Long-Wavelength Calcium Indicators

Calcium Green™ • Oregon Green® 488 BAPTA • Calcium Yellow™ • Calcium Orange™ • Calcium Crimson™

Introduction

The long-wavelength calcium indicators Calcium Green™, Oregon Green® 488 BAPTA, Calcium Yellow™, Calcium Orange™, and Calcium Crimson™ are visible light-excitable probes derived from fluorescein, Oregon Green® 488 dye, lucifer yellow, tetramethylrhodamine, and Texas Red®, respectively. Molecular Probes prepares these indicators in the water-soluble potassium salt form, cell-permeant acetoxymethyl (AM) ester form, and in some cases as compartmentalization-resistant dextran conjugates (Table 1). Upon binding to calcium, these indicators exhibit an increase in fluorescence emission intensity with little shift in wavelength (Figure 1). The spectral characteristics of the long-wavelength indicators have three major advantages: 1) Their emissions are in regions of the spectrum where cellular autofluorescence and scattering backgrounds are often less of a problem; 2) The energy of the excitation light is low, reducing the potential for cellular photodamage; and 3) The wavelengths required for optimal excitation are compatible with those produced by laser-based instrumentation, such as confocal laser scanning microscopes. Long-wavelength Ca^{2+} indicators also provide increased options for simultaneous measurements of other physiological parameters, such as pH and membrane potential,[1,2] and for experiments involving photoactivatable "caged" probes.[3]

Although Calcium Green™-1 is structurally similar to fluo-3, it is more fluorescent at low calcium concentrations, facilitating the determination of baseline Ca^{2+} levels and increasing the visibility of resting cells (Figure 1A). Calcium Green™-1 and Oregon Green® 488 BAPTA-1 are currently the indicators of choice for multiphoton excitation imaging of calcium dynamics in brain slice preparations[4] and intact live brains.[5]

In contrast to Calcium Green™-1, Calcium Green™-2 has two fluorescent reporter groups, which are believed to quench one another in the absence of calcium. Calcium Green™-2 undergoes a much larger increase in fluorescence emission upon calcium binding than does Calcium Green™-1 and its lower affinity for calcium makes it particularly suited to measuring relatively high spikes of calcium, up to 25 µM (Table 1 and Figure 1B).

Oregon Green® 488 BAPTA-1 and -2 have generally similar properties to Calcium Green™-1 and -2, respectively. The major difference is that both the fluorescence excitation and emission maxima of the Oregon Green® 488 BAPTA indicators are shorter by ~10 nm (Table 1), resulting in more efficient 488 nm excitation by argon-ion lasers. The extinction coefficient for Oregon Green® 488 BAPTA-1 absorption at 488 nm is ~93% of its maximum value, whereas for Calcium Green™-1 it is only about 45% of maximum.

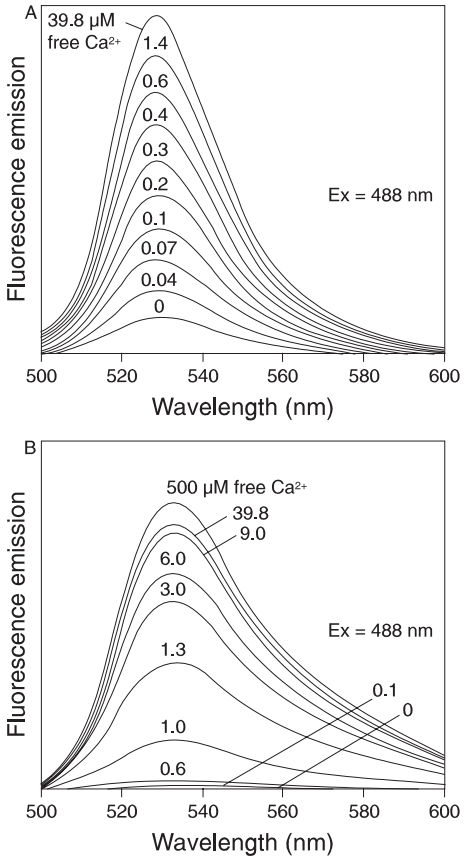

Figure 1. Emission spectra of A) Calcium Green™-1 and B) Calcium Green™-2.

Cell-impermeant Calcium Yellow™ has an exceptionally large Stokes shift, with excitation/emission maxima of ~435/550 nm. Like Calcium Green™-1, Calcium Yellow™ is fluorescent at low Ca^{2+} concentrations. This indicator has a high affinity for Ca^{2+} (K_d = 185 nM) and exhibits an approximately 19-fold increase in fluorescence intensity upon binding calcium in buffer solutions. Calcium Yellow™ is compatible with standard filter sets suitable for lucifer yellow.

Calcium Orange™ and Calcium Crimson™ are spectrally similar to tetramethylrhodamine and Texas Red® (Table 1). The long-wavelength spectral characteristics of these indicators allow them to be used in combination with fluorescein and ultraviolet-excitable dyes.[6] The wavelength separation between the excitation spectrum of Calcium Orange™ and the action spectrum of genetically-altered rhodopsin pigments has enabled researchers to detect and image light-induced Ca^{2+} transients in *Drosophila* photoreceptors.[7]

Table 1. *Spectral characteristics of long-wavelength calcium indicators.*

Indicator	Salt *	AM †	Dextran ‡	Ex/Em § (nm)	F_{Ca}/F_{free} **	K_d ††
Calcium Green™-1	C3010	C3011, C3012	C3713, C3714, C6765, C6766	506/531	~14	190 nM
Calcium Green™-2	C3730	C3732		503/536	~100	550 nM
Calcium Green™-5N	C3737	C3739		506/532	~38	14 µM
Oregon Green® 488 BAPTA-1	O6806	O6807	O6798	494/523	~14	170 nM
Oregon Green® 488 BAPTA-2	O6808	O6809		494/523	~100	580 nM
Oregon Green® 488 BAPTA-6F	O23990	O23991		494/524	~14	3 µM
Oregon Green® 488 BAPTA-5N	O6812	O6813		494/521	~44	20 µM
Calcium Yellow™	C36202			435/550	~19	185 nM
Calcium Orange™	C3013	C3015		549/576	~3	185 nM
Calcium Crimson™		C3018		590/615	~2.5	185 nM

***** Catalog numbers for water-soluble potassium salt forms. **†** Catalog numbers for cell-permeant acetoxymethyl (AM) esters. **‡** Catalog numbers for dextran conjugates. **§** Excitation and Emission maxima for Ca^{2+}–bound indicator. ****** Fluorescence intensity of the Ca^{2+}–bound indicator relative to its Ca^{2+}–free form. All these indicators exhibit fluorescence enhancement on binding Ca^{2+} with essentially no change in excitation or emission wavelengths. **††** Dissociation constant for Ca^{2+} determined *in vitro* at 22°C in 100 mM KCl, 10 mM MOPS, pH 7.2. K_d values depend on temperature, ionic strength, pH, presence of other ions (e.g. Mg^{2+}) and other factors. Values reported are determined using the acid form of the indicator. K_d values for dextran conjugates may vary between production lots; lot-specific values are printed on the vial in most cases.

Calcium Green™-5N, Oregon Green® 488 BAPTA-5N, and Oregon Green® 488 BAPTA-6F have the lowest Ca^{2+} binding affinities in this series of indicators (Table 1) and are not expected to respond to normal changes in intracellular magnesium. These low-affinity indicators can be used to detect high-amplitude calcium transients, such as those evoked by neurotransmitters [8] and IP_3.[9] The high Ca^{2+} dissociation rates of these low-affinity indicators are advantageous for rapid kinetic tracking of Ca^{2+} release.[10-12]

High molecular weight dextran conjugates can be loaded into cells using our Influx™ pinocytic cell-loading reagent (I14402), by direct injection via a recording electrode, by electroporation, or by simply delivering the conjugate just outside cell bodies by pressure or electrophoretic application, which leads to spontaneous uptake.[13, 14] The dextran conjugates show a dramatic reduction in both leakage and compartmentalization—a particular problem for the acidic forms of these probes in plant cells.[15] We offer Calcium Green™-1 conjugated to 3000, 10,000, 70,000, and 500,000 MW dextrans. Our 500,000 MW dextran conjugate of Calcium Green™-1 should be effectively excluded from cellular organelles, including the nucleus.

Materials

These products are provided as lyophilized solids and should be stored at ≤–20ºC, desiccated, and protected from light until use. Allow products to warm to room temperature before opening.

The salts may be reconstituted in aqueous buffers or distilled water; store aqueous stock solutions at ≤–20°C and protected from light. The salts are stable for at least six months in most physiological buffers.

AM esters are susceptible to hydrolysis, particularly in solution. They should be reconstituted just before use in high-quality, anhydrous dimethylsulfoxide (DMSO). DMSO stock solutions may be stored desiccated at ≤–20°C and protected from light. Under these conditions, AM esters should be stable for several months. To prevent repeated exposure to moisture, AM esters are supplied in 500 µg units prealiquoted into ten vials of 50 µg each so that the entire contents do not have to be dissolved at the same time. Dilute aqueous solutions of the AM esters for cell loading should be used on the same day that they are prepared.

Application

The following AM ester loading protocol is provided as an introductory guide only. AM ester loading protocols for a variety of cell types and vesicle preparations can be obtained from the literature.[16]

Suggested AM Ester Loading Protocol

1. Prepare a 2 to 5 mM stock solution of the AM ester in high-quality, anhydrous DMSO, as described in *Materials*; the molecular weight (MW) is indicated on the product label. The nonionic detergent Pluronic® F-127 is sometimes used to increase the aqueous solubility of AM esters. For the convenience of our customers, Molecular Probes offers Pluronic F-127 in three forms: 1 mL of a 20% (w/v) solution in DMSO (P3000MP), 30 mL of a sterile 10% (w/v) solution in water (P6866), and 2 g of the solid (P6867). A 20% Pluronic F-127 solution can be used in place of DMSO to prepare solutions of these calcium indicators; however, long-term storage of AM esters in the presence of Pluronic F-127 is not recommended.

2. On the day of the experiment, either dissolve the lyophilized solid in DMSO or thaw an aliquot of the indicator stock solution to room temperature. Prepare a working solution of 1 to 10 µM in the buffer of choice; the exact concentration of indicator required for cell loading must be determined empirically. To avoid calcium buffering, toxicity and other artifacts of overloading, one should generally use the lowest probe concentration that yields sufficient signal.

3. Incubate cells with the AM ester for 20 minutes to one hour at or below room temperature. Adherent cultures do not need to be lifted for loading. Some investigators report that decreasing the loading temperature reduces indicator compartmentalization. The zwitterionic Calcium Crimson™ has been found to be particularly susceptible to this problem.

4. Wash cells to remove excess probe that either has not been loaded or may be noncovalently associated with the membrane. This step is important when labeling with Calcium Orange™ and Calcium Crimson™, which are fluorescent prior to ester cleavage.

Response Calibration

The excitation (λ_{EX}) and emission (λ_{EM}) wavelengths and calcium responses of these long-wavelength calcium indicators are shown in Table 1. The following bandpass filter sets are recommended for fluorescence microscopy applications:

- Oregon Green® 488 BAPTA indicators: Omega sets XF100 or XF23; Chroma sets 41001 or 31001
- Calcium Green™ indicators: Omega sets XF104 or XF23; Chroma sets 41028 or 31001
- Calcium Yellow™ indicators: Omega sets XF14-2, XF15, XF77-2; Chroma sets 31010 or 31038
- Calcium Orange™ indicators: Omega sets XF108 or XF32; Chroma sets 41002 or 31002
- Calcium Crimson™ indicators: Omega sets XF102 or XF43; Chroma sets 41004 or 31004

Omega® filters are supplied by Omega Optical Inc. (www.omegafilters.com). Chroma filters are supplied by Chroma Technology Corp. (www.chroma.com).

Calibration is typically accomplished using the acid form of the indicator in solutions of precisely known free calcium concentrations, such as those generated using Molecular Probes' Calcium Calibration Buffer Kits (C3008MP, C3009, C3723) and Calcium Calibration Standard Kit (C6775). The Calcium Calibration Buffer Kits, which contain CaEGTA solutions that have been precisely prepared using Roger Tsien's "pH-metric" method,[17] can be used to obtain calibration curves and K_ds for high- to moderate-affinity calcium indicators. In contrast, our Calcium Calibration Standard Kit provides 11 solutions, containing from zero to 1 mM calcium, for calibrating our low-affinity calcium indicators such as Calcium Green™-5N and Oregon Green® 488 BAPTA-5N.

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

$$\left[Ca^{2+}\right]_{free} = K_d \left[\frac{F - F_{min}}{F_{max} - F}\right]$$

where F is the fluorescence of the indicator at experimental calcium levels, F_{min} is the fluorescence in the absence of calcium and F_{max} is the fluorescence of the calcium-saturated probe. The dissociation constant (K_d) is a measure of the affinity of the probe for calcium.

The Ca^{2+} -binding and spectroscopic properties of fluorescent indicators vary quite significantly in cellular environments compared to calibration solutions. *In situ* response calibrations of intracellular indicators typically yield K_d values significantly higher than *in vitro* determinations.[18] *In situ* calibrations are performed by exposing loaded cells to controlled Ca^{2+} buffers in the presence of ionophores such as A-23187 (A1493), 4-bromo A-23187 (B1494) and ionomycin (I24222).[19] Alternatively, cell permeabilization agents such as digitonin or Triton® X-100 can be used to expose the indicator to the controlled Ca^{2+} levels of the extracellular medium.[16] Calcium Green™-1, Oregon Green® 488 BAPTA-1, Calcium Orange™, and Calcium Crimson™ are quite fluorescent at low free calcium concentrations, making visualization of resting cells and determination of F_{min} values easier than with fluo-3.

References

1. Biophys J 68, 739 (1995); **2.** Cell Calcium 19, 337 (1996); **3.** Neuron 15, 755 (1995); **4.** Methods 18, 231 (1999); **5.** Nature 385, 161 (1997); **6.** Methods 18, 204 (1999); **7.** Neuron 13, 837 (1994); **8.** Neurosci Lett 163, 149 (1993); **9.** J Physiol 476, 17 (1994); **10.** Biophys J 70, 222 (1996); **11.** Biophys J 70, 896 (1996); **12.** Biophys J 70, 1006 (1996); **13.** Real-Time Imaging 8, 175 (2002); **14.** Neuron 27, 25 (2000); **15.** J Microscopy 166, 57 (1992); **16.** *Cell Biology: A Laboratory Handbook, Second Edition*, Volume 3, Celis J.E., Ed., Academic Press (1998) pp. 363-374; **17.** Methods Enzymol 172, 230 (1989); **18.** Cell Calcium 28, 213 (2000); **19.** Cell Calcium 21, 233 (1997).

Product List *Current prices may be obtained from our Web site or from our Customer Service Department.*

Cat #	Product Name	Unit Size
C3018	Calcium Crimson™, AM *cell permeant* *special packaging*	10 x 50 µg
C3011MP	Calcium Green™-1, AM *cell permeant*	500 µg
C3012	Calcium Green™-1, AM *cell permeant* *special packaging*	10 x 50 µg
C6765	Calcium Green™-1 dextran, potassium salt, 3000 MW, anionic	5 mg
C3713	Calcium Green™-1 dextran, potassium salt, 10,000 MW, anionic	5 mg
C3714	Calcium Green™-1 dextran, potassium salt, 70,000 MW, anionic	5 mg
C6766	Calcium Green™-1 dextran, potassium salt, 500,000 MW, anionic	5 mg
C3010MP	Calcium Green™-1, hexapotassium salt *cell impermeant*	500 µg
C3732	Calcium Green™-2, AM *cell permeant* *special packaging*	10 x 50 µg
C3730	Calcium Green™-2, octapotassium salt *cell impermeant*	500 µg
C36202	Calcium Yellow™, tetrapotassium salt *cell impermeant*	500 µg
C3739	Calcium Green™-5N, AM *cell permeant* *special packaging*	10 x 50 µg
C3737	Calcium Green™-5N, hexapotassium salt *cell impermeant*	500 µg
C3015	Calcium Orange™, AM *cell permeant* *special packaging*	10 x 50 µg
C3013	Calcium Orange™, tetrapotassium salt *cell impermeant*	500 µg
O6807	Oregon Green® 488 BAPTA-1, AM *cell permeant* *special packaging*	10 x 50 µg
O6798	Oregon Green® 488 BAPTA-1 dextran, potassium salt, 10,000 MW, anionic	5 mg
O6806	Oregon Green® 488 BAPTA-1, hexapotassium salt *cell impermeant*	500 µg
O6809	Oregon Green® 488 BAPTA-2, AM *cell permeant*	10 x 50 µg
O6808	Oregon Green® 488 BAPTA-2, octapotassium salt *cell impermeant*	500 µg
O6813	Oregon Green® 488 BAPTA-5N, AM *cell permeant* *special packaging*	10 x 50 µg
O6812	Oregon Green® 488 BAPTA-5N, hexapotassium salt *cell impermeant*	500 µg
O23990	Oregon Green® 488 BAPTA-6F, hexapotassium salt *cell impermeant*	500 µg
O23991	Oregon Green® 488 BAPTA-6F, AM *cell impermeant**special packaging*	10 x 50 µg

Contact Information

Further information on Molecular Probes products, including product bibliographies, is available from your local distributor or directly from Molecular Probes. Customers in Europe, Africa and the Middle East should contact our office in Paisley, United Kingdom. All others should contact our Technical Service Department in Eugene, Oregon.

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