cal-520® AM can be readily loaded to a guinea pig's heart and stays there for a few hours in the absence of probenecid. The calcium signal can be readily monitored with Cal-520® AM while it is difficult to observe the calcium signal under the same conditions with other green calcium dyes, such as Fluo-3 AM and Fluo-4 AM.

Table 2.5 Spectral Comparison of Fluo-3, Fluo-4, Fluo-8®, Cal-520® & Calbryte™ 520

Dye	Ex (nm)	Em (nm)	QY*
Calbryte™ 520	492	514	**0.75**
Cal-520®	492	514	**0.75**
Fluo-3	506	525	0.15
Fluo-4	493	515	0.16
Fluo-8®	490	514	0.16

*QY = Fluorescence Quantum Yield in the presence of 5 mM calcium citrate.

Key Features of Cal-520® AM

- ***Better Intracellular Retention,*** Cal-520® AM is better retained in live cells than Fluo-3 AM and Fluo-4 AM.
- ***Higher Sensitivity,*** Cal-520® AM has much higher signal-to-background ratio than Fluo-3 AM and Fluo-4 AM in cells.
- ***Convenient,*** Cal-520® AM has almost identical spectra to those of Fluo-4 AM.

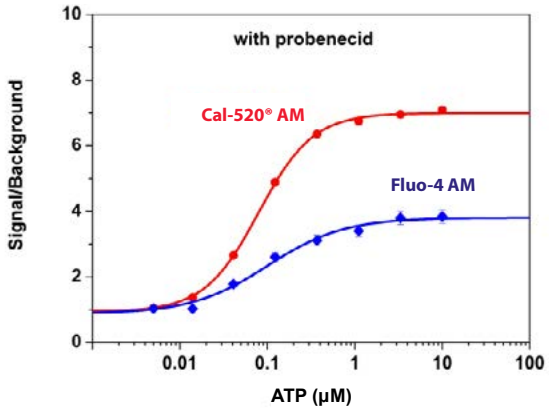

Figure 2.6 ATP-stimulated calcium responses of endogenous P2Y receptor in CHO-K1 cells incubated with Cal-520® AM (red curve, Cat# 21131), or Fluo-4 AM (blue curve) respectively with probenecid under the same conditions. CHO-K1 cells were seeded overnight at 50,000 cells/100 µL/well in a Costar 96-well black wall/clear bottom plate. 100 µL of 5 µM Fluo-4 AM or Cal-520® AM in HHBS with 2.5 mm probenecid was added into the cells, and the cells were incubated at 37 °C for 2 hours.

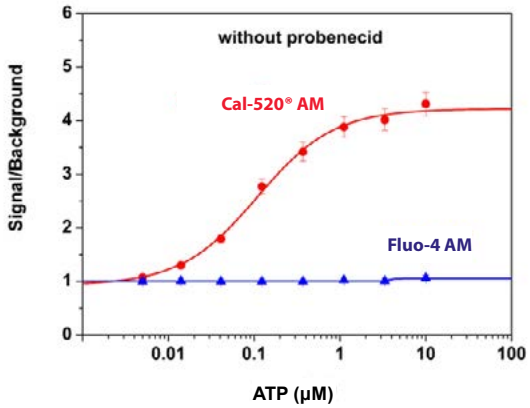

Figure 2.7 ATP-stimulated calcium responses of endogenous P2Y receptors in CHO-K1 cells incubated with Cal-520® AM (red curve, Cat# 21131), or Fluo-4 AM (blue curve, Cat# 20551) respectively, without probenecid under the same conditions. CHO-K1 cells were seeded overnight at 50,000 cells/100 µL/well in a Costar 96-well black wall/clear bottom plate. 100 µL of 5 µM Fluo-4 AM or Cal-520® AM in HHBS was added into the cells, and the cells were incubated at 37 °C for 2 hours.

Table 2.6 Cal-520® Calcium Indicators

Cat #	Product Name	Size	Ex (nm)	Em (nm)	K_d
21131	Cal-520®, AM	1 mg	492	514	320 nM
21141	Cal-520®, potassium salt	1 mg	492	514	320 nM
21136	Cal-520®, sodium salt	1 mg	492	514	320 nM
20606	Cal-520®-Biocytin Conjugate	5x50 µg	492	514	N/D
20605	Cal-520®-Biotin Conjugate	5x50 µg	492	514	N/D
20600	Cal-520®-Dextran Conjugate *MW 3,000*	1 mg	492	514	N/D
20601	Cal-520®-Dextran Conjugate *MW 10,000*	5 mg	492	514	N/D
20610	Cal-520® Maleimide	100 µg	492	514	N/D
20609	Cal-520®, NHS ester	100 µg	492	514	N/D
21142	Cal-520FF™ AM	1 mg	492	514	9.8 µM
21144	Cal-520FF™, potassium salt	10x50 µg	492	514	9.8 µM
21146	Cal-520N™, AM	10x50 µg	492	514	90 µM
21147	Cal-520N™, potassium salt	10x50 µg	492	514	90 µM

Calbryte™ 520 Calcium Indicators

The Calbryte™ series is a family of the brightest fluorescent dyes with the highest signal-to-background ratio developed to monitor intracellular calcium. It includes three novel calcium indicators: Calbryte™ 520, Calbryte™ 590 and Calbryte™ 630.

Followed by Fluo-3 being introduced in 1989, Fluo-4, Fluo-8 and Cal-520® were later developed with improved signal/background ratio, and became the widely used Ca^{2+} indicators for confocal microscopy, flow cytometry and high throughput screening applications. However, there are still a few severe problems with Fluo-4. For example, as for Fluo-3, in all most all the intracellular calcium assays with Fluo-4 AM, probenecid is required to prevent the cell-loaded Fluo-4 from leaking out of cells. The use of probenecid with Fluo-4-based calcium assays compromises the assay results since probenecid is well-documented to have a variety of complicated cellular effects. Calbryte™ 520 AM is a new fluorescent and cell-permeable calcium indicator. Like other dye AM cell loading, Calbryte™ 520 AM ester is non-fluorescent and once gets inside cells, it is hydrolyzed by intracellular esterase and gets activated. The activated indicator is a polar molecule that is no longer capable of freely diffusing through cell membrane, essentially trapped inside cells. Upon binding Ca^{2+} ions, Calbryte™ 520 produces bright fluorescence signal with extremely high signal/background ratio. In addition, Calbryte™ 520 demonstrates greatly improved intracellular retention. It has the identical excitation and emission wavelength as Fluo-4, thus the same Fluo-4 assay settings can be readily applied to Calbryte™ 520-based calcium assays. Calbryte™ 520 is a new generation of fluorescent indicators for the measurement of intracellular calcium. Its greatly improved signal/background ratio and intracellular retention properties make Calbryte™ 520 AM the most robust indicator for evaluating GPCR & calcium channel targets as well as for screening their agonists and antagonists in live cells.

Key Features of Calbryte™ 520 AM

- Exceptionally brighter than any other calcium indicators under the same condition
- Greatly improved signal to background ratio than Fluo-3 AM and Fluo-4 AM in cells
- Significantly enhanced intracellular retention (decrease or even eliminate the use of probenecid)
- Faster cell loading (Room temperature is ok.)

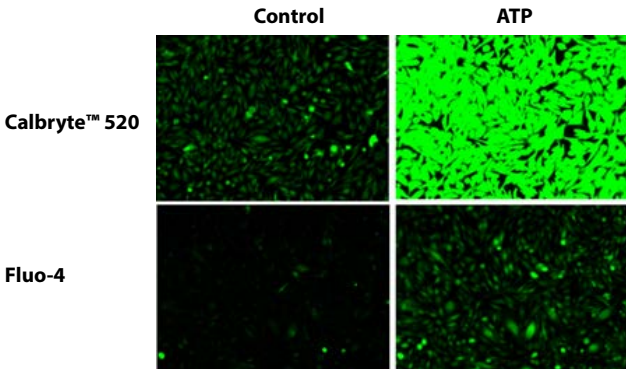

Figure 2.8 Response of endogenous P2Y receptor to ATP in CHO-K1 cells. CHO-K1 cells were seeded overnight at 40,000 cells/100 µL/well in a 96-well black wall/clear bottom Costar plate. 100 µL of Fluo-4 AM (Cat# 20551) or Calbryte™ 520 AM (Cat# 20651) in HHBS with probenecid were added into the wells, and the cells were incubated at 37 °C for 45 minutes. The dye loading solution was replaced with 200 µL HHBS, 50 µL of 50 µM ATP was added. The cells were imaged with a fluorescence microscope (Keyence) using FITC channel.

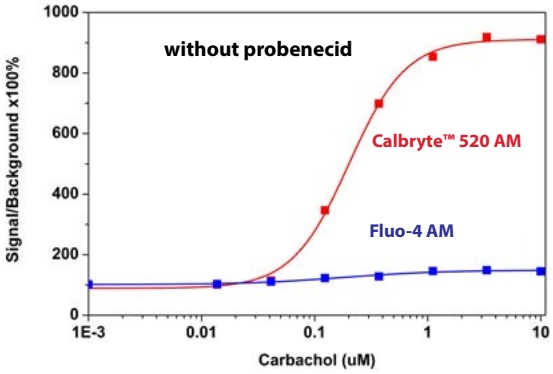

Figure 2.9 Carbachol-stimulated calcium response of exogenous M1 receptor in CHO-M1 cells measured with Calbryte™ 520 AM (Cat# 20651) or Fluo-4 AM (Cat# 20551). CHO-M1 cells were seeded overnight at 40,000 cells/100 µL/well in a 96-well black wall/clear bottom Costar plate. 100 µL of Fluo-4 AM or Calbryte™ 520 AM without probenecid was added into the cells, and the cells were incubated at 37 °C for 45 minutes. Carbachol (50 µL/well) was added by FlexStation® 3 to achieve the final indicated concentrations.

Table 2.7 Calbryte™ 520 Calcium Indicators

Cat #	Product Name	Size	Ex (nm)	Em (nm)	K_d (μM)
20651	Calbryte™ 520, AM	10x50 μg	492	514	1.2
20658	Calbryte™ 520, potassium salt	10x50 μg	492	514	1.2
20640	Calbryte™ 520L, AM	10x50 μg	492	524	91
20650	Calbryte™ 520L, potassium salt	10x50 μg	492	524	91

Red Fluorescent Calcium Indicators

Cal-590™ Calcium Indicators

Rhod-2 is the most commonly used red fluorescent calcium indicators. However, Rhod-2 AM (Cat# 21064) is only moderately fluorescent in live cells upon esterase hydrolysis, and has very small cellular calcium responses. Moreover, Rhod-2 is concentrated inside mitochondria and is not homogenously localized inside cells upon loading.

Cal-590™ has been developed to improve Rhod-2 AM cell loading and calcium response while maintaining the similar spectral wavelengths of Rhod-2 AM, making it compatible with TRITC/Cy3® filter set. In CHO and HEK cells, the cellular calcium response of Cal-590™ is much more sensitive than that of Rhod-2 AM. The spectra of Cal-590™ is well separated from those of FITC, Alexa Fluor® 488 and GFP, making it an ideal calcium probe for multiplexing intracellular assays with GFP cell lines or FITC/Alexa Fluor® 488 labeled antibodies.

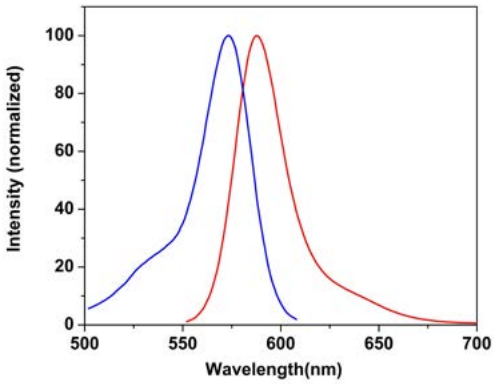

Figure 2.10 The excitation and emission spectra of Cal-590™ in the presence of calcium chloride (5 mM).

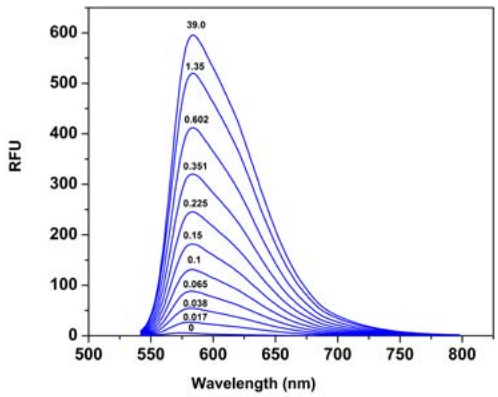

Figure 2.11 Fluorescence emission spectra of Cal-590™ in solutions containing 0 to 39 μM free Ca^{2+}.

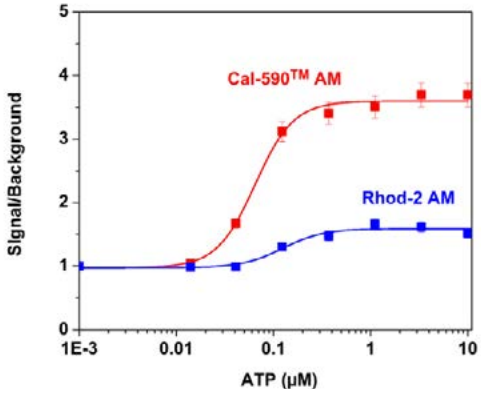

Figure 2.12 ATP-stimulated calcium response of endogenous P2Y receptor in CHO-K1 cells incubated with Cal-590™ AM (red, Cat# 20510) and Rhod-2, AM (blue, Cat# 21064) under the same conditions. CHO-K1 cells were seeded overnight at the cell density of 50,000 cells/100 μL/well in a 96-well black wall/clear bottom plate. 100 μL of 5 μg/mL Cal-590™ AM or Rhod-2 AM with 2.5 mM probenecid was added into the cells, and the cells were incubated at 37 °C for 1 hour. ATP (50 μL/well) was added by FlexStation® (Molecular Devices) to achieve the final indicated concentrations.

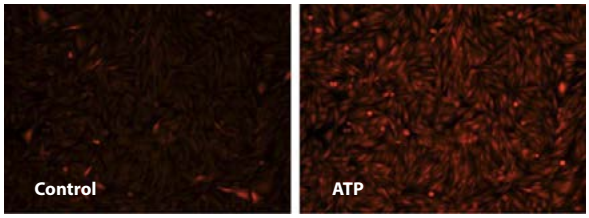

Figure 2.13 Responses of endogenous P2Y receptor to ATP in CHO-K1 cells. CHO-K1 cells were seeded overnight at 40,000 cells/100 μL/well in a Costar 96-well black wall/clear bottom plate. 100 μL of 4 μM Cal-590™ AM (Cat# 20510) in HHBS with 1 mM probenecid was added into the wells, and the cells were incubated at 37 °C for 2 hours. The dye loading solution was replaced with 100 μL HHBS and 1 mM probenecid. The cells were imaged with a fluorescence microscope (Olympus IX71) using TRITC channel before and after adding 50 μL of 300 μM ATP .

Table 2.8 Cal-590™ Calcium Indicators

Cat #	Product Name	Size	Ex (nm)	Em (nm)	K_d (nM)
20511	Cal-590™, AM	10x50 μg	573	588	561
20518	Cal-590™, potassium salt	5x50 μg	573	588	561
20515	Cal-590™, sodium salt	5x50 μg	573	588	561
20508	Cal-590™-Dextran Conjugate *MW 3,000*	1 mg	573	588	N/D
20509	Cal-590™-Dextran Conjugate *MW 10,000*	1 mg	573	588	N/D

Calbryte™ 590 Calcium Indicators

Calbryte™ 590 is our upgrade for orange-red fluorescent indicators such as Calcium Orange™ and Rhod-2. This dye has an excitation maximum at 580 nm and is well excited by the 555 nm laser line. It has an emission maximum at 592 nm, making it compatible with TRITC/Cy3® filter sets. Calbryte™ 590 is approximately ten times more sensitive for calcium than Rhod-2 under comparable conditions. Moreover, unlike Rhod-2 which primarily localizes in mitochondria, Calbryte™ 590 retains well in the cytosol of cells.

Key Features of Calbryte™ 590 AM

- A red-shifted calcium indicator compatible with GFP
- A superior replacement for Calcium Orange™ and Rhod-2
- Ten times more sensitive than Rhod-2
- Greatly improved signal to background ratio than Rhod-2 and Cal-590™ in cells
- Significantly enhanced intracellular retention
- Homogeneous cytosolic location

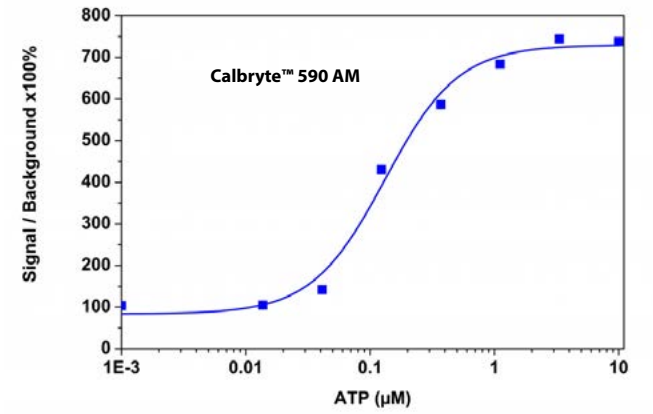

Figure 2.14 ATP dose response was measured in CHO-K1 cells with Calbryte™ 590 AM (Cat# 20701). CHO-K1 cells were seeded overnight at 50,000 cells/100 µL/well in a 96-well black wall/clear bottom Costar plate. 100 µL of 10 µg/mL Calbryte™ 590 AM in HH Buffer with probenecid was added and incubated for 60 minutes at 37°C. Dye loading solution was removed and replaced with 200 µL HH Buffer/well. ATP (50 µL/well) was added by FlexStation® 3 to achieve the final indicated concentrations.

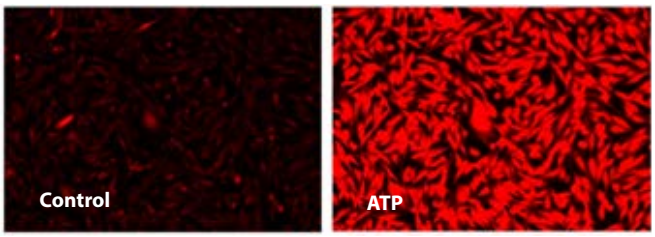

Figure 2.15 Response of endogenous P2Y receptor to ATP in CHO-K cells. CHO-K cells were seeded overnight at 40,000 cells/100 µL/well in a 96-well black wall/clear bottom Costar plate. 100 µL of Calbryte™ 590 AM (Cat# 20701) in HHBS with 2 mM probenecid was added into the wells, and the cells were incubated at 37 °C for one hour. The dye loading solution was replaced with 200 µL HHBS, treated with 50 µL of 50 µM ATP, and imaged with a fluorescence microscope (Keyence) using TRITC channel.

Table 2.9 Calbryte™ 590 Calcium Indicators

Cat #	Product Name	Size	Ex (nm)	Em (nm)	K_d (µM)
20701	Calbryte™ 590, AM	10x50 µg	573	588	1.4
20706	Calbryte™ 590, potassium salt	5x50 µg	573	588	1.4

Cal-630™ Calcium Indicators

X-Rhod-1 is commonly used as a red fluorescent calcium indicator. However, X-Rhod-1 is only moderately fluorescent in live cells upon esterase hydrolysis, and has very small cellular calcium responses. In addition, X-Rhod-1 is mostly localized in mitochondria, thus giving low signal/background ratio. Cal-630™ has been developed to improve X-Rhod-1 cell loading and calcium response while maintaining the similar spectral wavelengths of X-Rhod-1, making it compatible with Texas Red® filter set. In CHO and HEK cells, the cellular calcium response of Cal-630™ is much more sensitive than that of X-Rhod-1. The maximum emission wavelength of Cal-630™ is well separated from those of FITC, Alexa Fluor® 488 and GFP, making it an ideal calcium probe for multiplexing intracellular assays with GFP cell lines or FITC/Alexa Fluor® 488 labeled antibodies.

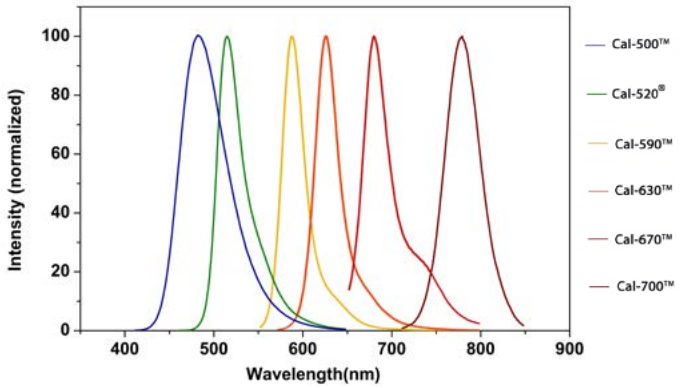

Figure 2.16 Normalized emission spectra of Cal-500™, Cal-520®, Cal-590™, Cal-630™, Cal-670™ and Cal-700™.

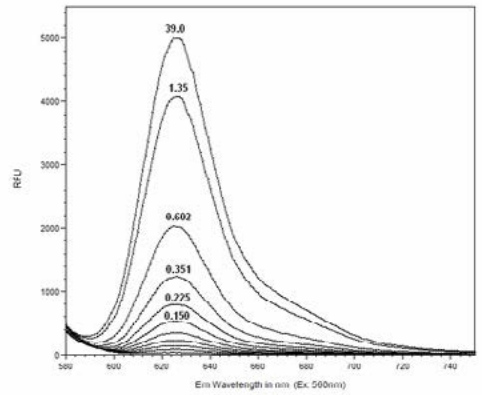

Figure 2.17 Fluorescence emission spectra of Cal-630™ in solutions containing 0 to 39 µM free Ca^{2+}.

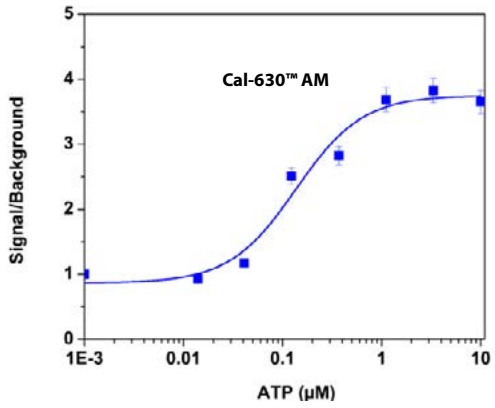

Figure 2.18 ATP-stimulated calcium response of endogenous P2Y receptor in CHO-K1 cells measured with Cal-630™ AM (Cat# 20530). CHO-K1 cells were seeded overnight at the cell density of 50,000 cells per 100 µL per well in a 96-well black wall/clear bottom plate. 100 µL of 10 µg/mL Cal-630™ AM with 2.0 mM probenecid was added into the cells, and the cells were incubated at 37 ºC for 2 hours. ATP (50 µL/well) was added by FlexStation® (Molecular Devices) to achieve the final indicated concentrations.

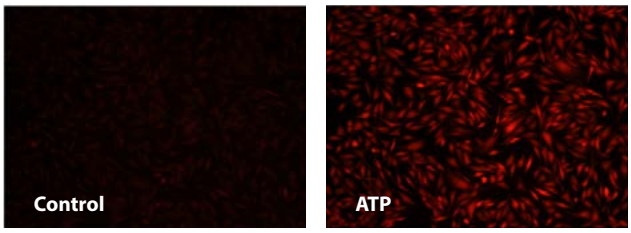

Figure 2.19 Responses of endogenous P2Y receptor to ATP in CHO-K1 cells. CHO-K1 cells were seeded overnight at 40,000 cells per 100 µL per well in a 96-well black wall/clear bottom plate. 100 µL of 4 µM Cal-630™ AM (Cat# 20530) in HHBS with 1 mM probenecid were added into the wells, and the cells were incubated at 37 °C for 2 hours. 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 .

Table 2.10 Cal-630™ Calcium Indicators

Cat #	Product Name	Size	Ex (nm)	Em (nm)	K_d (nM)
20531	Cal-630™, AM	10x50 µg	608	626	792
20538	Cal-630™, potassium salt	5x50 µg	608	626	792
20535	Cal-630™, sodium salt	5x50 µg	608	626	792
20545	Cal-630™-Dextran Conjugate *MW 3,000*	1 mg	608	626	N/D
20546	Cal-630™-Dextran Conjugate *MW 10,000*	1 mg	608	626	N/D

Calbryte™ 630 Calcium Indicators

Calbryte™ 630 is our upgrade for red & deep-red fluorescent indicators such as X-Rhod-1. This dye has an excitation maximum at 608 nm, which aligns well with the 594 nm laser line. This dye has an emission maximum at 624 nm and is compatible with common Texas® Red filter sets. Because of its distance from the green region of the spectrum, Calbryte™ 630 is well suited for multiplex with a green fluorescent label such as iFluor™ 488, Alexa Fluor® 488 or GFP. Moreover, Calbryte™ 630's long emission wavelength makes it well suited for study of deep tissue. This is because longer wavelength dyes have an easier time penetrating through many cell layers, whereas short-wavelength dyes cannot.

Key Features of Calbryte™ 630 AM

- A red-shifted calcium indicator compatible with GFP
- A superior replacement for X-Rhod-1 and Cal-630™
- Significantly enhanced intracellular retention
- Well suited for multiplex with a green fluorescent label such as iFluor™ 488, Alexa Fluor® 488 or GFP

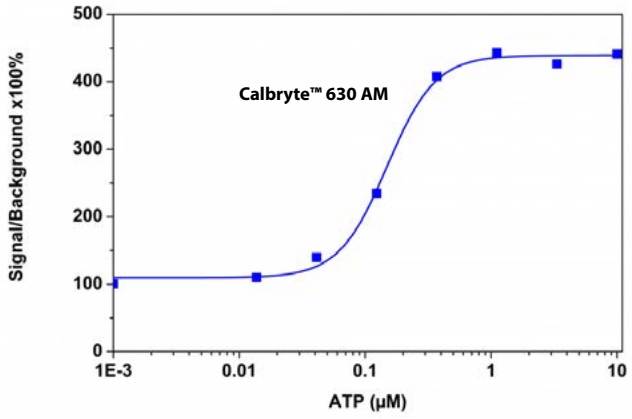

Figure 2.20 ATP dose response was measured in CHO-K1 cells with Calbryte™630 AM (Cat# 20721). CHO-K1 cells were seeded overnight at 50,000 cells/100 µL/well in a 96-well black wall/clear bottom Costar plate. 100 µL of 10 µg/mL Calbryte™630 AM in HH Buffer with probenecid was added and incubated for 60 minutes at 37°C. Dye loading solution was then removed and replaced with 200 µL HH Buffer/well. ATP (50 µL/well) was added by FlexStation® 3 to achieve the final indicated concentrations.

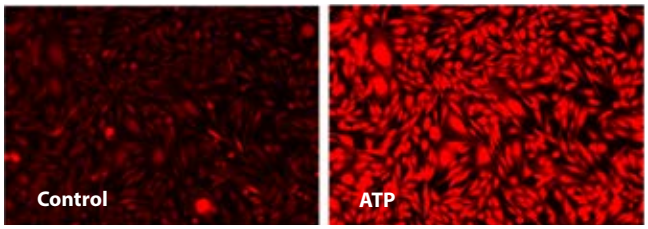

Figure 2.21 Response of endogenous P2Y receptor to ATP in CHO-K cells. CHO-K cells were seeded overnight at 40,000 cells/100 µL/well in a 96-well black wall/clear bottom costar plate. 100 µL of Calbryte™ 630 AM (Cat# 20721) in HHBS with 2 mM probenecid were added into the wells, and the cells were incubated at 37 °C for one hour. The dye loading solution was replaced with 200 µL HHBS, treated with 50 µL of 50 µM ATP, and imaged with a fluorescence microscope (Keyence) using Texas Red® Channel.

Table 2.11 Calbryte™ 630 Calcium Indicators

Cat #	Product Name	Size	Ex (nm)	Em (nm)	K_d (µM)
20721	Calbryte™ 630, AM	10x50 µg	608	626	1.2
20727	Calbryte™ 630, potassium salt	5x50 µg	608	626	1.2

Rhod-4™ Calcium Indicators

Rhod-2 is the most commonly used red fluorescent calcium indicators. However, Rhod-2 AM (Cat# 21064) is only moderately fluorescent in live cells upon esterase hydrolysis, and has very small cellular calcium responses. Moreover, Rhod-2 is concentrated inside mitochondria and is not homogenously localized inside cells upon loading. Rhod-4™ has been developed to improve the cell loading and calcium response while maintaining the spectral wavelength of Rhod-2. In CHO and HEK cells, the cellular calcium response of Rhod-4™ AM (Cat# 21112) is 10 times more sensitive than that of Rhod-2 AM. Our in-house research indicated that Rhod-4™ AM can detect calcium transients in stem cell cardiomyocytes that was not detected with Rhod-2 AM under the same conditions. The higher sensitivity of Rhod-4™ AM might be due to its higher cell loading efficiency than that of Rhod-2 AM.

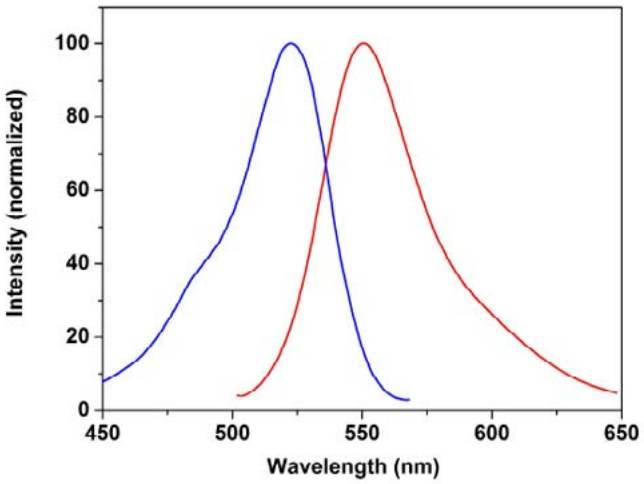

Figure 2.22 The excitation and emission spectra of Rhod-4™ in PBS buffer (pH 7.2) in the presence of 5 mM calcium chloride.

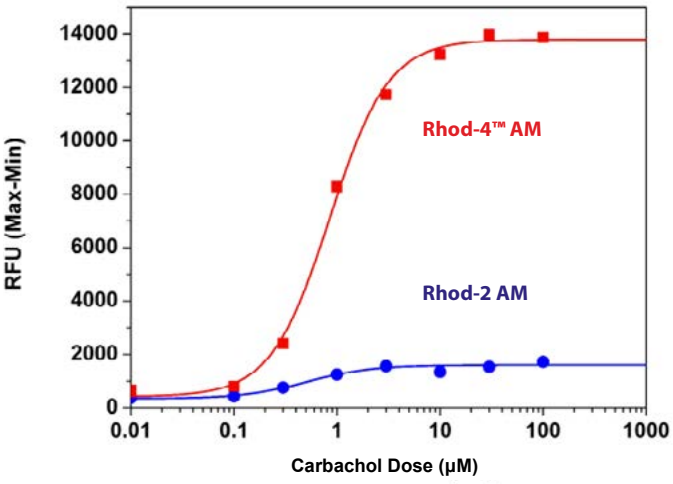

Figure 2.23 Carbachol dose responses were measured in HEK-293 cells with Rhod-4™ AM (red curve, Cat# 21120) and Rhod-2 AM (blue curve, Cat# 21064). HEK-293 cells were seeded overnight at 40,000 cells/100 µL/well in a Costar 96-well black wall/clear bottom 96-well plate. The growth medium was removed, and the cells were incubated with 100 µL Rhod-4™ AM dye loading solution, or 100 µL Rhod-2 AM dye loading solution (5 µM) for 1 hour at room temperature. Carbachol (25 µL/well) was added by NOVOstar (BMG Labtech) to achieve the final indicated concentrations. The EC_{50} of carbachol with Rhod-4™ AM was about 0.8 µM.

Table 2.12 Rhod-4™ and Related Calcium Indicators

Cat #	Product Name	Size	Ex (nm)	Em (nm)	K_d
21064	Rhod-2, AM *UltraPure grade"	20 x 50 µg	549	578	570 nM
21067	Rhod-2, tripotassium salt	1 mg	549	578	570 nM
21068	Rhod-2, trisodium salt	1 mg	549	578	570 nM
21112	Rhod-4™, AM	10 x 50 µg	524	551	451 nM
21129	Rhod-4™, potassium salt	5 x 50 µg	524	551	451 nM
21128	Rhod-4™, sodium salt	5 x 50 µg	524	551	451 nM
21070	Rhod-5N, AM	1 mg	551	577	0.3 mM
21072	Rhod-5N, tripotassium salt	1 mg	551	577	0.3 mM
21078	Rhod-FF, AM	10 x 50 µg	549	578	19 µM
21076	Rhod-FF, tripotassium salt	10 x 50 µg	549	578	19 µM

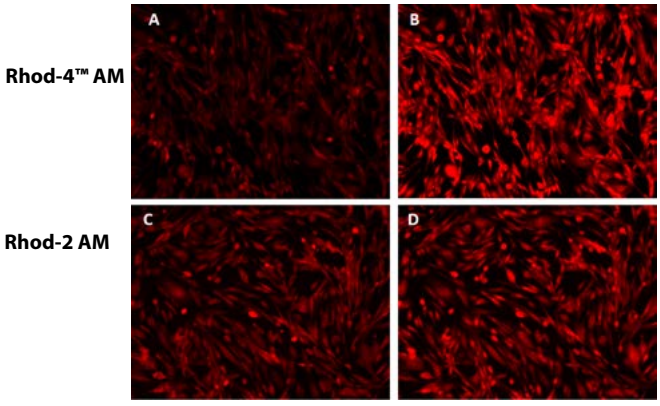

Figure 2.24 ATP-stimulated calcium responses of endogenous P2Y receptors were measured in CHO-K1 cells with Rhod-4™ AM (Cat# 21120) and Rhod-2 AM (Cat# 21064). CHO-K1 cells were seeded overnight at 50,000 cells/100 µL/well in a Costar 96-well black wall/clear bottom plate. The growth medium was removed, and the cells were incubated with 100 µL of dye loading solution using Rhod-4™ AM (4 µM, A and B) or Rhod-2 AM (4 µM, C and D) for 1 hour in a 37 °C, 5% CO_2 incubator. The cells were washed twice with 200 µL HHBS, and imaged before (A and C) and after (B and D) ATP treatment with a fluorescence microscope (Olympus IX71) using TRITC channel.

Cal-670™ Calcium Indicators

Cal-670™ is a far-red fluorescent calcium indicator with excitation at 650 nm and emission at 675 nm. It can be conveniently detected using Cy5® detection setup. Upon binding to calcium, Cal-670™ enhances its fluorescence by 125 folds.

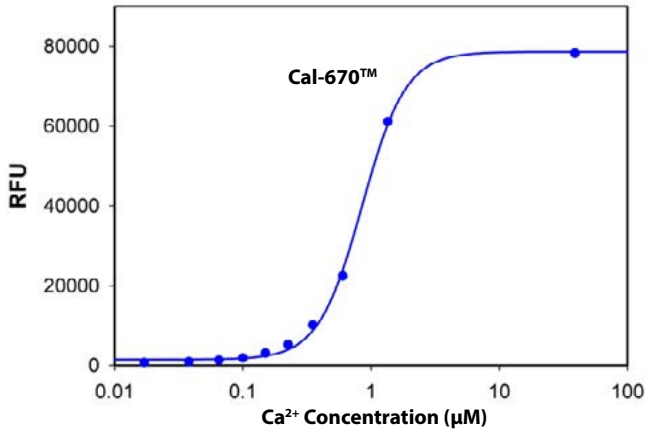

Figure 2.25 Cal-670™ was incubated with buffer that contains different concentration of free Ca^{2+}. The fluorescence was monitored using a fluorometer (Gemini XS, Molecular Devices). The K_d of Cal-670™ is 853 nM.

NIR Fluorescent Calcium Indicators

Far-red to near-infrared (NIR) fluorescent calcium indicators show greater tissue penetration in *in vivo* and *ex vivo* studies, have less overlap with the spectrum of background autofluorescence, and exhibit less phototoxicity. Furthermore, far-red to NIR fluorescent calcium indictors are likely to be separated from other fluorescence indicators and markers, including genetically expressed fluorescent proteins, and thus has potential for multicolor imaging.

Cal-770™ Calcium Indicators

Cal-770™ is a NIR fluorescent calcium indicator with excitation at 750 nm and emission at 775 nm. It is the only fluorescent calcium indicator with excitation and emission longer than 700 nm with a moderate calcium affinity of K_d ~850 nM. Cal-770™ is one of the very few calcium indicators that can be potentially used for *in vivo* imaging since it has NIR fluorescence.

Table 2.13 NIR Fluorescent Calcium Indicators

Cat #	Product Name	Size	Ex (nm)	Em (nm)	K_d (nM)
20455	Cal-670™, potassium salt	10x50 µg	650	675	853
20456	Cal-670™-Dextran Conjugate *MW 3,000*	1 mg	650	675	ND*
20457	Cal-670™-Dextran Conjugate *MW 10,000*	1 mg	650	675	ND*
20460	Cal-770™, potassium salt	10x50 µg	750	775	850
20461	Cal-770™-Dextran Conjugate *MW 3,000*	1 mg	750	775	ND*
20462	Cal-770™-Dextran Conjugate *MW 10,000*	1 mg	750	775	ND*

*The K_d value was not determined.