Ratiometric Fluorescent Calcium Indicators

I. Introduction

Among the ratiometric calcium indicators, Fura-2 and Indo-1 are most commonly used. Fura-2 is excitation-ratio able while Indo-1 is emission-ratio able. Fura-2 is preferred for ratio-imaging microscopy, in which it is more practical to change excitation wavelengths than emission wavelengths. Upon binding Ca2+, Fura-2 exhibits an absorption shift that can be observed by scanning the excitation spectrum between 300 and 400 nm, while monitoring the emission at ~510 nm. AAT has recently developed Fura-8™ to further improve the calcium response of Fura-2. Fura-8 has its emission shifted into longer visible wavelength that is compatible with the common filter sets. It has comparable calcium affinity (to Fura-2). Fura-8™ AM is more sensitive to calcium than Fura-2 AM with higher signal/background ratio than that of Fura-2 AM. Most ratiometric indicators require anion transporter inhibitor such as probenecid in order to achieve better dye retention in cells. Thus, AAT Bioquest has recently developed Fura-10™. A greatly improved signal/background ratio and intracellular retention properties make Fura-10™ the most robust calcium ratiometric and UV light—excitable indicator for evaluating GPCR and calcium channel targets. We have also developed a new 488 nm-excitable ratiometric fluorescence calcium indicator Cal Red™ R525/650. The emission signal is increased at 525 nm and decreased at 650 nm when excited at 488 nm. The excitation and emission wavelength 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. AM ester form of all these dyes can be loaded into live cells noninvasively.

Table 1. Spectral and Ca^{2+}–Binding Properties of Ratiometric Calcium Detection Reagents

Ca^{2+} Indicator	Catalog Numbers		Excitation	Emission	K_d of Ca^{2+}-Binding
	Salt	AM Ester			
Cal Red™ R525/650	20588	20590, 20591	492 nm	525/650 nm	330 nM
Fura-2	21025, 21026	21020, 21021 21022, 21023	340/380 nm	510 nm	140 nM
Fura FF	21028	21027	340/380 nm	510 nm	5.5 μM
Fura-8™	21057, 21058	21055, 21056	354/415 nm	524 nm	260 nM
Fura-8 FF	20621	20620	354/415 nm	524 nm	6 μM
Fura-10™	21110, 21111	21114, 21115	354/415 nm	524 nm	260 nM
Fura Red	21045, 21047	21046, 21048	436/471 nm	630/652	400 nM
Indo-1	21040, 21044	21030, 21032 21033, 21036	355 nm	400/475 nm	230 nM

II. Storage Conditions

Store at –20 ^{0}C, protected from light. Expiration date is 12 months from the date of receipt.

III. Use of Calcium indicator AM Esters

1. Load Cells with Calcium Indicator AM Esters:

AM esters are the non-polar esters that readily cross live cell membranes, and rapidly hydrolyzed by cellular esterases inside live cells. AM esters are widely used for loading a variety of polar fluorescent probes into live cell non-invasively. However, cautions must be excised when AM esters are used since they are susceptible to hydrolysis, particularly in solution. They should be reconstituted in high-quality, anhydrous dimethyl sulfoxide (DMSO). DMSO stock solutions should be stored desiccated at -20 °C and protected from light. Under these conditions, AM esters should be stable for several months. Among ratiometric calcium ion indicators, Fura-2 and Indo-1 are the two most popular ones. However, there are still a few challenges for using these two calcium ion indicators, in particular, for live cells. UV-excitation of Fura 2 caused fast photo bleaching. Fura-8™ was introduced a few years ago to shift the excitation closer to visible light. Although Fura-8™ demonstrated significant improvement in the ratio of signal/background, it is not well retained in live cells just like Fura-2. Fura-10™ has

recently been introduced to address this cellular retention issue. As shown in Figure 2, Fura 10™ demonstrated dramatic improvement in the ratio of signal/background in the absence of probenecid.

Following is our recommended protocol for loading AM esters into live cells. This protocol only provides a guideline, and should be modified according to your specific needs.

a) Prepare a 2 to 5 mM AM esters stock solution in high-quality, anhydrous DMSO.
b) On the day of the experiment, either dissolve calcium indicators solid in DMSO or thaw an aliquot of the indicator stock solutions to room temperature. Prepare a working solution of 1 to 20 µM in the buffer of your choice (such as Hanks and Hepes buffer) with 0.04% *Pluronic® F-127*. For most cell lines we recommend the final concentration of calcium indicators be 4-5 µM. The exact concentration of indicators required for cell loading must be determined empirically. To avoid any artifacts caused by overloading and potential dye toxicity, it is recommended to use the minimal probe concentration that can yield sufficient signal strength.
Note: The nonionic detergent Pluronic® F-127 is sometimes used to increase the aqueous solubility of calcium indicator AM esters. A variety of Pluronic® F-127 solutions can be purchased from AAT Bioquest.
c) If your cells (such as CHO cells) containing the organic anion-transports, probenecid (2–5 mM) or sulfinpyrazone (0.2–0.5 mM) may be added to the dye working solution (final in well concentration will be 1-2.5 mM for probenecid, or 0.1 -0.25 mM for sulfinpyrazone) to reduce the leakage of the de-esterified indicators.
Note: A variety of ReadiUse™ probenecid including water soluble sodium salt and stabilized solution can be purchased from AAT Bioquest.
Note: For Fura-10™ AM, no probenecid or lower concentration of (0.2-1 mM) probenecid can be used for the cell loading step.
d) Add equal volume of the dye working solution (from step b or c) into your cell plate.
e) Incubate the dye-loading plate room at temperature or 37 °C for 20 minutes to 2 hours, and then incubate the plate at room temperature for another 30 minutes.
Note: Decreasing the loading temperature might reduce the compartmentalization of the indictor.
Note: Incubation time should be optimized as per the cells type.
f) Replace the dye working solution with HHBS or buffer of your choice (containing an anion transporter inhibitor, such as 1 mM probenecid, if applicable) to remove excess probes.
g) Run the experiments at desired Ex/Em wavelengths (see Table 1).

2. Measure Intracellular Calcium Responses:

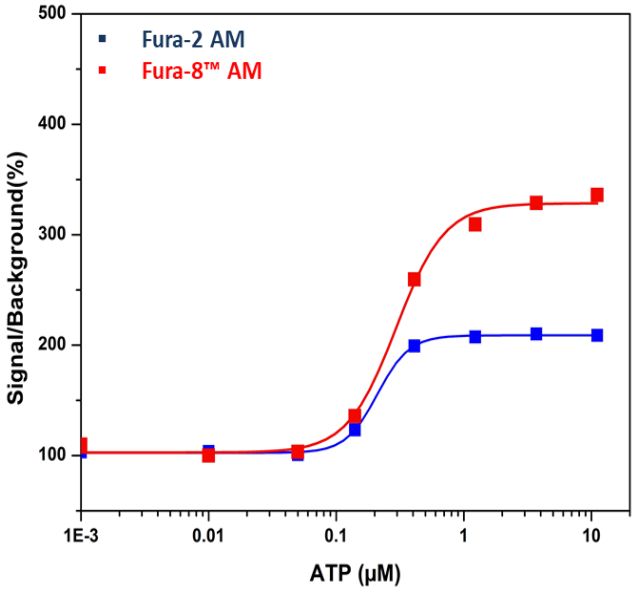

Figure 1. **ATP-stimulated calcium response of endogenous P2Y receptor in CHO-K1 cells measured with Fura-2 AM and Fura-8™ AM.** CHO-K1cells were seeded overnight in 50,000 cells per 100 µL per well in a 96-well black wall/clear bottom costar plate. 100 µL of 5 µM Fura-2 AM (Blue) or Fura-8™ AM (Red) with probenecid (2.5 mM) was added into the cells, and the cells were incubated at 37 °C for 45 minutes and RT for 30 minutes. ATP (50µL/well) was added by FlexStation (Molecular Devices) to achieve the final indicated concentrations.

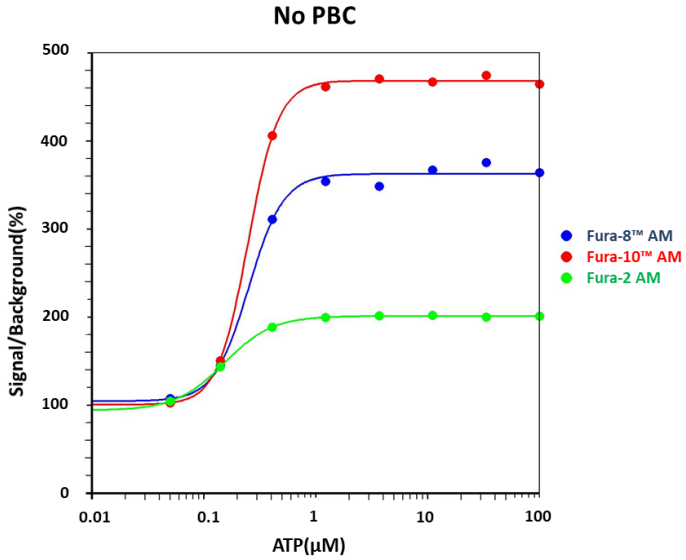

Figure 2. **ATP-stimulated calcium response of endogenous P2Y receptor in CHO-K1 cells measured with Fura-2 AM, Fura-8™ AM and Fura-10™ AM in the absence of Probenecid.** CHO-K1cells were seeded overnight in 50,000 cells per 100 µL per well in a 96-well black wall/clear bottom costar plate. 100 µL of 5 µM Fura-2 AM or Fura-8™ AM or Fura-10™ AM without probenecid was added into the cells, and the cells were incubated at 37 °C for 45 minutes and RT for 30 minutes. ATP (50µL/well) was added by FlexStation (Molecular Devices) to achieve the final indicated concentrations.

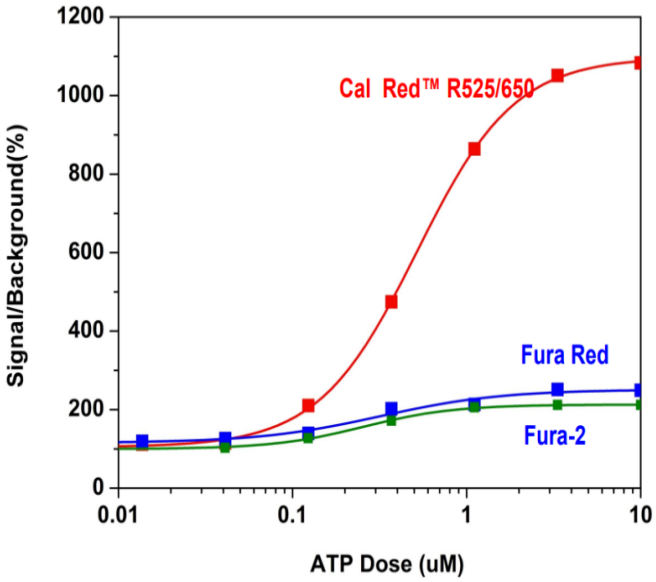

Figure 3. **ATP-stimulated calcium response of endogenous P2Y receptor in CHO-K1 cells measured with Fura-2 AM, Fura Red AM and Cal Red™ R525/650 AM.** CHO-K1cells were seeded overnight in 50,000 cells

per 100 µL per well in a 96-well black wall/clear bottom costar plate. 100 µL of 5 µM Fura-2 AM, Fura Red AM and Cal Red™ R525/650 AM was added into the cells, and the cells were incubated at 37 °C for 45 minutes and RT for 30 minutes. ATP (50µL/well) was added by FlexStation (Molecular Devices) to achieve the final indicated concentrations.

IV. Use of Calcium indicator Salts

In contrast to single-wavelength indicators such as Fluo-4, the absorption (or fluorescence excitation) maximum of Fura indicators shifts from 380 nm (Fura-2), 415 nm (Fura-8™ and Fura-10™) for the Ca^{2+}-free chelator to about 340 nm (Fura-2), 355 nm (Fura-8™ and Fura-10™) for the Ca^{2+}-bound . The wavelength of maximum fluorescence emission is relatively independent of Ca^{2+} concentration. The largest dynamic range for Ca^{2+}-dependent fluorescence signals is obtained by using excitation at 340 nm and 380 nm (for Fura-2), 355 nm and 415 nm (for Fura-8™ and Fura-10™) and ratioing the fluorescence intensities detected at ~510 nm (Fura-2), 525 nm (Fura-8™ and Fura-10™). From this ratio, the level of intracellular Ca^{2+} can be estimated, using dissociation constants (Kd) that are derived from calibration curves. By using the ratio of fluorescence intensities produced by excitation at two wave lengths, factors such as uneven dye distribution and photo bleaching are minimized because they should affect both measurements to the same extent. Calibration solutions should be initially free of heavy metal ions such as manganese, which may affect both its fluorescence and its affinity for calcium.

Once the indicator has been calibrated with solutions of known Ca^{2+} concentrations (see below), the following equation can be used to relate the intensity ratios to Ca^{2+} levels:

$$[Ca+2] = \mathrm{Kd}\ \mathrm{Q}\frac{(\mathrm{R}-\mathrm{Rmin})}{(\mathrm{Rmin}-\mathrm{R})}$$

Where, R represents the fluorescence intensity ratio $F_{\lambda 1}/F_{\lambda 2}$, in which λ1 (~340 nm for Fura-2, and 355 nm for Fura-8™ and Fura-10™) and λ2 (~380 nm fro Fura-2, and 415 nM for Fura-8™ and Fura-10™) are the fluorescence detection wavelengths for the ion-bound and ion-free indicator, respectively. Ratios corresponding to the titration end points are denoted by the subscripts indicating the minimum and maximum Ca^{2+} concentration. Q is the ratio of Fmin to Fmax at λ2 (~380 nm, for Fura-2, and 415 nM for Fura-8™ and Fura-10™). Kd is the Ca^{2+} dissociation constant of the indicator. Calibrating fura indicators requires making measurements for the completely ion-free and ion-saturated indicator (to determine the values for Fmin, Fmax, Rmin, and Rmax) and for the indicator in the presence of known Ca^{2+} concentrations (to determine Kd).