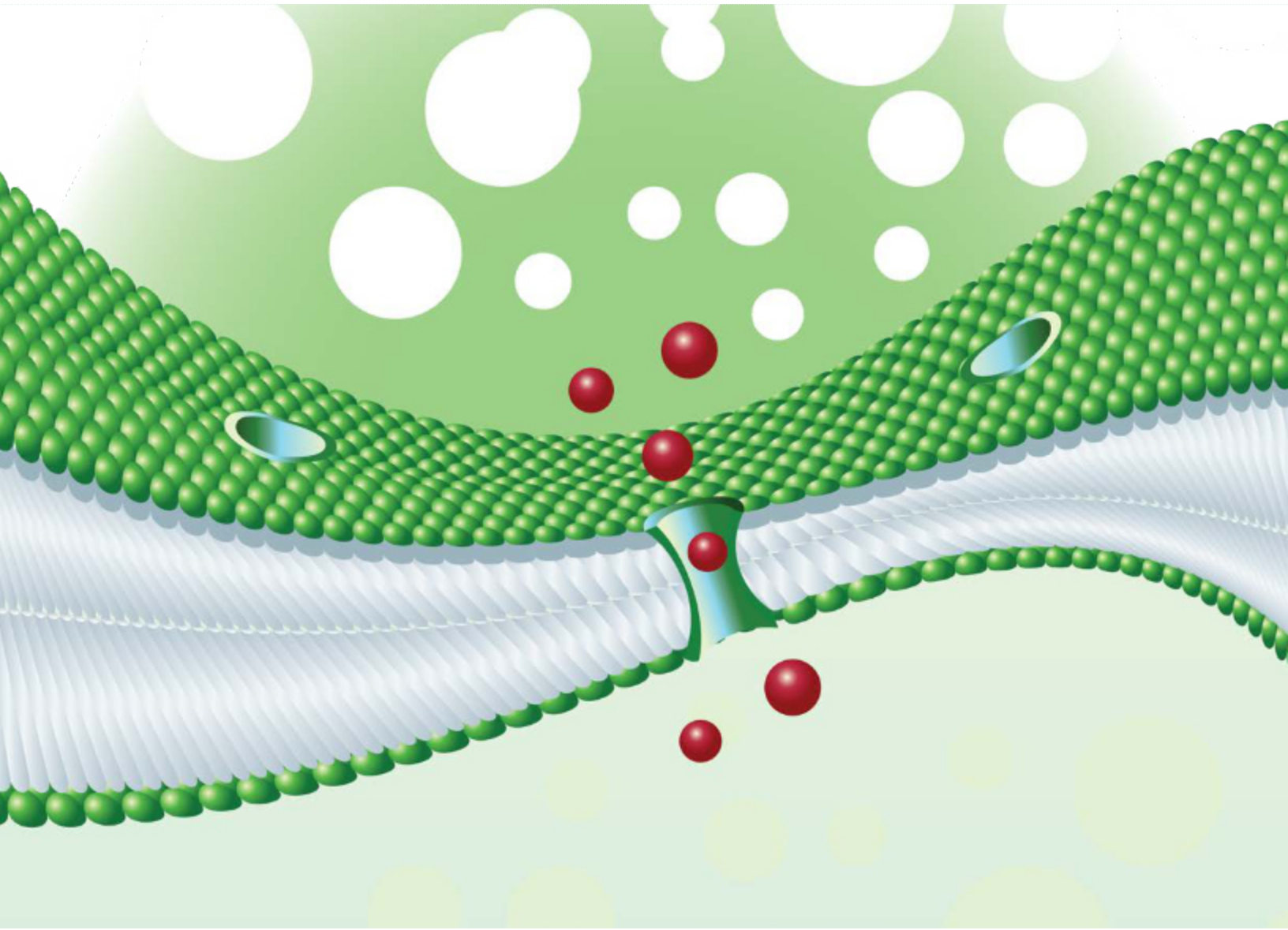
Calcium Detection Probes & AssayKits



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

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AAT Bioquest®

Our Mission

AAT Bioquest® is committed to constantly meet or exceed its customer's requirements by providing consistently high quality products and services, and by encouraging continuous improvements in its long-term and daily operations. Our core value is Innovation and Customer Satisfaction.

Our Story

AAT Bioquest®, Inc. (formerly ABD Bioquest, Inc.) develops, manufactures and markets bioanalytical research reagents and kits to life sciences research, diagnostic R&D and drug discovery. We specialize in photometric detections including absorption (color), fluorescence and luminescence technologies. The Company's superior products enable life science researchers to better understand biochemistry, immunology, cell biology and molecular biology. AAT Bioquest offers a rapidly expanding list of enabling products. Besides the standard catalog products, we also offer custom services to meet the distinct needs of each customer. Our current services include custom synthesis of biological detection probes, custom development of biochemical, cell-based and diagnostic assays and custom high throughput screening of drug discovery targets.

It is my greatest pleasure to welcome you to AAT Bioquest. We greatly appreciate the constant support of our valuable customers. While we continue to rapidly expand, our core value remains the same: Innovation and Customer Satisfaction. We are committed to being the leading provider of novel biological detection solutions. We promise to extend these values to you during the course of our service and to continue to support you with our new products and services. It is our greatest honor to receive valuable feedbacks and suggestions from you so that we can better serve your projects.

Very truly yours,

Zhenjun Diwu, Ph.D.
President

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Trademarks of AAT Bioquest

AAT Bioquest®
Amplite™
Cal-500™
Cal-520®
Cal-520FF™
Cal-590™
Cal-630™
Cal-670™
Cal-770™
Cal Green™
Cal Red™
Calbryte™
Calcium Blue™
Cell Meter™
Fluo-8®
Fluo-8E™
Fluo-8FF™
Fluo-8H™
Fluo-8L™
Fura-8™
Fura-8FF™
iFluor™
Phenol Red Plus™
Quest Fluor™
ReadiUse™
Rhod-4™
Rhod Red™
Screen Quest™
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Trademarks of Other Companies

Alexa Fluor®(Thermo Fisher)
Calcium Green™(Thermo Fisher)
Calcium Orange™(Thermo Fisher)
CLARIOstar®(BMG Labtech)
Cy3®(GE Healthcare)
FDSS®(Hamamatsu)
FlexStation®(Molecular Devices)
FLIPR®(Molecular Devices)
Fluo-4 Direct™(Thermo Fisher)
NovoCyte®(ACEA Biosciences)
Pluronic®(BASF Corporation)
SpectraMax®(Molecular Devices)
Texas Red®(Thermo Fisher)

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CUSTOMER SERVICE & ORDERING INFORMATION

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sales@aatbio.com (quote request)
support@aatbio.com (technical support)

International Distributors:
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TERMS AND CONDITIONS OF SALE

1. Prices, Orders and Changes: Prices shown are in US currency. Please call us for current prices if you require this information prior to placing your order. We guarantee our written quotations for 60 days. You may not cancel purchase orders unless such cancellation is expressly agreed by us. In such event, you will be advised of the total charge for such cancellation. You agree to pay such charges, including, but not limited to, storage and shipment costs, costs of producing non-standard materials, costs of purchasing non-returnable materials, cancellation costs imposed on us by our suppliers, and any other cost resulting from cancellation of this order.

2. Delivery: In most cases, we use standard overnight or two-day Federal Express delivery (or equivalent). All shipping charges billed are the responsibility of the customer and are normally prepaid by AAT Bioquest, Inc. and added to the invoice. We reserve the right to make delivery in installments, all such installments to be separately invoiced and paid for when due per invoice, without regard to subsequent deliveries. Partial shipments of available items are made when another item is backordered. Please inspect your packages upon receipt. If the goods have been damaged in transit, we can assist you in filing a claim with the carrier. You shall notify us in writing of any claims for shortages, defects or damages and shall hold the goods for our written instructions concerning disposition. Any claims for such errors must be made within 10 business days. If it is our error, we will do whatever is necessary to ship the correct products as soon as possible. If you shall fail to notify us any defects within 10 days after the goods have been received, such goods shall conclusively be deemed to conform to the terms and conditions and to have been irrevocably accepted by the buyer.

3. Payment: Terms of sale are net 30 days of date of invoice that is sent to you within 24 hours of shipping the order. The amount received must be sufficient to cover both the invoiced amount and any bank charges that may be incurred. Late charges may be added to invoices not paid within the 30-day time period. Late charges must be paid before subsequent orders can be shipped.

4. Warranties: The products shipped by AAT Bioquest are warranted to conform to the chemical or biological descriptions provided in our publications. This warranty is exclusive, and we make no other warranty, expressor implied, including any implied warranty of merchantability or fitness for any particular purpose. Our sole and exclusive liability and your exclusive remedy with respect to products proved to our satisfaction to be defective or nonconforming shall be replacement of such products without charge or refund of the purchase price, in our sole discretion, upon the return of such products in accordance with our instructions. We will not be liable for any incidental, consequential or contingent damages involving their use.

5. Returns: We must authorize any returns. We will not accept return shipments unless we have given prior written permission and shipping instructions. Goods may not be returned for credit except with our permission, and then only in strict compliance with our return shipment instructions. Any returned items may be subject to a 20% restocking fee. In many cases, items ordered in error cannot be returned because of the sensitive nature of many of our products and the difficulty and expense of requalifying returned items. If items are accepted for return, they must be in new, unopened, unused and undamaged condition, and you will be charged a per-unit 20% restocking charge.

6. Use of Our Products: Our products are used ONLY for laboratory research and development purposes. We realize that, since our products are, unless otherwise stated, intended primarily for research purposes, they may not be on the Toxic Substances Control Act (TSCA) inventory. You assume responsibility to assure that the products purchased from us are approved for use under TSCA, if applicable. You have the responsibility to verify the hazards and to conduct any further research necessary to learn the hazards involved in using products purchased from us. You also have the duty to warn your customers and any auxiliary personnel (such as freight handlers, etc.) of any risks involved in using or handling the products.

7. Patent Disclaimer: We do not warrant that the use or sale of our products will not infringe the claims of any United States or other patents covering the product itself or the use thereof in combination with other products or in the operation of any process.

8. Miscellaneous: We reserve the right to discontinue our products or change specifications or prices of our products and to correct any errors or omissions at any time without incurring obligations.

Custom Products and Services

Our Technologies

Amplite™enzyme-baseddetection platform is optimized for measuring horseradish peroxidase (HRP),alkaline phosphates, luciferase, beta-galactosidase, lactamase, oxidase, protein kinases,protein phosphatases, phosphodiesterases, proteases, cytochrome P450,histone deacetylase (HDAC)and cell signaling molecules such asNAD/NADH, NADP/NADPH,IP_3, cAMP and cGMPetc.

CellExplorer™celllabeling platform is a complete set of tools for tracking live cells. This platform is also widely used for sorting mixed populations of cells.

CellNavigator™cell staining platform is a complete set of tools for selective labeling subcellular structures of live, fixed and dead cells.

CellMeter™cellular functional assayplatform is a complete set of tools for functional analysisof cellular events and real time-monitoring of cell functions.

iFluor™superiorfluorescent labeling dyes are optimized for labeling proteins and nucleic acids. This group of dyes span from UVto infrared wavelength with good photostability and brightness.

mFluor™superiorfluorescent labeling dyes are optimized for flow cytometry applications.

PhosphoWorks™detectionplatform is a set of tools for detection of ATP,ADP,AMP,phosphate, pyrophosphate, phosphoproteins and phosphopeptides.

QuestView™colorimetric protease platform is a sensitive and robust tool for rapid detection of protease and glycosidase biomarkers. This technology platform has been licensed by a few diagnostic companies for developing rapid diagnostic tests.

RatioWorks™superior cellular dyes are a sensitive and robust tool set for ratio imaging and real time monitoring of cellular functions (such aspH and ions) in live cells.

ScreenQuest™assaykits are a set of HTS-readytools for high throughput screening of biochemical and cellular targets such as protein kinases, proteases, HDAC,cell apoptosis and cytotoxicity, GPCR,ionchannels, ADMEand transporters.

Tide Fluor™***and Tide Quencher***™superior labeling dyes are specially optimized for labeling nucleotides and peptides. This platform offers the best value in the industry. It is second to none in terms of performance and cost. This technology platform has been licensed by a few diagnostic companies for developing IVDdiagnostic tests.

trFluor™superiorfluorescent labeling dyes are optimized for developing time-resolved fluorescence-based assays. It has been used for developing HTSassaytechnologies for many drug discovery targets.

Our Services

Besidesthe catalog products we also offer custom servicesto meet the distinct needsof each customer. Our current services include custom synthesisof biological detection probes, custom development of biochemical, cell-based and diagnostic assays,custombioconjugation and custom high throughput screening of drug discovery targets.

Custom AssayDesign and Development

At AAT Bioquest we not only make probes and assaykits, but also use them extensively ourselves. Scientists at AAT Bioquest are experts on assaydesign and have developed a wide variety of tests that range from biochemical detection to cellular functions. Our assayoptions include:

- Enzyme activities
- Binding assays
- Cell-based assays
- Microplate assays
- Flow cytometric analysis
- Fluorescence imaging

Custom Conjugation

AAT Bioquest offers the best and the most rapid bioconjugation servicein the industry.

- Biotinylation
- Fluorescence labeling (iFluor™, mFluor™,Alexa Fluor®, APC,RPE,PerCP,and other fluorescent dyes)
- Enzyme labeling (AP and HRP)
- Small molecule conjugation

Custom Screening

AAT Bioquest offers on-demand high-throughput screening and pharmacology profiling assayswith multiple methodologies. Functional assaysare designed, validated and customized to the needs of our pharmaceutical and biotechnology industry clients. Theseassaysareaimed at assessingandmonitoring the efficacy, tolerability and safety parametersof candidate compounds for treating and/or diagnosing cancer, infectious disease,autoimmunity and transplantation. Our screening options include:

- Full assaydevelopment for a target of your choice
- Optimization of your assayprotocol for HTS
- Multiple assayplatforms and detection methods
- Custom data analysis

Custom Synthesis of Fluorophores and Luminophores

AAT Bioquest is recognized by the top pharmaceutical companies and diagnostic companies asa key provider of novel fluorescent dyes and luminescent probes. Over the years we have developed and synthesized many enabling fluorescent and luminescent probes for running a variety of challenging biological detection tasks.

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Non-Fluorescent Calcium Signaling Molecules & Chelators

Calcium ion (Ca^{2+}) impacts nearly every aspect of cellular life, e.g., Ca^{2+} signaling, from changes in protein conformations driven by Ca^{2+} to the mechanisms that control Ca^{2+} levels in the cytoplasm and organelles, the highly localized nature of Ca^{2+}-mediated signal transduction and its specific roles in excitability, exocytosis, motility, apoptosis, and transcription etc. Intracellular calibration of Ca^{2+} indicators may be achieved either by manipulating Ca^{2+} levels inside cells using an ionophore or by releasing the indicator into the surrounding medium of known Ca^{2+} concentration via detergent lysis of the cells. Besides the fluorescent and luminescent calcium detection reagents, we also offer several non-fluorescent compounds for measuring and manipulating intracellular and extracellular Ca^{2+}.

NAADP

Nicotinic acid adenine dinucleotide phosphate (NAADP, Cat#20999) is a secondary messenger that plays a key role in calcium signaling pathways. NAADP is functionally distinct from cADPR and IP_3. Unlike the latter, NAADP does not mobilize calcium from ER. Rather, it mobilizes calcium from the recently discovered acidic calcium stores located throughout the cytoplasm. These acidic calcium stores include subcellular compartments such as endosomes, lysosomes, secretory granules and Golgi bodies. More specifically, recent research suggests that NAADP targets a family of membrane bound ion-channels, called two-pore channels (TPC), in order to stimulate calcium release.

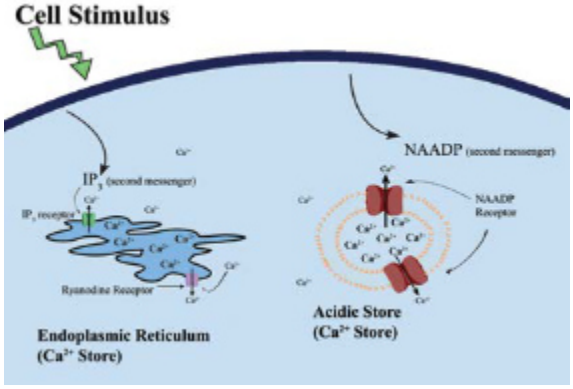

Figure 1.1 Inositol triphosphate (IP_3) and NAADP are second messenger molecules that transfer a chemical stimulus received by the cell. IP_3 binds to IP_3 ligand-gated Ca^{2+} channels causing an influx of Ca^{2+} into the cytosol from the endoplasmic reticulum. NAADP triggers an influx of Ca^{2+} from acidic vesicles into the cytosol.

While Lee and colleagues first discovered the presence of NAADP in 1987, it was not until 1995, almost a decade later, that its structure was determined. NAADP has become the focus of intense research in recent years. It has been proposed as a pharmacological target for a variety of diseases affecting the pancreas, heart and nervous system. Experiments with NAADP have shown it to be an extremely potent calcium mobilizer as well as a modulating agent for other cellular pathways, such as those involving inositol trisphosphate (IP_3).

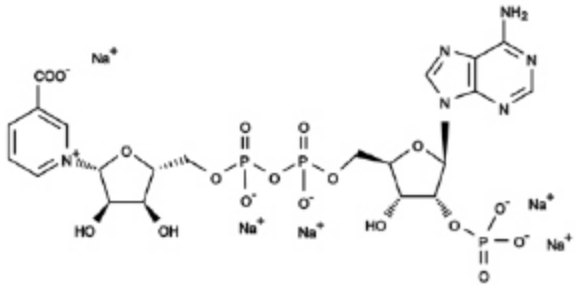

Figure 1.2 The chemical structure of NAADP (Cat# 20999).

NAADP-AM

As interests in NAADP are rapidly growing, scientists have begun to look for better tools for studying NAADP. In recent years, the research process has been significantly aided by the development of two separate compounds: NED-19 and NAADP-AM (Cat# 20997).

NED-19 is a NAADP antagonist that was first developed through virtual chemical screening of NAADP analogs. It acts specifically to block both NAADP-mediated Ca^{2+} response as well as NAADP binding.

The second important development in the study of NAADP is the synthesis of a cell permeable NAADP analog, NAADP-AM. Prior to its development, studies with NAADP had to utilize invasive cellular techniques such as microinjections or electroporation in order to load NAADP into cells. There are several well-documented problems with these methods. At the very least, normal cellular function is disrupted due to the disruption of the cell membrane. In the case of microinjections, the process is very time-intensive as it is limited to single cells. For electroporation, common problems include low loading efficiency and high rates of cell death.

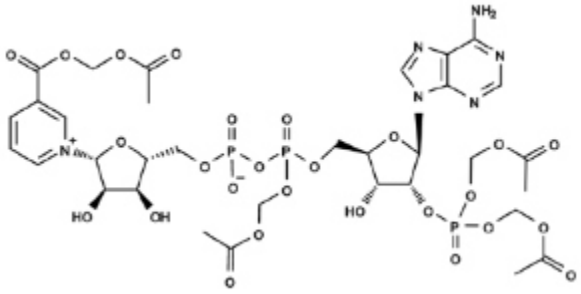

Figure 1.3 The chemical structure of NAADP-AM (Cat# 20997).

The usage of acetoxymethyl esters (AM esters) resolves many of the problems faced by prior loading techniques. This is particularly true in the case of NAADP because it is negatively charged. What this means is that while NAADP is well-retained in cells, it has an especially difficult time passing through cell membranes. By chemically adding AM esters to it, thus synthesizing NAADP-AM, NAADP not only loses its negative charge but also becomes hydrophobic. This change in chemical properties allows NAADP-AM to easily pass through the phospholipid membrane of cells. Once inside, the AM ester is cleaved by intracellular esterases, thus returning the compound to its original NAADP form. In this manner, through the use of AM esters, NAADP can be easily loaded into a population of cells without the need for invasive cellular techniques.

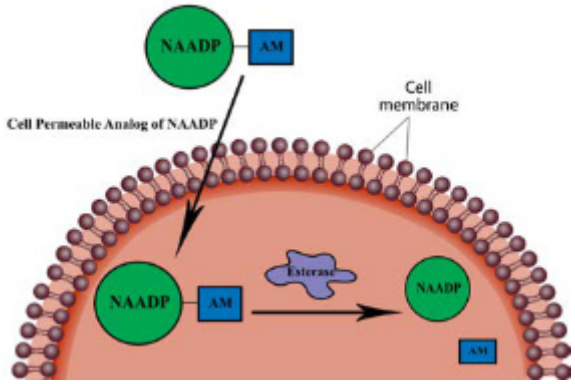

Figure 1.4 NAADP-AM (Cat# 20997) is a cell permeant analog of NAADP. NAADP-AM is taken into a cell's cytosol where it is hydrolyzed by esterase enzymes. The resulting influx of NAADP second messengers induces NAADP-mediated calcium signaling.

Fluorimetric cADP-Ribose Assay

cADP-ribose (cADPR) is a Ca^{2+} messenger derived from NAD^+. ADP-riboxyl cyclase (ADPRC) catalyzes the synthesis of cADPR from NAD^+, but the reaction can be reversed in the presence of high concentration of nicotinamide, producing NAD^+ from cADPR stoichiometrically. The resultant NAD^+ can be detected using our newly developed NAD sensor, Quest Fluor™ NAD reagent. The assay makes monitoring cADPR in tissues and cell cultures possible in the low nM range.

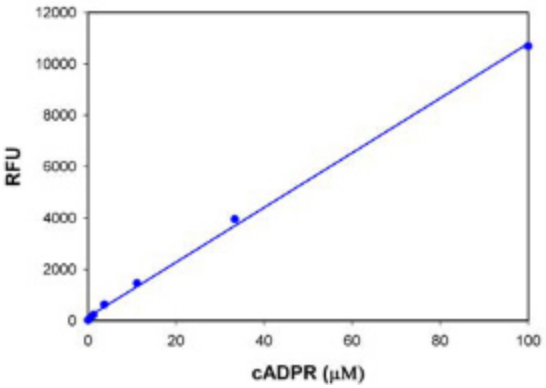

Figure 1.5 The concentration of cADPR was detected using Amplite™ Fluorimetric cADP-Ribose Assay Kit (Cat# 20305). Different concentrations of cADPR were incubated with ADPRC reaction mix for 1 hour at room temperature before NAD detection reagent was added. The lowest detected concentration of cADPR is 100 nM.

The NAD^+ detection using Quest Fluor™ NAD reagent is specific to NAD^+ and has no response to NADH. The fluorescence signal can be readily detected at Ex/Em = 420/480 nm. This assay can be performed in a convenient 96-well or 384-well microtiter plate format.

BAPTA AM

BAPTA is a calcium-specific aminopolycarboxylic acid. The presence of four carboxylic acid functional groups makes possible the binding of two calcium ions. The extensive flexibility of the carboxylate ligands is critical to the coordination of calcium and other metal ions.

BAPTA AM (Cat# 21002) is cell-permeable version of BAPTA (1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid), a cell-permeable calcium chelator. This BAPTA derivative is used for adjusting calcium concentrations in cells and tissues.

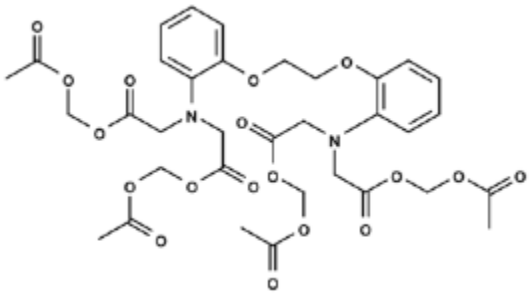

Figure 1.6 The chemical structure of BAPTAAM (Cat# 21002).

EGTA AM

EGTA is an aminopolycarboxylic acid, a chelating agent. Compared to EDTA, EGTA has a lower affinity for magnesium, making it more selective for calcium ions. It is useful in buffer solutions that resemble the environment in living cells where calcium ions are usually at least a thousand fold less concentrated than magnesium. The pK_a for binding of calcium ions by tetrabasic EGTA is 11.00, but the protonated forms do not significantly contribute to binding, so at pH 7, the apparent pK_a becomes 6.91.

EGTA AM (Cat# 21005) is the cell-permeable version of EGTA (ethylene glycol tetraacetic acid), a cell-permeable calcium chelator. This EGTA derivative is used for adjusting calcium concentrations in cells and tissues.

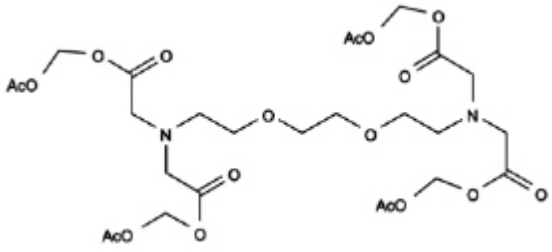

Figure 1.7 The chemical structure of EGTAAM (Cat# 21005).

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Pluronic®F-127

Pluronic®F-127 (Cat# 20050) is a nonionic surfactant that is 100% active and relatively non-toxic to cells at low concentrations, and frequently used with dye AM esters such as Indo-1 AM, Fura-2 AM, Calcein AM, Fluo-3 AM, Fluo-4 AM, Fluo-8®AM, Cal-520®,Calbryte™ 520 and Rhod-4™AM,etc.to improve their water solubility. Pluronic® F-127 may also be useful for dispersing other lipophilic probes. Appropriate controls should be performed to make certain that Pluronic®F-127isnot altering the membrane properties of the cells.

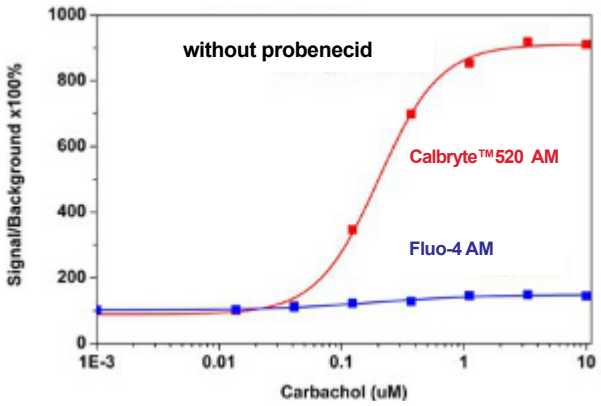

Figure 1.8 Carbachol-stimulated calcium response of exogenous M1 receptor in CHO-M1 cellsmeasured with Calbryte™520 AM (Cat#20651) or Fluo-4 AM (Cat#20551). CHO-M1 cellswere seeded overnight at 40,000cells/100 µL/well in a 96-well black wall/clear bottom costar plate. 100 µLof Fluo-4 AM or the Calbryte™520AM without probenecid was added into the cells, and the cells were incubated at 37 °C for 45min. Carbachol (50 µL/well) wasadded by FlexStation3 to achievethe final indicated concentrations.

Probenecid

Probenecid(Cat#20060)isan inhibitor of organic-anion transporters located in cell membranes. These transporters often extrude fluorescent indicators from cells, and therefore contribute to poor dye retention. This phenomenon usually causeshigh background in the assaysthat require the good retention of the dye indicators inside cells.Theuseof probenecid to inhibit the transporter activity, and thus to reduce leakage of the intracellular dye indicators is a common method for reducing fluorescencebackground of calcium assays.Thecommonly used free acid form of probenecid requires the useof 1 M NaOHto dissolveit due to its poor water solubility in neutral water.

AAT Bioquest offers the convenient ReadiUse™water-soluble and heat-stable probenecid in the format of powder, solution or tablet. They are convenient to use and are aseffective asthe free acid form at the sameconcentration.

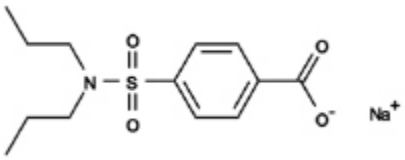

Figure 1.9 The chemical structure of Readiuse™Probenecid (Cat# 20062).

Table 1.1 Non-Fluorescent Calcium Detection Reagents and Assay Kit

Cat #	Product Name	Size
20305	Amplite™ Fluorimetric cADP-RiboseAssayKit	100 tests
21001	BAPTAAM	25 mg
21002	BAPTAAM *UltraPure grade*	25 mg
21003	BAPTA,tetrapotassium salt	100 mg
21004	BAPTA,tetrasodium salt	100 mg
21005	EGTAAM	10 mg
21006	EGTAAM *10 mM DMSOsolution*	1 mL
21008	EGTA,tetrasodium salt *10 mM aqueous solution*	10 mL
21007	EGTA,tetrasodium salt *UltraPure grade*	1 g
20999	NAADP[Nicotinic acid adenine dinucleotide phosphate sodium salt]	1 mg
20997	NAADP-AM	2x50 µg
20053	Pluronic®F-127*10% solution in water*	10 mL
20052	Pluronic®F-127 *20% solution in DMSO*	10 mL
20050	Pluronic®F-127*cell culture tested*	10 g
20060	Probenecid*cell culture tested*	10x72 mg
20062	ReadiUse™Probenecid*25 mM stabilized aqueous solution*	10x10 mL
20061	ReadiUse™Probenecid,sodium salt *water-soluble*	10x77 mg

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	OG488BAPTA-1, AM [equivalent to Oregon Green®488 BAPTA-1, AM]	500 µg	494	523	170 nM
20506	OG488BAPTA-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

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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.

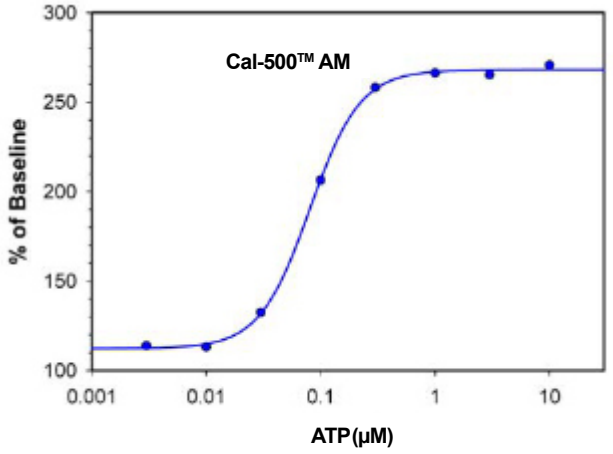

Figure 2.1 The ATP dose dependent intracellular calcium release was measured by Cal-500™ AM (Cat# 20410) in CHO-K1 cells. Cells were incubated with Cal-500™ AM dye for 60 minutes at 37 °C before different concentration of ATP was added into the cells. The response was measured over time on FlexStation®.

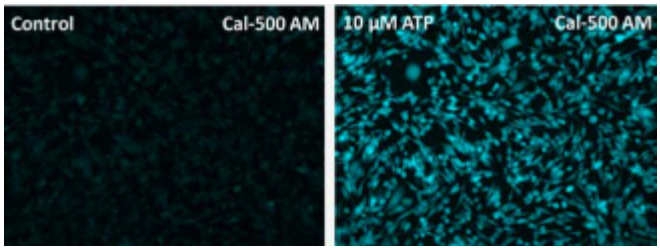

Figure 2.2 Response of endogenous P2Y receptor to ATP in CHO-K1 cells. CHO-K1 cells were seeded overnight at 40,000 cells per 100 µL per well in a 96-well black wall/clear bottom Costar plate. 100 µL of Cal-500™ AM in HHBS with probenecid were added into the wells, and the cells were incubated at 37 °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.

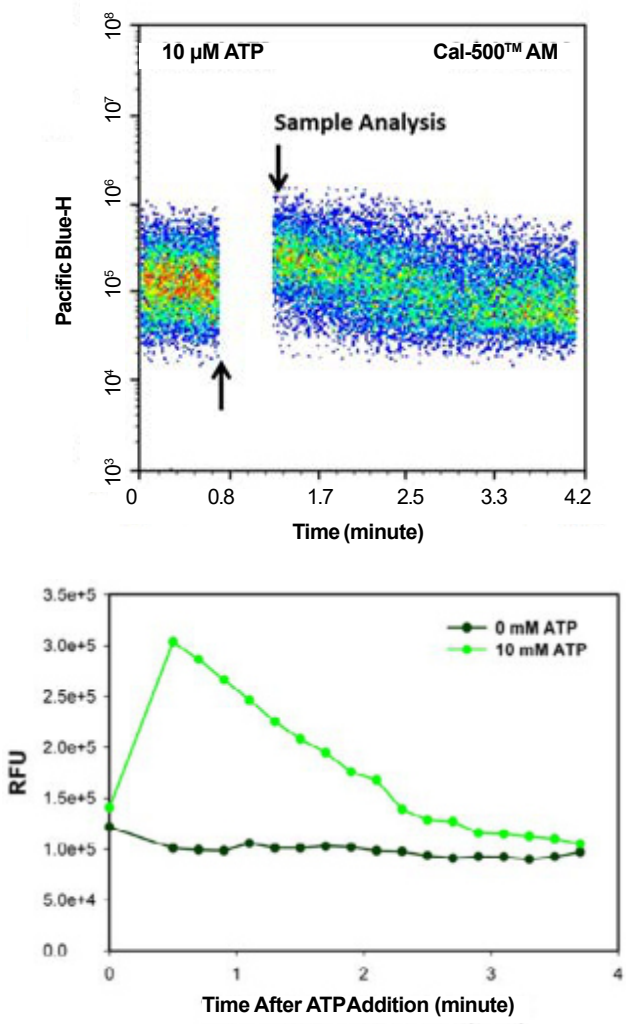

Figure 2.3 The ATP dependent intracellular calcium release was measured by Cal-500™ AM (Cat# 20410) in CHO-K1 cells. Cells were incubated with Cal-500™ AM dye for 60 minutes at 37 °C before 10 µM ATP was added into the cells. **Top:** The baseline was acquired and the rest of the cells were analyzed 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.

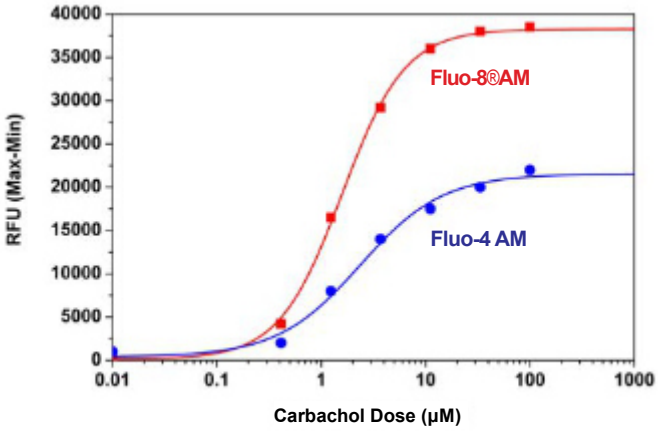

Figure 2.4 Carbachol dose responses were measured in HEK-293 cells with Fluo-8® AM (Cat# 21082) and Fluo-4 AM (Cat# 20551). HEK-293 cells were seeded overnight at 40,000 cells/100 µL/well in a 96-well black wall/clear bottom Costar plate. The growth medium was removed, and the cells were incubated with 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

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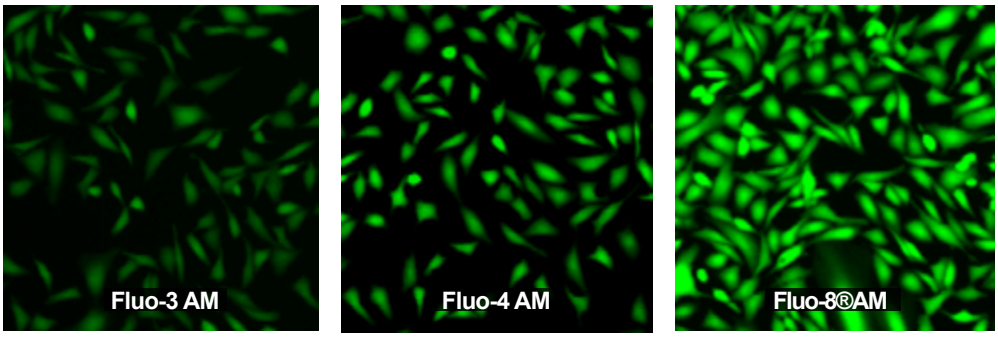

Figure 2.5 U2OScellswere seeded overnight at 40,000cellsper 100 µL per well in a Costarblack wall/clear bottom 96-well plate. The growth medium was removed, and the cells were incubated with 100 µL of 4 µM Fluo-3 AM, Fluo-4 AM and Fluo-8®AM in HHBSat 37 °Cfor 1 hour.The cells were washed twice with 200 µL HHBS,and imaged with Olympus IX71 using FITCchannel.

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 GPCRandcalcium 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®AMwhile 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 Comparisonof 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= FluorescenceQuantum Yield in the presence of 5 mM calcium citrate.

Key Features of Cal-520®AM

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

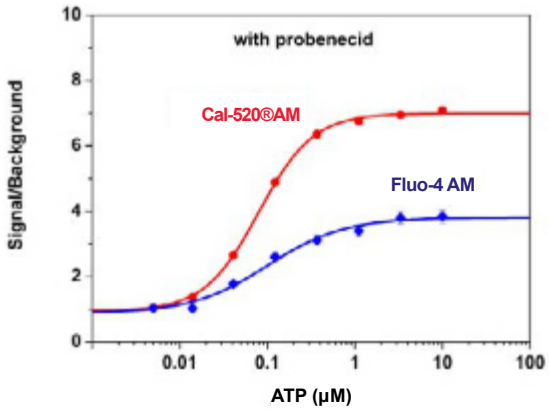

Figure 2.6 ATP-stimulated calcium responses of endogenous P2Yreceptor in CHO-K1 cells incubated with Cal-520®AM (red curve, Cat# 21131), or Fluo-4 AM (blue curve) respectively with probenecid under the same conditions. CHO-K1cells were seeded overnight at 50,000cells/100 µL/well in a Costar96-well black wall/clear bottom plate. 100 µL of 5 µM Fluo-4 AM or Cal-520®AMin HHBSwith 2.5 mm probenecid was added into the cells,and the cellswere incubated at 37 °Cfor 2 hours.

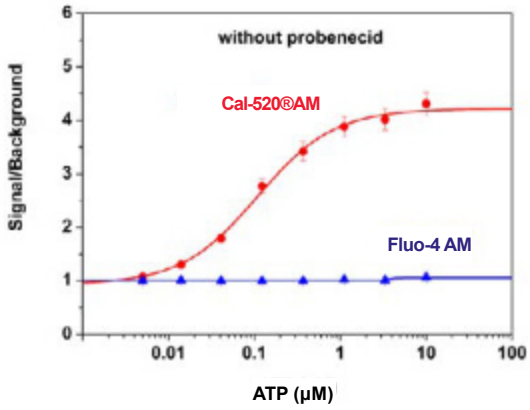

Figure 2.7 ATP-stimulated calcium responses of endogenous P2Yreceptors in CHO-K1 cellsincubated with Cal-520®AM(red curve, Cat#21131), or Fluo-4 AM(blue curve, Cat# 20551)respectively, without probenecid under the same conditions. CHO-K1cellswere seeded overnight at 50,000cells/100 µL/well in aCostar 96-well black wall/clear bottom plate. 100 µL of 5 µM Fluo-4 AM or Cal-520®AM in HHBSwas added into the cells, and the cellswere incubated at 37 °Cfor 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 *MW3,000*	1 mg	492	514	N/D
20601	Cal-520®-Dextran Conjugate *MW10,000*	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.)

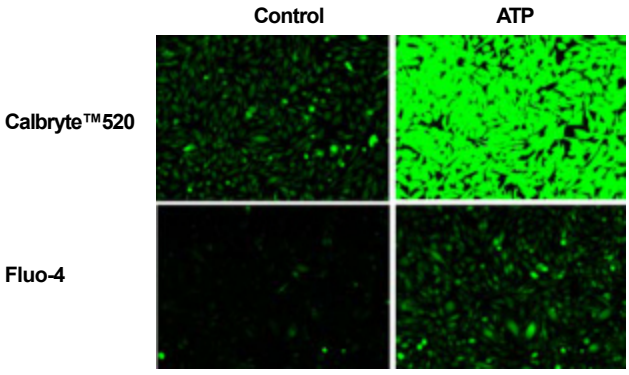

Figure 2.8 Response of endogenous P2Y receptor to ATP in CHO-K1 cells. CHO-K1 cells were seeded overnight at 40,000 cells/100 µL/well in a 96-well black wall/clear bottom Costar plate. 100 µL of Fluo-4 AM (Cat# 20551) or Calbryte™ 520 AM (Cat# 20651) in HHBS with probenecid were added into the wells, 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.

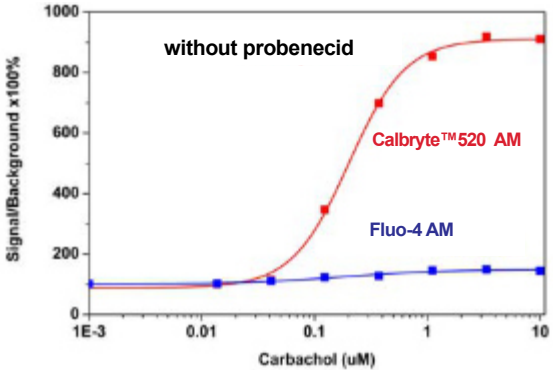

Figure 2.9 Carbachol-stimulated calcium response of exogenous M1 receptor in CHO-M1 cells measured with Calbryte™ 520 AM (Cat# 20651) or Fluo-4 AM (Cat# 20551). CHO-M1 cells were seeded overnight at 40,000 cells/100 µL/well in a 96-well black wall/clear bottom Costar plate. 100 µL of Fluo-4 AM or Calbryte™ 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.

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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/AlexaFluor®488 labeled antibodies.

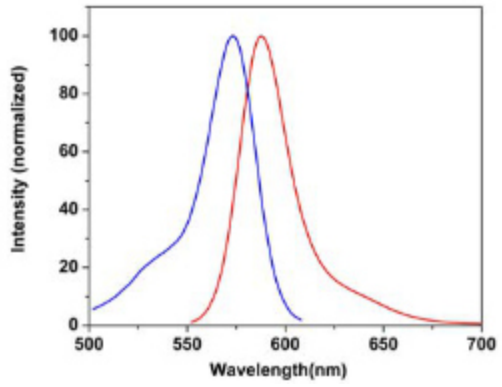

Figure 2.10 The excitation and emission spectra of Cal-590™ in the presence of calcium chloride (5 mM).

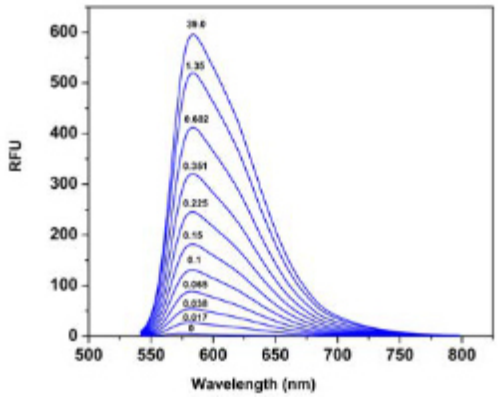

Figure 2.11 Fluorescence emission spectra of Cal-590™ in solutions containing 0 to 39 μM free Ca^{2+}.

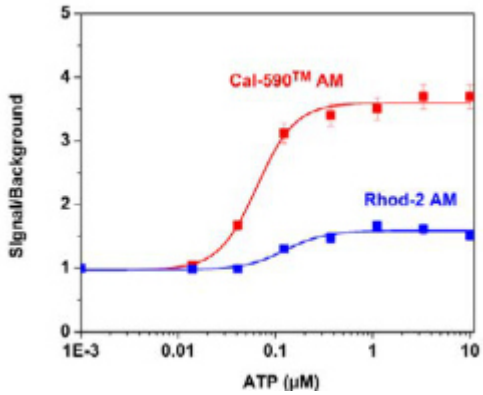

Figure 2.12 ATP-stimulated calcium response of endogenous P2Y receptor in CHO-K1 cells incubated with Cal-590™ AM (red, Cat# 20510) and Rhod-2, AM (blue, Cat# 21064) under the same conditions. CHO-K1 cells were seeded overnight at the cell density of 50,000 cells/100 μL/well in a 96-well black wall/clear 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.

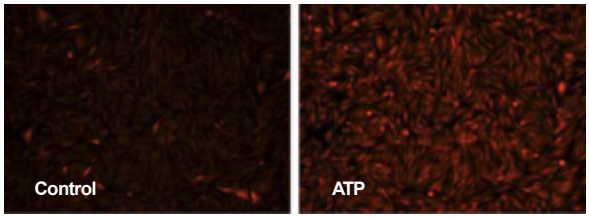

Figure 2.13 Responses of endogenous P2Y receptor to ATP in CHO-K1 cells. CHO-K1 cells were seeded overnight at 40,000 cells/100 μL/well in a Costar 96-well black wall/clear bottom plate. 100 μL of 4 μM Cal-590™ AM (Cat# 20510) in HHBS with 1 mM probenecid was added into the wells, and the cells were 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®filtersets.Calbryte™590 isapproximately ten timesmore sensitive for calcium than Rhod-2 under comparable conditions. Moreover, unlike Rhod-2 which primarily localizesin mitochondria, Calbryte™590retains 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 enhancedintracellular retention
- Homogeneous cytosolic location

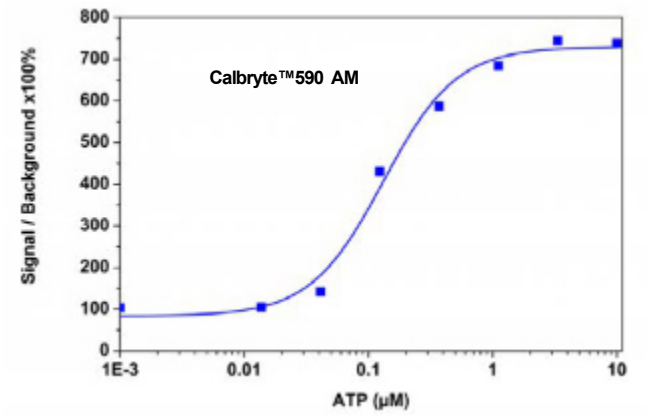

Figure 2.14 ATPdose response was measured in CHO-K1cells with Calbryte™590 AM (Cat# 20701). CHO-K1cells were seeded overnight at 50,000 cells/100 µL/well in a 96-well black wall/clear bottom Costarplate. 100 µLof 10 µg/mL Calbryte™590AM in HH Buffer with probenecid was added and incubated for 60 minutes at 37°C.Dye loading solution was removed and replaced with 200 µL HH Buffer/well. ATP(50 µL/well) was added by FlexStation®3to achieve the final indicated concentrations.

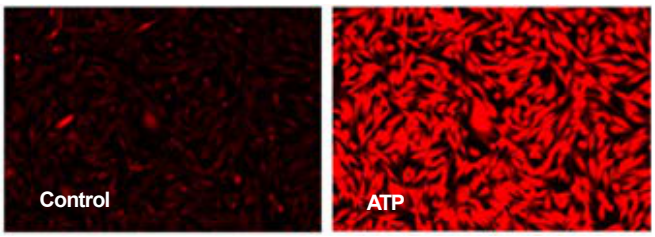

Figure 2.15 Responseof endogenous P2Yreceptor to ATPin CHO-Kcells. CHO-Kcells were seeded overnight at 40,000cells/100 µL/well in a 96-well black wall/clear bottom Costar plate. 100 µL of Calbryte™590 AM (Cat#20701) in HHBSwith 2 mM probenecid was added into the wells, and the cells were incubated at 37 °Cfor 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 TRITCchannel.

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-1is commonly used asa red fluorescent calcium indicator. However, X-Rhod-1is 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™hasbeen 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 HEKcells, 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 assayswith GFPcell lines or FITC/Alexa Fluor®488 labeled antibodies.

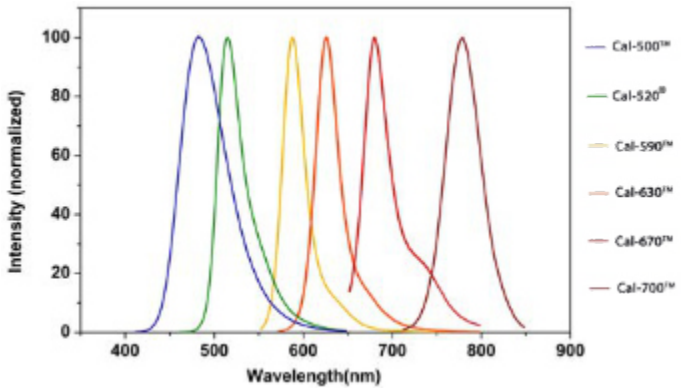

Figure 2.16 Normalized emission spectra of Cal-500™,Cal-520®, Cal-590™,Cal-630™, Cal-670™and Cal-700™.

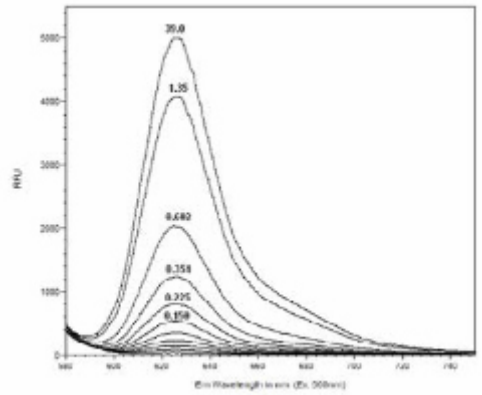

Figure 2.17 Fluorescence emission spectra of Cal-630™in solutions containing 0 to 39 µM free Ca^{2+}.

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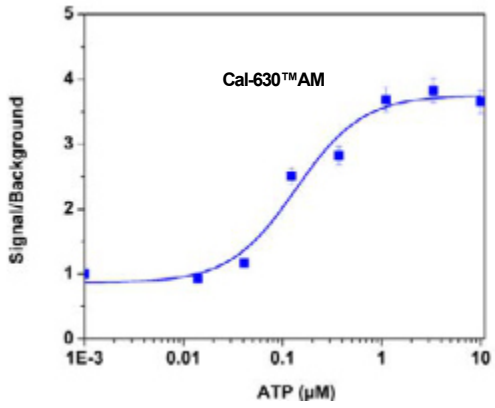

Figure 2.18 ATP-stimulated calcium response of endogenous P2Y receptor in CHO-K1 cells measured with Cal-630™AM (Cat# 20530). CHO-K1 cells were seeded overnight at the cell density of 50,000 cells per 100 µL per well in a 96-well black wall/clear bottom plate. 100 µL of 10 µg/mL Cal-630™AM with 2.0 mM 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.

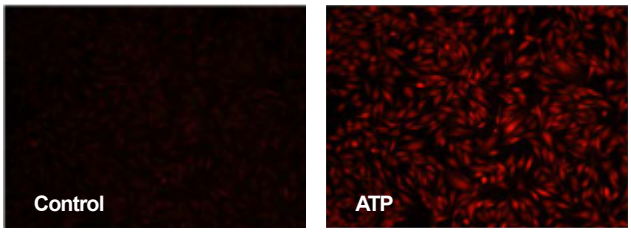

Figure 2.19 Responses of endogenous P2Y receptor to ATP in CHO-K1 cells. CHO-K1 cells were seeded overnight at 40,000 cells per 100 µL per well in a 96-well black wall/clear bottom plate. 100 µL of 4 µM Cal-630™AM (Cat# 20530) in HHBS with 1 mM probenecid were added into the wells, and the cells were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

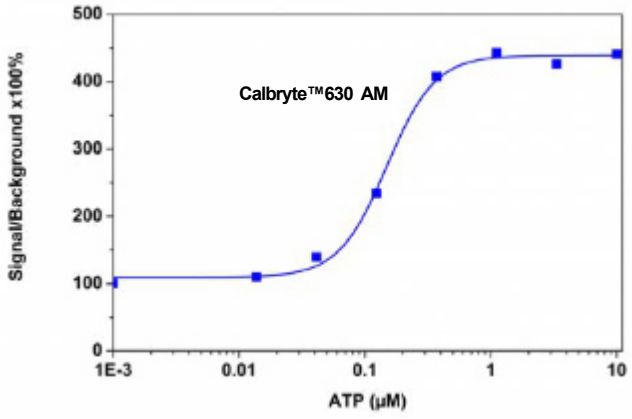

Figure 2.20 ATP dose response was measured in CHO-K1 cells with Calbryte™630 AM (Cat# 20721). CHO-K1 cells were seeded overnight at 50,000 cells/100 µL/well in a 96-well black wall/clear bottom Costar plate. 100 µL of 10 µg/mL Calbryte™630 AM in HH Buffer with probenecid was added and incubated for 60 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.

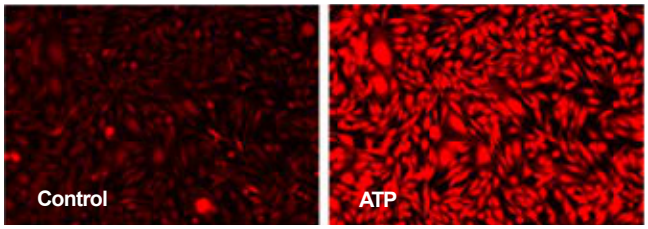

Figure 2.21 Response of endogenous P2Y receptor to ATP in CHO-K cells. CHO-K cells were seeded overnight at 40,000 cells/100 µL/well in a 96-well black wall/clear bottom costar plate. 100 µL of Calbryte™630 AM (Cat# 20721) in HHBS with 2 mM probenecid were added into the wells, and the cells were incubated 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.

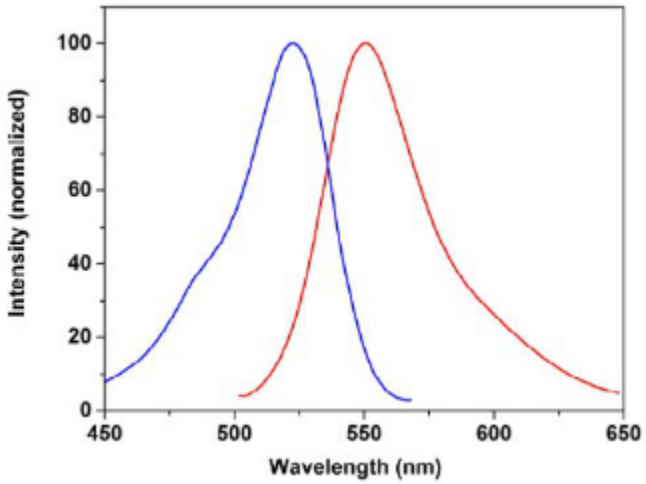

Figure 2.22 The excitation and emission spectra of Rhod-4™ in PBS buffer (pH 7.2) in the presence of 5 mM calcium chloride.

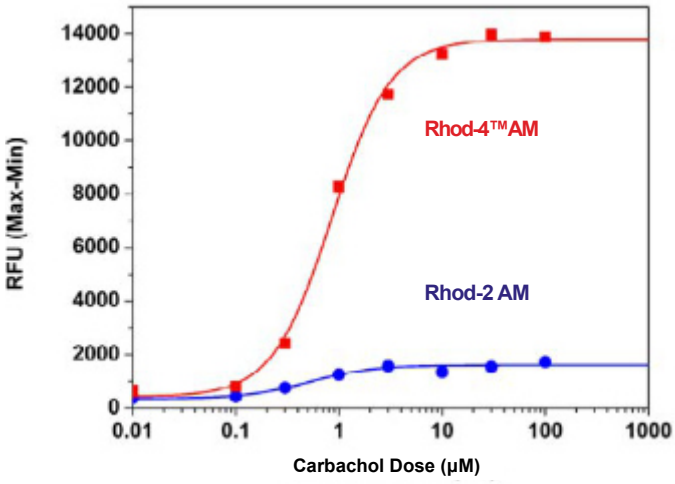

Figure 2.23 Carbachol dose responses were measured in HEK-293 cells with Rhod-4™ AM (red curve, Cat# 21120) and Rhod-2 AM (blue curve, Cat# 21064). HEK-293 cells were seeded overnight at 40,000 cells/100 µL/well in a Costar 96-well black wall/clear bottom 96-well plate. The growth medium was removed, 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

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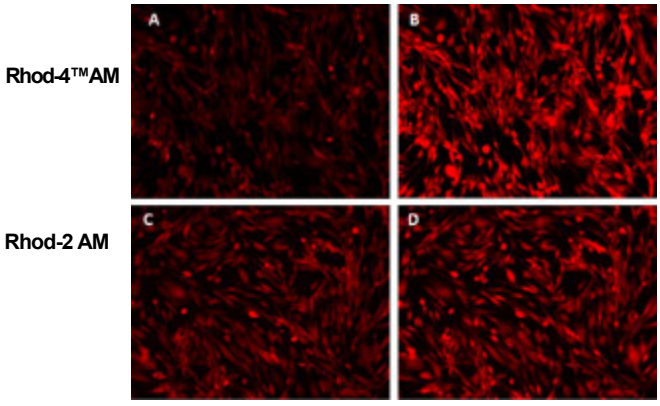

Figure 2.24 ATP-stimulated calcium responses of endogenous P2Y receptors were measured in CHO-K1cells with Rhod-4™AM(Cat# 21120) and Rhod-2 AM (Cat# 21064). CHO-K1cellswere seeded overnight at 50,000cells/100 µL/well in aCostar 96-well black wall/clear bottom plate. The growth medium was removed, and 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, Cand D) for 1 hour in a 37 °C,5% CO_2incubator.The cellswere washed twice with 200 µL HHBS,andimaged before (A and C)and after (Band D) ATPtreatment with a fluorescence microscope (Olympus IX71) using TRITCchannel.

Cal-670™Calcium Indicators

Cal-670™isafar-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.

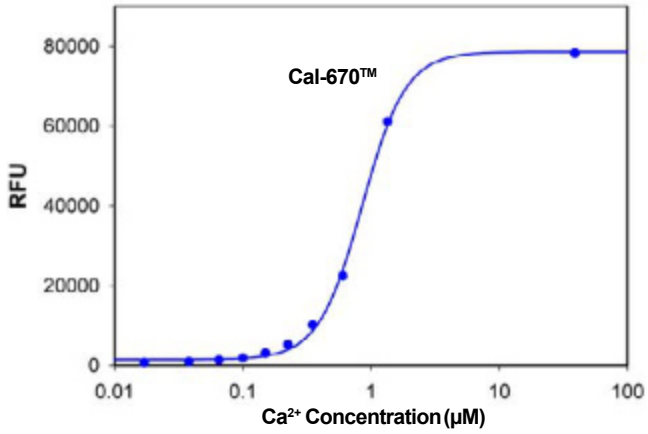

Figure 2.25 Cal-670™wasincubated with buffer that contains different concentration of free Ca^{2+}.Thefluorescence wasmonitored using a fluorometer (Gemini XS,Molecular Devices). The K_d of Cal-670™is 853 nM.

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 separatedfrom other fluorescence indicators and markers,including genetically expressedfluorescent proteins, and thus haspotential 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™isone of the very few calcium indicators that can be potentially used for *in vivo* imaging since it has NIRfluorescence.

Table 2.13 NIRFluorescent CalciumIndicators

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

*The $K_d$value wasnot determined.

Fluorescent Ratiometric Calcium Indicators

BTC

Among the ratiometric calcium indicators, Fura-2 and Indo-1 are most commonly used. BTC is another excitation-ratioable calcium indicator. However, BTC can only be used for high calcium level detection due to its low affinity to calcium. In recent years, BTC has been increasingly used for monitoring potassium channels since BTC demonstrated an excellent fluorescence enhancement responseupon binding thallium ion that selectively passesthrough potassium channels.

Fura-2

Fura-2is a ratiometric fluorescent dye which binds free intracellular calcium. It was the first widely-used dye for calcium imaging, and remains very popular. Fura-2 is excited at 340 nm and 380 nm, and the ratio of the emissions at those wavelengths is directly correlated to the amount of intracellular calcium. Regardlessof the presence of calcium, Fura-2 emits at 510 nm. The use of the ratio automatically cancels out confounding variables, such as variable dye concentration and cell numbers, making Fura-2one of the most appreciated tools to quantify calcium levels. Fura-2is preferred for ratio-imaging microscopy, in which it is more practical to change excitation wavelengths than emission wavelengths.

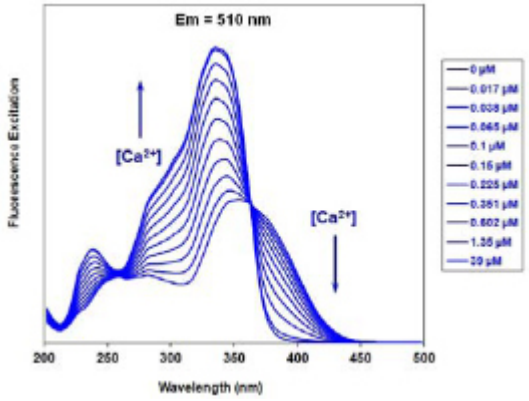

Figure 3.1 Fluorescenceexcitation spectra of Fura-2in solutionscontaining 0 to 39 µM free Ca^{2+}.

Fura-8™

Although Fura-2 has been widely used as the preferred excitation-ratioable calcium indicator, it has certain limitations, e.g., lower sensitivity compared to the single wavelength calcium dyes, such as Fluo-8® and Cal-520®. AAT Bioquest has recently developed Fura-8™ to improve the calcium response of Fura-2. As demonstrated in Figures 3.2 & 3.3, Fura-8™ AM is more sensitive to calcium than Fura-2 AM. In addition, Fura-8™ has its emission shifted to longer wavelength (Em = 525 nm). Fura-8™ might be also used for the flow cytometric analysisof calcium in cells due to its excellent excitation at 405 nm that perfectly matches the violet laser line.

Key Features of Fura-8™

- The same calcium response as Fura-2
- Red-shifted dual excitation wavelengths (354 nm/415 nm)
- Better excited at 405 nm for flow cytometric applications
- Compatible with common filter sets
- Higher signal/background ratio than that of Fura-2

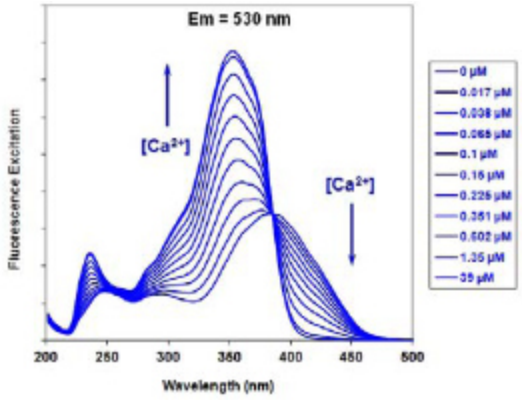

Figure 3.2 Fluorescence excitation spectra of Fura-8™ in solutions containing 0 to 39 µM free Ca^{2+}.

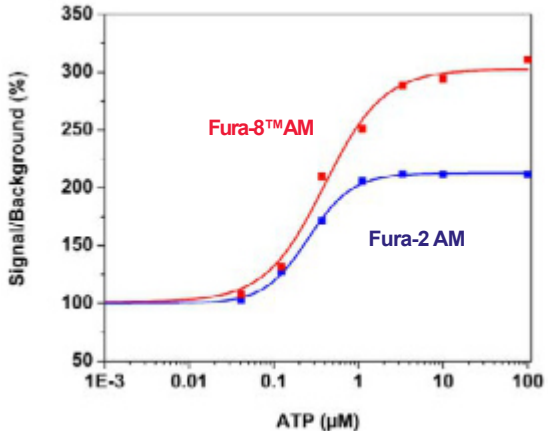

Figure 3.3 ATP dose responses in CHO-K1 cells measured with Fura-2 AM (blue curve, Cat# 21021) and Fura-8™ AM (red curve, Cat# 21056) respectively. CHO-K1 cells were seeded overnight at 40,000 cells/100 µL/well in a Costar 96-well black wall/clear bottom plate. The cells were incubated with Fura-2 AM or Fura-8™ AM calcium assay dye-loading solution for 1 hour at room temperature. ATP (50 µL/well) was added by FlexStation®.

Indo-1

In contrast to Fura-2, Fura-8™ and BTC, Indo-1 is the preferred emission-ratioable dye for flow cytometry, where it is more practical to use a single laser for excitation (usually the 351–364 nm spectral lines of the argon-ion laser). The emission maximum of Indo-1 shifts from ~475 nm in Ca^{2+}-free medium to ~400 nm when the dye is

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saturated with Ca^{2+} (see Figure 20). While Indo-1 is not cell permeant, its pentaacetoxymethyl ester, Indo-1 AM, enters the cell where it is cleaved by intracellular esterases to give Indo-1.

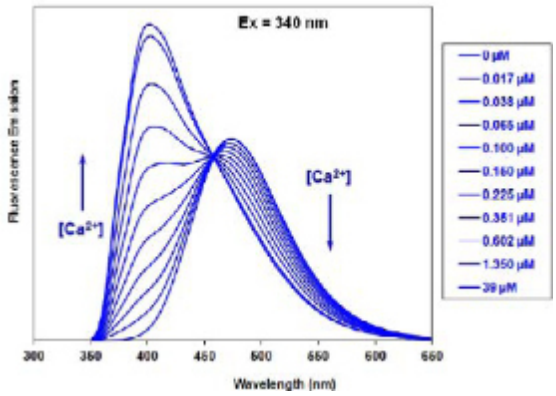

Figure 3.4 Fluorescence emission spectra of Indo-1 in solutions containing 0 to 39 µM free Ca^{2+}.

Cal Red™ R525/650

The most popular ratiometric calcium indicators (such as Fura-2 and Indo-1) have certain limitations such as lower sensitivity, UV excitation, and are not compatible with HTS screening filter sets. Cal Red™ R525/650 has been developed as a new 488 nm-excitable ratiometric fluorescence calcium indicator. It is a chelating agent that, when bound to calcium, will have an emission signal which increases at 525 nm and decreases at 650 nm upon excitation at 488 nm. The excitation and emission wavelengths of Cal Red™ R525/650 are compatible with common filter sets with minimal damage to cells, making it a robust tool for evaluating and screening GPCR agonists and antagonists as well as calcium channel targets.

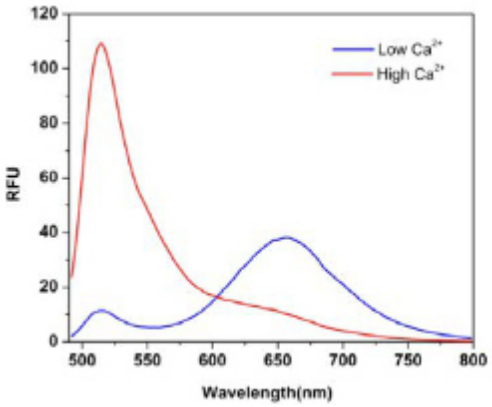

Figure 3.5 Fluorescence emission spectra of Cal Red™ R525/650 (calcium bound).

Table 3.1 Ratiometric Fluorescent Calcium Indicators

Cat #	Product Name	Size	Zero Calcium		High Calcium		K_d
			Ex (nm)	Em (nm)	Ex (nm)	Em (nm)	
21054	BTCAM	1 mg	464	533	401	529	7,000 nM
21053	BTC, tetrapotassium salt	1 mg	464	533	401	529	7,000 nM
20590	Cal Red™ R525/650 AM	1 mg	492	650	492	525	330 nM
20588	Cal Red™ R525/650, potassium salt	5x50 µg	492	650	492	525	330 nM
21021	Fura-2 AM *UltraPure grade*	1 mg	363	512	335	505	145 nM
21025	Fura-2, pentapotassium salt	1 mg	363	512	335	505	145 nM
21026	Fura-2, pentasodium salt	1 mg	363	512	335	505	145 nM
21055	Fura-8™, AM	1 mg	386	532	354	524	260 nM
21057	Fura-8™, potassium salt	1 mg	386	532	354	524	260 nM
21058	Fura-8™, sodium salt	1 mg	386	532	354	524	260 nM
20620	Fura-8FF™, AM	10x50 µg	386	532	354	415	6 µM
20621	Fura-8FF™, potassium salt	10x50 µg	386	532	354	415	6 µM
21027	Fura-FF, AM [Fura-2FF, AM]	10x50 µg	363	512	363	512	5.5 µM
21028	Fura-FF, pentapotassium salt	10x50 µg	363	512	363	512	5.5 µM
21048	Fura Red, AM	10x50 µg	490	656	458	597	400 nM
21047	Fura Red, potassium salt	10x50 µg	490	656	458	597	400 nM
21032	Indo-1 AM *UltraPure grade*	1 mg	346	475	330	401	230 nM
21040	Indo-1, pentapotassium salt	1 mg	346	475	330	401	230 nM
21044	Indo-1, pentasodium salt	1 mg	346	475	330	401	230 nM
21050	Quin-2 AM	1 mg	353	495	333	495	60 nM
21052	Quin-2, tetrapotassium salt	5 mg	353	495	333	495	60 nM

Luminescent Calcium Indicators

The aequorin complex comprises a 22,000-dalton apoaequorin protein, molecular oxygen and the luminophore coelenterazine. When three Ca^{2+} ions bind to this complex, coelenterazine is oxidized to coelenteramide, with a concomitant release of carbon dioxide and blue light. The approximately third-power dependence of aequorin's bioluminescence on Ca^{2+} concentration allows the measurement of Ca^{2+} concentrations with a broad detection range from ~ 0.1 μM to >100 μM. Unlike fluorescent Ca^{2+} indicators, Ca^{2+}-bound aequorin can be detected without illuminating the sample, thereby eliminating the interference from autofluorescence.

AAT Bioquest offers coelenterazine and several synthetic coelenterazine analogs for reconstituting aequorin in cells that have been transfected with apoaequorin cDNA. In addition to native coelenterazine, we also offer a few derivatives of coelenterazine that confer different Ca^{2+} affinities and spectral properties on the aequorin complex. Recombinant apoaequorin reconstituted with coelenterazine hcp is reported to have the best luminescence overall, with both a high quantum yield and a fast response time. However, intracellular reconstitution of aequorin from coelenterazine analogs can be relatively slow. Aequorins containing the cp, f or h form of coelenterazine exhibit 10–20 times stronger luminescence than that of apoaequorin reconstituted with native coelenterazine. Coelenterazine h has been predominantly used in HTS screening assay for GPCRs.

Besides the standalone coelenterazine products, AAT Bioquest offers a luminescent calcium assay kit. The kit uses a highly calcium-sensitive and membrane-permeable coelenterazine analog as a calcium indicator for the cells transfected with apoaequorin gene. Our coelenterazine–based kit is much more sensitive than the fluorescence-based calcium assay kits (such as Fluo-4, Fluo-3, Calcium-3 and Calcium-4). This kit provides an optimized assay method for monitoring 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.

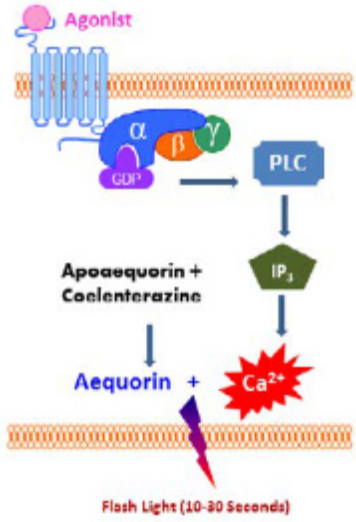

Figure 4.1 The aequorin-based calcium assay principle. Coelenterazine h is the preferred luminophore used in the luminescence-based calcium assays.

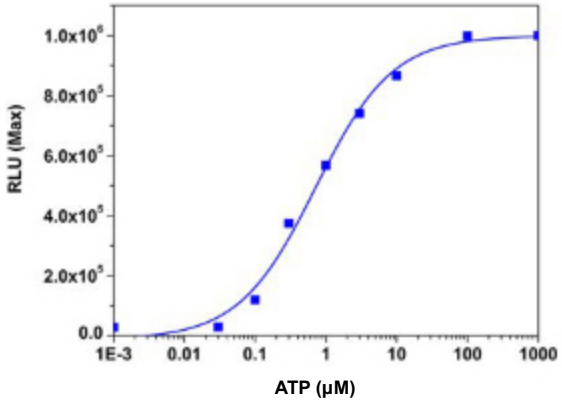

Figure 4.2 ATP dose responses on CHO-aeq cells. CHO cells stably transfected with apoaequorin were seeded overnight at 50,000 cells/100 μL/well in a Costar white wall/clear bottom 96-well plate. The growth medium was removed and the cells were incubated with 100 μL of dye-loading solution using the Screen Quest™ Luminometric Calcium Assay Kit (Cat# 36305) for 3 hours at room temperature and protected from light. ATP (25 μL/well) was added by NOVOstar (BMG Labtech) to achieve the final indicated concentrations. The EC_{50} of ATP is about 0.8 μM.

Table 4. 1 Luminescent Fluorescent Calcium Indicators*

Cat #	Product Name	Size	Em (nm)	RL	HRT (ms)
21150	Coelenterazine *UltraPure grade*	250 μg	466	1	6-30
21151	Coelenterazine cp *UltraPure grade*	250 μg	442	28	2-5
21152	Coelenterazine f *UltraPure grade*	250 μg	472	20	6-30
21153	Coelenterazine h *UltraPure grade*	250 μg	466	16	6-30
21154	Coelenterazine hcp *UltraPure grade*	250 μg	445	500	2-5
21155	Coelenterazine n *UltraPure grade*	250 μg	468	0.15	6-30
36305	Screen Quest™ Luminometric Calcium Assay Kit	10 plates	466	16	6-30

* Notes: a). RL= relative luminescence; HRT= half rise time in milli seconds; b). Data from O. Shimomura, et al. (1993). The relative rate of aequorin regeneration from apoaequorin and coelenterazine analogues. Biochem J296 (Pt 3), 549-51.

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Live Cell Calcium Assays

FLIPR Calcium Assays

Calcium flux assays are preferred methods in drug discovery for screening G protein coupled receptors (GPCRs). Screen Quest™ Calcium Assay Kits provide a homogeneous fluorescence-based assay for detecting the intracellular calcium mobilization. Cells expressing a GPCR of interest that signals through calcium are pre-loaded with our proprietary Fluo-8®AM, Calbytre™520, Calbryte™ 590 or Rhod-4™AM which can cross cell membrane. The assays can be performed in a convenient 96-well or 384-well microtiter-plate format and easily adapted to automation.

Screen Quest™ Fluo-8 NW Calcium Assay Kit (Cat#36315) provides a homogeneous fluorescence-based assay for detecting the intracellular calcium mobilization. Fluo-8®NW is the brightest green calcium indicator available for HTS screening. The characteristics of its long wavelength, high sensitivity, and 100-250 times fluorescence increases (when it forms complexes with calcium) make Fluo-8®NW an ideal indicator for measurement of cellular calcium.

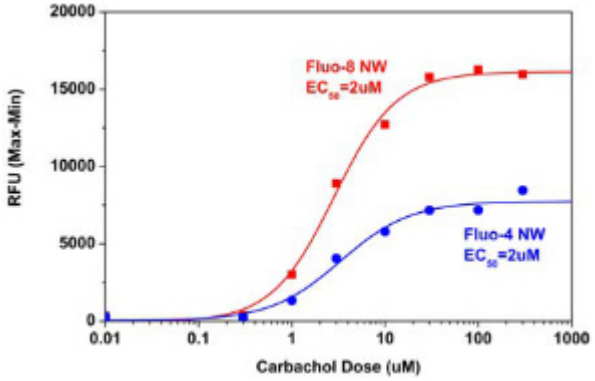

Figure 5.1 Carbachol dose responses were measured in HEK-293 cells with Screen Quest™ Fluo-8®No Wash Calcium Assay Kit (blue, Cat# 36315) and Fluo-4 No Wash Calcium Assay Kit (red, Cat# 36325). HEK-293 cells were seeded overnight at 40,000 cells/100µL/well in a Costar 96-well black wall/clear bottom 96-well plate. The cells were incubated with 100 µL of dye-loading solution using Screen Quest™ Fluo-8®No Wash Calcium Assay Kit or Fluo-4 No Wash Kit for 1 hour at room temperature. Carbachol (50 µL/well) was added by NOVOstar to achieve the final indicated concentrations.

Screen Quest™ Calbryte-520 Probenecid-Free and Wash-Free Calcium Assay Kit (Cat# 36318) provides the most robust homogeneous fluorescence-based assay for detecting the intracellular calcium mobilization. Cells expressing a GPCR of interest that signals through calcium are pre-loaded with our proprietary Calbryte™-520NW which can cross cell membrane. Once inside the cell, the lipophilic blocking groups of Calbryte™-520NW are cleaved by non-specific cell esterase, resulting in a negatively charged fluorescent dye that stays inside cells, and its fluorescence is greatly enhanced upon binding to calcium. When cells stimulated with screening compounds, the receptor signals release of intracellular calcium, which greatly increase the fluorescence of Calbryte™-520NW. The characteristics of its excellent cell retention, high sensitivity, and 100-250 times fluorescence increases (when it forms complexes with calcium) make Calbryte™-520NW an exceptionally good indicator for measurement of cellular calcium. Calbryte™-520NW is the only calcium dye that does not require probenecid for better cellular retention. This Screen Quest™ Calbryte-520 Probenecid-Free and Wash-Free Calcium Assay Kit provides the most optimized assay method for monitoring G-protein-coupled receptors (GPCRs) and calcium channels with fragile or difficult cell lines.

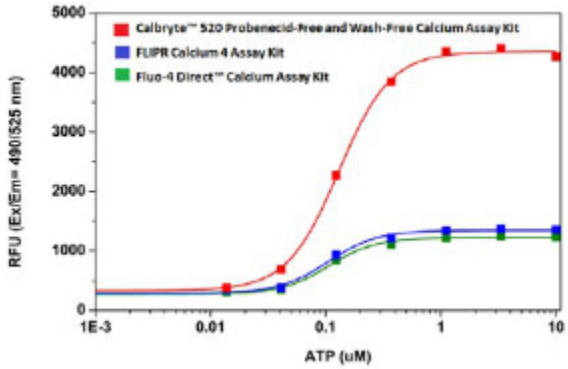

Figure 5.2 Comparison of fluorescent signal response of endogenous P2Y receptor to ATP in CHO-K1 cells. CHO-K1 cells were seeded overnight at 50,000 cells/100 µL/well in a 96-well black wall/clear bottom Costar plate. Calcium flux response was measured with Screen Quest™ Calbryte™520 Probenecid-Free and Wash-Free Calcium Assay Kit (red, Cat# 36318), FLIPR Calcium 4 Assay Kit (blue), and Fluo-4 Direct™ Calcium Assay kit (Green). ATP (50 µL/well) was added to achieve the final indicated concentrations.

The spectra of most common calcium indicators are in the green fluorescence range, but for green and yellow fluorescent cells and tissues studies, red-shifted wavelength calcium indicators are highly demanded. Although the rhodamine-based calcium indicators (such as Rhod-2 AM) are available, the higher staining background and undesired cellular localization (mostly in mitochondria) makes the rhodamine calcium dyes less sensitive when binding with Ca^{2+}. The new red calcium indicators, Calbryte™590, has been developed for monitoring calcium ions with Ex/Em = 581/593 nm, which is more red-shifted wavelength range than Rhod-2 indicators. When the non-fluorescent Calbryte™590AM enters the cells, the lipophilic AM blocking groups are cleaved by intracellular esterase resulting in a negatively charged red fluorescent dye that is retained in the cells. Calbryte™590 binds intracellular calcium and generates bright red fluorescence with no overlap with green fluorescent wavelength (FITC channel).

Screen Quest™ Calbryte-590 Probenecid-Free and Wash-Free Calcium Assay Kit (Cat# 36201) provides the most robust homogeneous red fluorescence-based assay for detecting intracellular calcium mobilization. It is the most optimized red fluorescence-based assay for monitoring GPCRs and calcium channels with fragile or difficult cell lines. The assay can be performed in a convenient 96-well or 384-well microtiter-plate format and easily adapted to automation.

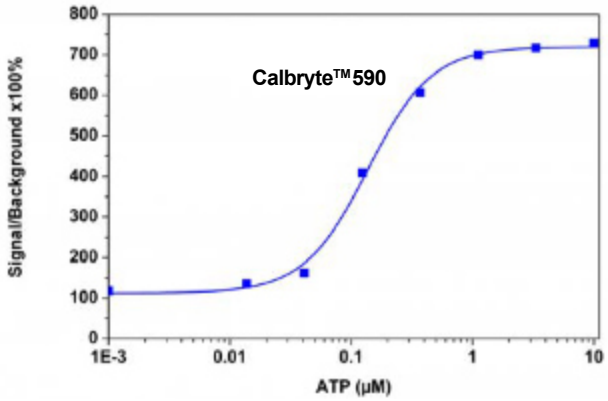

Figure 5.3 ATP dose response was measured in CHO-K1 cells with Screen Quest™ Calbryte-590 Probenecid-Free and Wash-Free Calcium Assay Kit (Cat# 36201). CHO-K1 cellswere seeded overnight. 100 µL dye loading solution wasadded and incubated for 45 minutesat 37°Cfollowed by 15 minutesat room temperature.

Rhod-4™AMis the brightest red calcium indicator available for HTS screening. Once inside the cell, the lipophilic blocking groups of Rhod-4™AM are cleaved by non-specific cell esterase, resulting in a negatively charged fluorescent dye that stays inside cells, and its fluorescence is greatly enhanced upon binding to calcium. When cells stimulated with screening compounds, the receptor signals the release of intracellular calcium, which greatly increases the fluorescence of Rhod-4™.Thecharacteristics of its long wavelength, high sensitivity, and >250 times fluorescence increases (when it forms complexes with calcium) make Rhod-4™AMan ideal indicator for the measurementof intracellular calcium.

Screen Quest™ Fura-2 No Wash Calcium Assay Kit (Cat# 36320) provides the only ratiometric FLIPR®calcium assay commercially available for screening GPCRsand calcium channel targets. The ratiometric characteristics of Fura-2 make this kit an ideal tool for more accurate measurement of cellular calcium concentration compared to Fluo-4 of the single wavelength.The kit usesexcitation ratio of 340/380 nm, monitoring emission at 510 nm. With a single addition, the assay is easy to perform and desirable in a high-throughput environment.

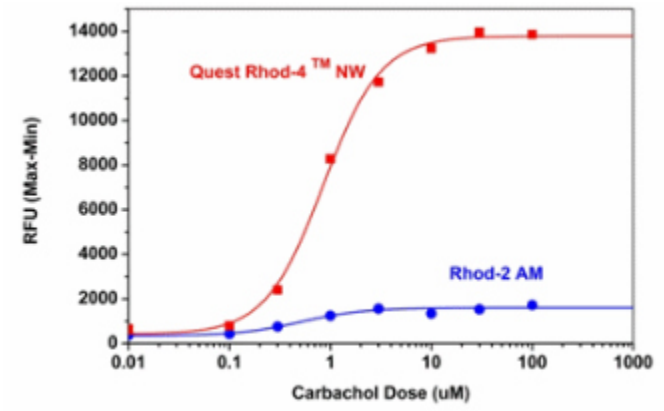

Figure 5.4 Carbachol dose responsewasmeasured in HEK-293cellswith ScreenQuest™ Rhod-4 NWAssay Kit (Cat#36334) and Rhod-2 AM. HEK-293cellswere seeded overnight at 40,000 cells/100 µL/well in a Costarblack wall/clear bottom 96-well plate. The cells were incubated with 100 µL of dye-loading solution using the ScreenQuest™Rhod-4 NW Calcium Assay Kit, or 100 µL of Rhod-2 AM solution (5 µM) for 1 hour at room temperature.The $EC_{50}$of Rhod-4 NWis about 0.6 µM.

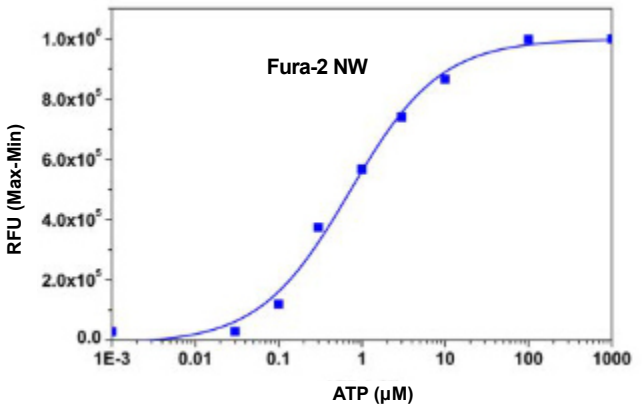

Figure 5.5 ATPdose responses were measured in CHOcells with Screen Quest™Fura-2 No WashCalcium Assay Kit (Cat# 36320). CHO-K1cells were seeded overnight at 40,000 cells/100 µL/well in a Costar 96-well black wall/clear bottom plate. The cells were incubated with 100 µL of Screen Quest™Fura-2No Wash Calcium Assay Kit for 1 hour at room temperature.

Table 5.1 Screen Quest™FLIPR Calcium Assays

Cat #	Product Name	Size	Ex (nm)	Em (nm)
36318	Screen Quest™Calbryte-520 Probenecid-Free and Wash-Free Calcium AssayKit	10 plates	490	525
36201	Screen Quest™Calbryte-590 Probenecid-Free and Wash-Free Calcium AssayKit	10 plates	573	588
36301	Screen Quest™10X Calcium AssayBuffer with Phenol Red Plus™	10 plates	N/A	N/A
36300	Screen Quest™10XCell Staining Buffer with Phenol Red Plus™	10 plates	N/A	N/A
36325	Screen Quest™Fluo-4 No Wash Calcium AssayKit	10 plates	490	525
36315	Screen Quest™Fluo-8 No Wash Calcium AssayKit	10 plates	490	525
36308	Screen Quest™Fluo-8 No Wash Calcium AssayKit *Medium Removal*	10 plates	490	525
36320	Screen Quest™Fura-2 No Wash Calcium Assay Kit	10 plates	340/380	510
36334	Screen Quest™Rhod-4 No Wash Calcium AssayKit	10 plates	530	590
36331	Screen Quest™Rhod-4 No WashCalcium AssayKit *Medium Removal*	10 plates	530	590

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Endpoint Calcium Assay

Cell Meter™ No Wash and Probenecid-Free Endpoint Calcium Assay Kit (Cat# 36312) enables homogeneous fluorescence-based assays for detecting intracellular calcium mobilization without the need to use kinetics reading mode. It can be used with conventional fluorescence microplate readers with bottom read mode that do not have a built-in liquid dispenser. After loading the Fluo-8E™ AM dye into cells of interest, one can simply add a calcium agonist by an external liquid dispenser or hand pipetting. The long lasting fluorescence signal of Fluo-8E™ makes it an ideal indicator for the measurement of cellular calcium with a conventional fluorescence microplate reader.

Flow Cytometric Calcium Assay

Cell Meter™ Flow Cytometric Calcium Assay Kit (Cat# 36310) provides a fluorescence-based assay for detecting intracellular calcium mobilization using a flow cytometer. It can be used for kinetic reading or for endpoint reading. After loading the Calbryte™ 520 AM dye into cells of interest, simply wash the cells and add the calcium agonist, one can then read the sample with a flow cytometer using FITC channel (Ex/Em= 490/525 nm). When the cells expressing GPCR of interest are stimulated with an agonist, the receptor signals the release of intracellular calcium, which significantly increases the fluorescence of Calbryte™ 520. The kit can be used for monitoring cellular calcium flux as well as cell sorting.

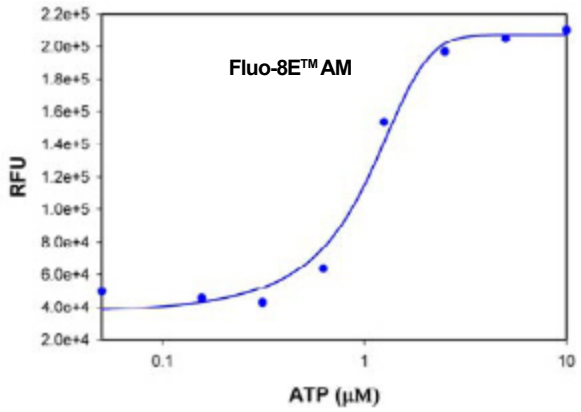

Figure 5.6 The ATP dose dependent intracellular calcium release was measured by Cell Meter™ No Wash and Probenecid-Free Endpoint Calcium Assay Kit (Cat# 36312) in CHO-K1 cells in a 96-well plate. 3 columns of cells were incubated with Fluo-8E™ AM dye loading solution for 1 hour at 37 °C before ATP was added into all 3 columns of the wells. The plate was read immediately after the addition of the ATP by CLARIOstar® with bottom reading and endpoint reading mode.

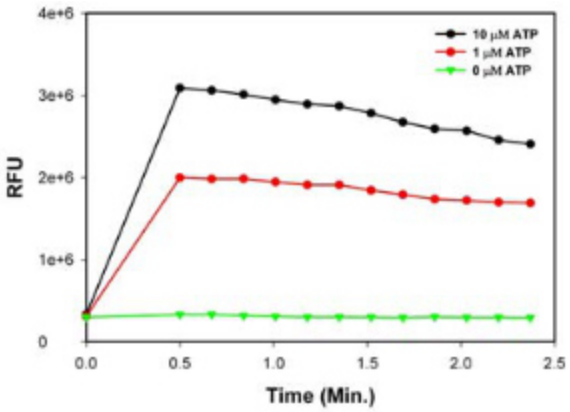

Figure 5.7 The ATP dose dependent intracellular calcium release was measured by Cell Meter™ Flow Cytometric Calcium Assay Kit (Cat# 36310) in CHO-K1 cells. Cells were incubated with Calbryte™ 520 AM dye for 30 minutes at 37 °C before ATP was added into the cells. The baseline was acquired and the rest of the cells were analyzed after the addition of ATP. The response was measured over time. The analysis was done on NovoCyte® 3000 flow cytometer.

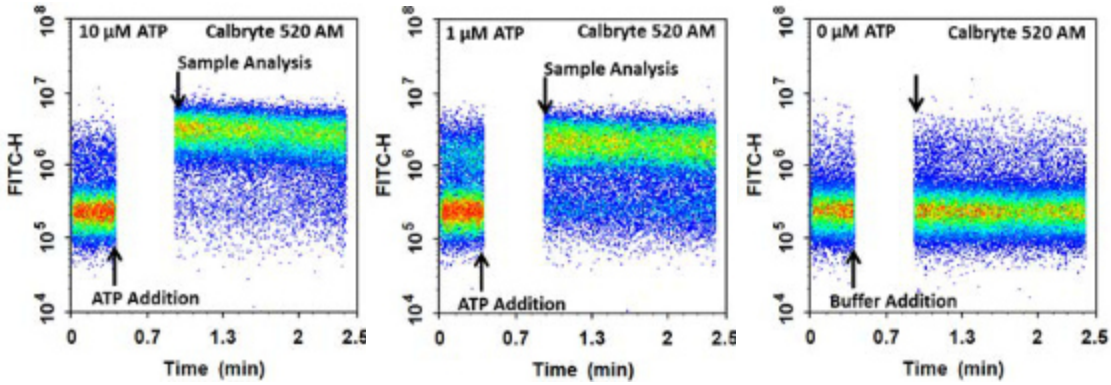

Figure 5.8 The ATP dose dependent intracellular calcium release was measured by Cell Meter™ Flow Cytometric Calcium Assay Kit (Cat# 36310) in CHO-K1 cells. Cells were incubated with Calbryte™ 520 AM dye for 30 minutes at 37 °C before ATP was added into the cells. The baseline was acquired and the rest of the cells were analyzed after the addition of ATP. The responses were measured over time. The analysis was done using a NovoCyte™ 3000 flow cytometer. 10 μM, 1 μM or 0 μM ATP were added to the cells. The arrows on the graph indicate the time (30 seconds) between the addition of ATP and the actual analysis.

Table 5.2 Endpoint & Flow Cytometric Calcium Assays

Cat #	Product Name	Size	Ex (nm)	Em (nm)
36310	Cell Meter™ Flow Cytometric Calcium Assay Kit	100 tests	492	514
36312	Cell Meter™ No Wash and Probenecid-Free Endpoint Calcium Assay Kit	100 tests	490	525

GPCRCell Lines for Calcium Assays

Screen Quest™ cell lines are a series of cells that have been successfully used in drug discovery and screening environments for studying G-protein-coupled receptors (GPCR) that do not conventionally couple through intracellular calcium. It have been effectively used with the FLIPR,FDSSSystemsin conjunction with non-Gq coupled members of many receptors such as chemokine, serotonin, glutamate, dopamine, opoid, vasopressin as well as α- and ß-adrenoceptor receptor families. Over 60% of the known GPCRssignal through pathways other than Gq which lead to an increase in intracellular calcium. Screen Quest™cell lines are used for investigating GPCRsthatdo not conventionally couple through intracellular calcium. Screen Quest™cell lines are based on a series of G-protein chimeras, including the promiscuous G-protein, $G_{\alpha 16}$. The chimeras consist of the alpha subunit of a Gq-protein complex whose 5 carboxy-terminal amino acids have been replaced with those from one of the other G-proteins (either $G_{\alpha s}$, $G_{\alpha i}$, $G_{\alpha o}$, or $G_{\alpha z}$). Theseamino acids are responsible for the coupling of the receptor to its G-protein. Co-expression of these chimeras with specific non-Gq-coupled receptors which normally act through the cAMP pathway may result in the generation of an intracellular calcium signal upon receptor stimulation. Activation of the specific non-Gq-coupled receptors in these cells by specific ligands can be detected using calcium sensitive dyes such as Calbryte™520 AM, Cal-520® AM, Fluo-8®AM, or Fluo-4 AM.

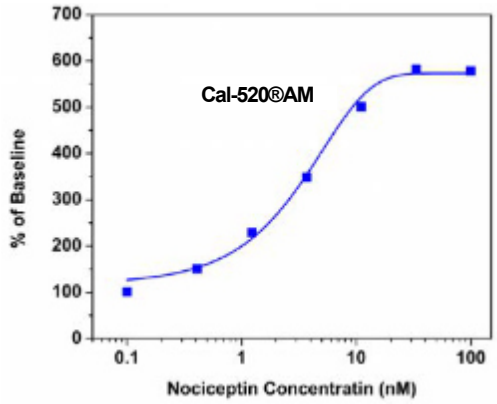

Figure 5.9 Nociceptin-stimulated calcium response was measured in CHO-Ga16-NOP cells with Cal-520®,AM (Cat#21130). CHO-Ga16-NOPcells were seeded overnight at 60,000cells/100 µL/well in aCostar black wall/clear bottom 96-well plate.The cellswere incubated with equal volume (100 µL) of 10 µM Cal-520®AMwith 2 mM probenecid in Hanks with 20 mM Hepes buffer (HHBS)at 37 °C for 1 hour. The Cal-520®AM loading solution was replaced with HHBSand 1 mM probenecid. Nociceptin was added by FlexStation®(Molecular Devices)to achieve the final indicated concentrations.

Live Cell cAMP assay

G protein coupled receptors (GPCR)areone of the largest receptor classestargeted by drug discovery programs. Calcium flux (coupled via Gq pathway) assayis a preferred method in drug discovery for screening GPCRtargets. However, over 60% of the known GPCRs signal through adenylyl cyclase activity coupled to cAMP. Most of the existing cAMPassaysnot only require cell lysisbut also lack both temporal and spatial resolution.

Screen Quest™Live Cell cAMP Assay Service Pack provides the real-time monitoring of intracellular cAMPchange in a high-throughput format without a cell lysis step. The assayworks through the cell lines that contain either an exogenous cyclic nucleotide-gated channel (CNGC)or the promiscuous G-protein, $G_{\alpha 16}$. The channel is activated by elevated levels of intracellular cAMP, resulting in ion flux and cell membrane depolarization which can be detected with either a fluorescent calcium (such as Calbryte™520 AM, Cal-520® AM and Fluo-8®AM) or a fluorescent membrane potential dye. Co-expressionof $G_{\alpha 16}$with specific non-Gq-coupled receptorswill result in the generation of an intracellular calcium signal upon receptor stimulation. The Screen Quest™ Live Cell cAMP Assay Service Pack provides both cell lines and reagents for the measurement of intracellular cAMP changes with a FLIPR®,a FDSS®or other equivalent fluorescence microplate readers. It has been successfully used to measure Gsand Gi coupled GPCRactivity.

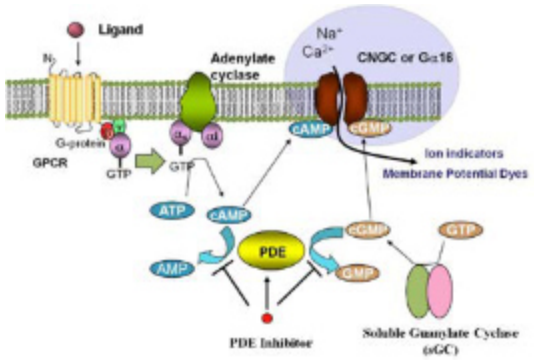

Figure 5.10 Screen Quest™Live Cell cAMP AssayPrinciple.

Table 5.3 Screen Quest™Cell Lines

Cat#	Product Name	Size
38000	Screen Quest™Amylin 3 Receptor	each
38001	Screen Quest™Cannabinoid Receptor	each
38002	Screen Quest™Chemokine (C-C) Receptor 2B	each
38003	Screen Quest™Chemokine (C-X-Cmotif) Receptor 4	each
38105	Screen Quest™CHO-Ga16Chimera Cell line	each
38101	Screen Quest™CHO-GqiChimera Cell line	each
38102	Screen Quest™CHO-GqoChimera Cell line	each
38104	Screen Quest™CHO-GqsChimera Cell line	each
38103	Screen Quest™CHO-GqzChimera Cell line	each
38004	Screen Quest™Dopamine Receptor 1 (DRD1)	each
38005	Screen Quest™Glucagon-like Receptor 1 (GLP1R)	each
38100	Screen Quest™Human Nociceptin Receptor Ga16 Coupled CHOCells (NOP-Ga16)	each
36382	Screen Quest™Live Cell cAMP Assay Service Pack	each
38006	Screen Quest™Opiate Receptor-like 1 (ORL1)	each

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cAMP & Phosphodiesterase (PDE) Assays

cAMP Assays

Cyclic adenosine monophosphate (cAMP) is an important second messenger in many biological processes. Monitoring levels of cAMP is one of the most common ways to screen for agonists and antagonists of GPCRs.

Screen Quest™ ELISAcAMP Assay Kits (Cat# 36371 & Cat# 36374) use HRP-labeled cAMP to compete with free cAMP for cAMP antibody binding in biochemical or cell-based assays. Compared to other ELISAcAMP assay kits, our kit eliminate the tedious acetylation step. Screen Quest™ ELISAcAMP Assay Kits provide the ready-to-use anti-cAMP Ab coated 96-well plate and HRP substrates Amplite™ Green (Colorimetric Assay, Cat# 36371) or Amplite™ Red (Fluorimetric Assay, Cat# 36374) to quantify the HRP activity.

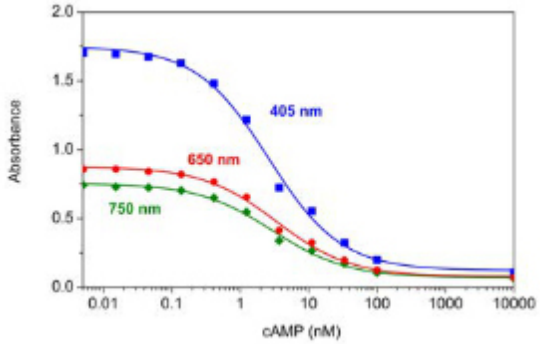

Figure 6.1 cAMP dose response was measured with Screen Quest™ Colorimetric ELISA cAMP Assay Kit (Cat# 36371) in a clear 96 well plate with a SpectraMax® microplate reader. As low as 0.1 nM cAMP can be detected in a 100 µL reaction volume at 405, 650 and 750 nm.

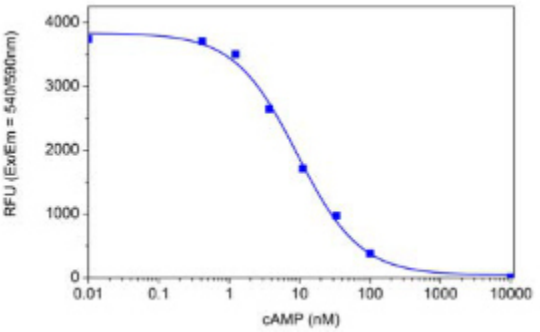

Figure 6.2 cAMP dose response was measured with Screen Quest™ Fluorimetric ELISA cAMP Assay Kit (Cat# 36374) in a solid black 96 well plate with a Gemini microplate reader. The kit can detect as low as 1 nM cAMP in a 100 µL reaction volume

Screen Quest™ FRET No Wash cAMP Assay Kit (Cat# 36380) provides a convenient assay method to monitor the activity of adenylyl cyclase in GPCR systems. Compared to other commercial ELISAcAMP assay kits, this homogenous cAMP assay kit does not require the wash steps or the acetylation step. The assay uses a fluorescent cAMP tracer to compete with free cAMP for anti-cAMP antibodies. The anti-cAMP antibody is labeled with our trFluor™ Eu while the cAMP tracer contains our trFluor™ 650. Upon binding, the generated FRET between the trFluor™ 650 labeled cAMP tracer and the trFluor™ Eu-labeled anti-cAMP antibody is proportional to the concentration of cAMP in a sample.

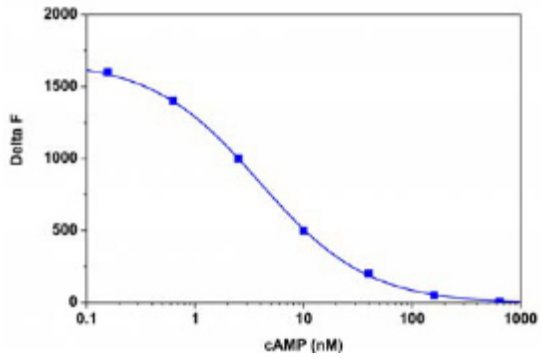

Figure 6.3 cAMP dose response was measured with Screen Quest™ FRET No Wash cAMP Assay Kit (Cat# 36380) using a CLARIOstar® microplate reader (BMG). The assay can detect as low as 1 nM cAMP.

Phosphodiesterase (PDE) Assay

Phosphodiesterase (PDE) is a group of enzymes that degrade the second messenger molecules: cyclic nucleotides cAMP and cGMP. They are important regulators of signal transduction mediated by these second messenger molecules. FAM-cAMP PDE IV (Cat# 13602) and TAMRA-cAMP PDE IV (Cat# 13603) substrates can be used to assay PDE IV activities; FAM-cGMP PDE V (Cat# 13604) and TAMRA-cGMP PDE V (Cat# 13605) substrates can be used to assay PDE V activities. Those substrates can be used in combination with anti-cAMP/cGMP antibodies in a FRET assay or FP assay.

Table 6.1 cAMP & Phosphodiesterase Assays

Cat#	Product Name	Size	Ex (nm)	Em (nm)
20300	cAMP AM	1 mg	N/A	N/A
13602	FAM-cAMP PDE IV Substrate *Green Fluorescence*	0.5 umol	492	515
13604	FAM-cGMP PDE V substrate *Green Fluorescence*	0.5 umol	492	515
36371	Screen Quest™ Colorimetric ELISAcAMP Assay Kit	10 plates	650	N/A
36374	Screen Quest™ Fluorimetric ELISAcAMP Assay Kit	10 plates	571	585
36380	Screen Quest™ FRET No Wash cAMP Assay Kit	10 plates	390	650
13603	TAMRA-cAMP PDE IV Substrate *Red Fluorescence*	0.5 umol	544	575
13605	TAMRA-cGMP PDE V substrate *Red Fluorescence*	0.5 umol	544	575

Measurement of Calcium **In Vitro**

Calcium is essential for all living organisms, particularly in cell physiology, where the movement of calcium ion into and out of the cytoplasm functions as a signal for many cellular processes. Calcium also plays an important role in mediating the constriction and relaxation of blood vessels, nerve impulse transmission, muscle contraction, and hormone secretion. The serum level of calcium is closely regulated (9 to 10.5 mg/dL) in the human body. Both hypocalcemia and hypercalcemia are serious medical disorders. Causes of low calcium levels include chronic kidney failure, vitamin D deficiency, and low blood magnesium levels.

Amplite™ Colorimetric Calcium Assay

Amplite™ Colorimetric Calcium Quantitation Kit (Cat# 36361) provides a simple method for detecting calcium in physiology solutions. The kit uses Calcium Blue™ as the chromogenic calcium indicator. Its absorbance changes in response to calcium binding. The absorbance signal can be easily read by an absorbance microplate reader at 600 or 650 nm. The kit can be performed in a convenient 96-well or 384-well microtiter-plate format within 5 minutes and easily adapted to automation without a separation step. With Amplite™ Colorimetric Calcium Quantitation Kit, the calcium detection linear range is from 0.1 to 7.5 nmoles in 100 µL final test volume (2.5 to 150 µM calcium).

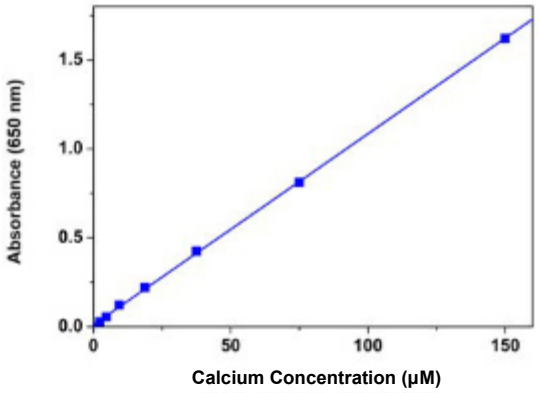

Figure 7.1 Calcium dose response was measured in a 96-well white wall/clear bottom plate with Amplite™ Colorimetric Calcium Quantitation Kit (Cat#36361). A slow as ~ 2.5 µM Ca^{2+} can be detected with 5 minutes incubation (n=3).

Amplite™ Fluorimetric Calcium Assay

Amplite™ Fluorimetric Calcium Quantitation Kit (Cat#36360) provides a simple method for detecting calcium in physiology solutions by using our proprietary red fluorescence probe. The fluorescence signal can be easily read with a fluorescence microplate reader at Ex/Em = 540/590 nm. The kit can be performed in a convenient 96-well or 384-well microtiter-plate format and easily adapted to automation. The assay can be completed within 30 minutes. With Amplite™ Fluorimetric Calcium Quantitation Kit, we have detected as little as 0.03 mM calcium. The kit has a broad dynamic range (30 µM to 10 mM). If more sensitive calcium detection is required, we recommend that Fluo-8® or Fluo-3 be used instead. Both Fluo-8® and Fluo-3 can be used for determining calcium in nM range.

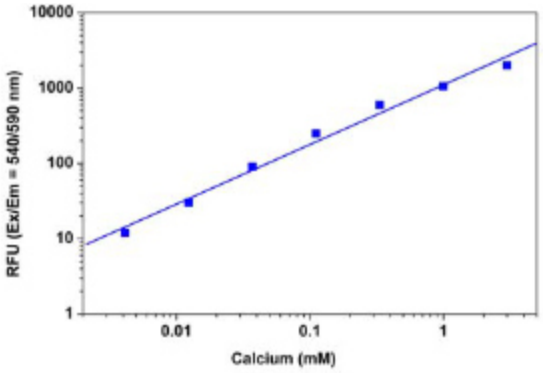

Figure 7.2 Calcium dose response was measured in a 96-well black solid plate with Amplite™ Fluorimetric Calcium Quantitation Kit (Cat#36360). A slow as 0.03 mM calcium can be detected with 5 minutes incubation (n=3).

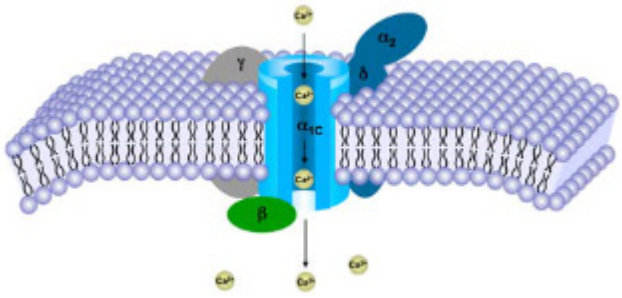

Figure 7.3 Examples of physiological effects of calcium ion: Excitation-secretion coupling; Excitation -contraction coupling in cardiac smooth muscles; Regulation of ion channel function; Activation of Ca^{2+}-dependent enzymes.

Table 7.1 *In Vitro* Calcium Assays

Cat #	Product Name	Size
36361	Amplite™ Colorimetric Calcium Quantitation Kit *Blue Color*	200 tests
36360	Amplite™ Fluorimetric Calcium Quantitation Kit *Red Fluorescence*	200 tests

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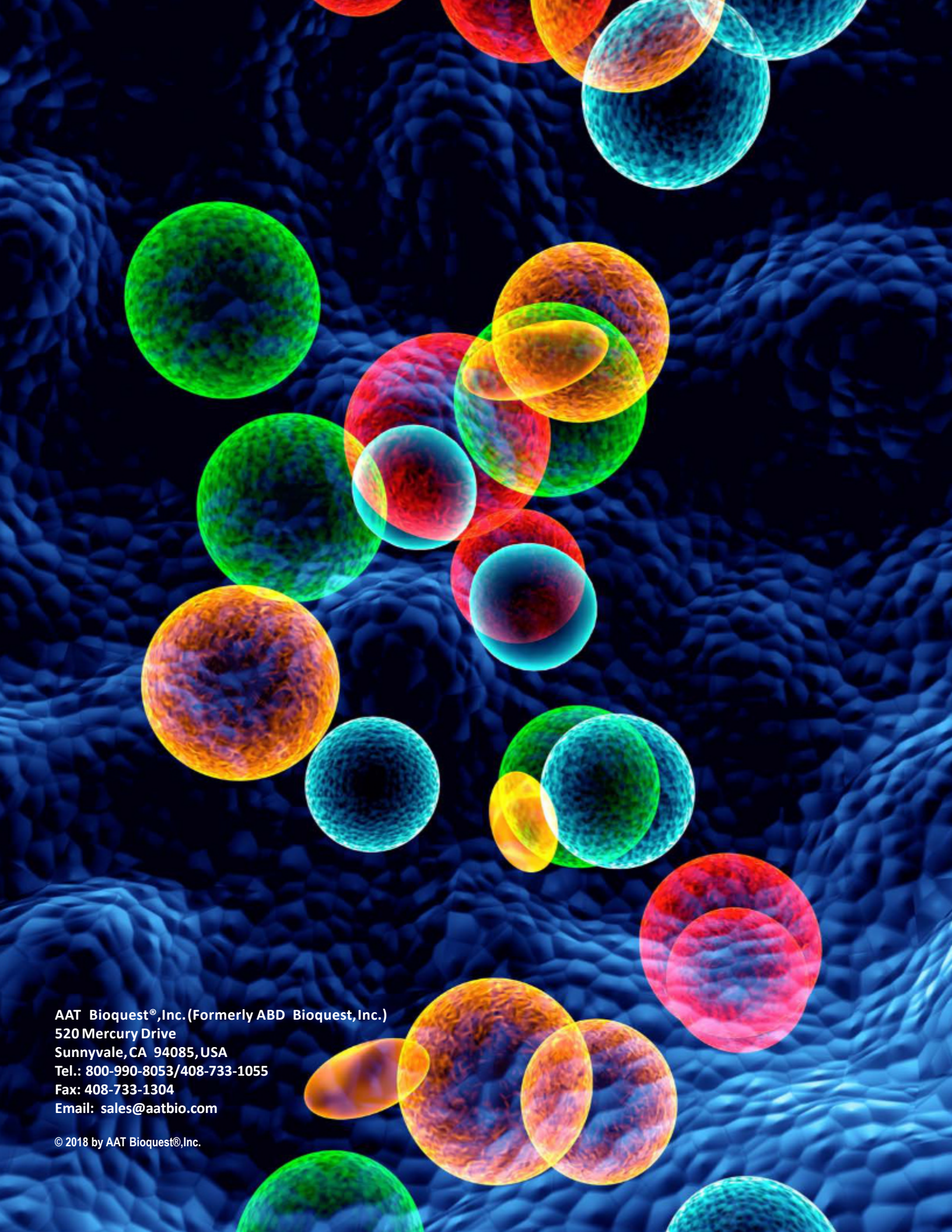

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