

Rhod-2 and X-rhod-1 Calcium Indicators

Introduction

Long-wavelength calcium indicators are valuable for experiments in cells and tissues with high levels of autofluorescence,^{1,2} and also for detecting calcium release generated by photoreceptors and photoactivatable chelators.³⁻⁵ Rhod-2, with fluorescence excitation and emission maxima at about 550 nm and 580 nm, respectively, was first introduced in 1989.⁶ More recently, variants with longer-wavelength excitation and emission (X-rhod-1) and lower Ca²⁺-binding affinity (rhod-5N, rhod-FF, etc.) have been developed at Molecular Probes. The AM ester forms of these rhodamine-based indicators are cationic, resulting in potential-driven uptake in mitochondria and a punctate staining pattern when loaded cells are viewed by fluorescence microscopy. This has led to the use of rhod-2 as a selective indicator for mitochondrial Ca²⁺,⁷⁻⁹ although the finding of localized subcellular accumulation, on which the data interpretation is based, is not universally supported.²

Materials

Contents

Rhod-2, rhod-FF, rhod-5N, X-rhod-1, X-rhod-5F, X-rhod-FF and X-rhod-5N are available as cell-permeant acetoxymethyl (AM) esters, and also as water-soluble salts. Rhod-2, AM is supplied either in 1 mg (R-1244) units or in a set of 20 vials containing 50 µg each (R-1245). Rhod-FF, AM, rhod-5N, AM, X-rhod-1, AM, X-rhod-5F, AM, X-rhod-FF, AM and X-rhod-5N, AM are supplied in sets of 10 vials containing 50 µg each (R-14208, R-23983, X-14210, X-23985, X-23987 and X-14212, respectively).

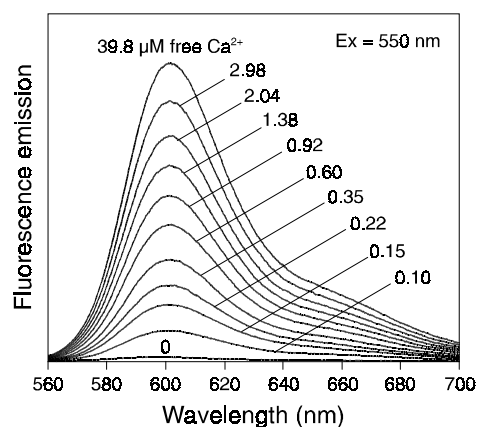


Figure 1. Fluorescence emission spectra of X-rhod-1 in solutions containing zero to 39.8 µM free Ca²⁺.

Table 1. Physical and spectroscopic properties of rhod and X-rhod indicators.

Product Name	Ex/Em Ca ²⁺ -free *	Ex/Em Ca ²⁺ -bound *	ε _{max} †	K _d (Ca ²⁺) ‡
rhod-2	549/none	552/581	82,000	570 nM
X-rhod-1	576/none	580/602	92,000	700 nM
rhod-FF	549/none	552/580	78,000	19 µM
rhod-5N	549/none	551/576	63,000	320 µM
X-rhod-FF	580/none	580/603	80,000	17 µM
X-rhod-5F	580/none	581/603	82,000	1.6 µM
X-rhod-5N	580/none	581/602	78,000	350 µM

* Fluorescence excitation and emission maxima, in nm. † Molar extinction coefficient at absorption maximum for Ca²⁺-bound indicator, in cm⁻¹ M⁻¹. ‡ Dissociation constants (K_d) determined at 22°C in 100 mM KCl, 10 mM MOPS pH 7.2. K_d values depend on pH, temperature, ionic strength and other factors and are usually significantly higher in cellular environments.

Storage

After receipt, the indicator salts can be stored desiccated and protected from light at room temperature, 4°C or -20°C without compromising stability. The acetoxymethyl (AM) esters should be stored desiccated and protected from light at -20°C; AM esters are susceptible to hydrolysis (particularly in solution) but can be stored at least six months in the vials as received. The AM esters should be reconstituted in anhydrous dimethylsulfoxide (DMSO) then used as soon as possible thereafter (within a week) to avoid decomposition with subsequent loss of cell loading capacity. Stock solutions of AM esters should be stored in the dark, frozen and desiccated. Stock solutions of the salts may be prepared in distilled water or aqueous buffers and stored frozen (-20°C) and protected from light; these solutions should be stable for at least six months.

To check for possible AM ester degradation, the following simple test may be performed in a fluorometer. Dilute a small aliquot of AM ester stock solution to a final concentration of about 1 µM in calcium-free buffer. Transfer the solution to a cuvette and measure the fluorescence intensity using appropriate wavelength settings (excitation at 550 nm, emission at 580 nm for rhod-2 and its derivatives; excitation at 575 nm, emission at 605 nm for X-rhod-1 and its derivatives). Add calcium to a saturating concentration (≥5 µM for rhod-2 and X-rhod-1; ≥1 mM for rhod-FF, X-rhod-5F and X-rhod-FF; ≥10 mM for rhod-5N and X-rhod-5N) and check fluorescence again. There should be

no significant change in fluorescence between the two readings (**Note:** Both intensity readings should be very low). Significantly increased fluorescence upon calcium addition (i.e., in the second reading) indicates partial hydrolysis of the AM ester.

Properties

Rhod-2, X-rhod-1 and their derivatives all exhibit large fluorescence intensity increases upon binding Ca^{2+} (Figure 1). The fluorescence intensity increase upon Ca^{2+} binding is typically >100-fold. Unlike the ultraviolet-excited indicators fura-2 and indo-1, there is no accompanying spectral shift. A summary of physical and spectroscopic properties is shown in Table 1.

Applications

Cell Loading Guidelines

The water-soluble salt forms of Ca^{2+} indicators may be loaded into cells by microinjection,¹⁰ infusion from a patch-pipette or using our Influx™ pinocytotic cell-loading reagent (I-14402). For loading using cell-permeant AM esters, the following protocols are provided as an introductory guide only; more detailed published procedures can be found elsewhere.^{11,12} Chemical reduction of rhod-2, AM with sodium borohydride prior to loading has been found to enhance mitochondrial localization of the indicator.⁹ A separate technical bulletin describing this procedure is available (Product Information Sheet X-1244).

1.1 Dilute an aliquot of DMSO stock solution (1–5 mM) to a final concentration of 1–5 μM in the buffered physiological medium of choice. Addition of the non-ionic detergent Pluronic® F-127 can assist in dispersion of the nonpolar AM ester in aqueous media. This can be conveniently accomplished by mixing the aliquot of AM ester stock solution in DMSO with an equal volume of 20% (w/v) Pluronic in DMSO (P-3000) before dilution into the loading medium, making the final Pluronic concentration about 0.02%. Molecular Probes also offers Pluronic F-127 in 30 mL units of a sterile 10% (w/v) solution in water (P-6866) and 2 g solid units (P-6867).

1.2. Cells are normally incubated with the AM ester for 15–60 minutes at 20–37°C (note **A**). Exact loading concentration, time and temperature will need to be determined empirically; in general it is desirable to use the minimum dye concentration required to yield adequate fluorescence signal to noise levels.

1.3 Before fluorescence measurements are commenced, cells should be washed in indicator-free medium to remove any dye that is nonspecifically associated with the cell surface, and then incubated for a further 30 minutes to allow complete de-esterification of intracellular AM esters. Prolonged incubation (12–24 hours) after washing eliminates cytosolic staining produced by rhod-2, AM, whereas mitochondrial staining is retained.⁹

Response Calibration

Fluorescence excitation and emission wavelength maxima are shown in Table 1. The following bandpass filter sets are recommended for use in fluorescence microscopy applications:

- rhod-2, rhod-FF and rhod-5N: Omega XF108 or XF32, Chroma 41002 or 31002

- X-rhod-1 X-rhod-5F, X-rhod-FF and X-rhod-5N: Omega XF102 or XF43; Chroma 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).

Response calibration can be carried out by measuring the fluorescence intensity of the tetracarboxylate form of the indicator in solutions with precisely known free Ca^{2+} concentrations. Solutions based on EGTA Ca^{2+} buffering^{13,14} suitable for calibrating rhod-2 and X-rhod-1 are supplied in a variety of convenient formats in Molecular Probes' Calcium Calibration Buffer Kits. Calibration of the ultra-low affinity indicators rhod-5N and X-rhod-5N requires a free Ca^{2+} concentration range from about 10 μM to 10 mM. The following equation is used to determine the ion dissociation constant (K_d):

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

where F_{min} is the fluorescence intensity of the indicator in the absence of calcium, F_{max} is the fluorescence of the calcium-saturated indicator and F is the fluorescence at intermediate calcium levels. When K_d is known, the same equation is used to obtain $[\text{Ca}^{2+}]_{\text{free}}$ for experimental samples from measured values of F .

It is important to recognize that the calcium-binding and spectroscopic properties of fluorescent indicators can vary quite markedly in cellular environments. In addition, BAPTA-based indicators such as rhod-2 and X-rhod-1 bind various heavy metal cations (e.g., Mn^{2+} , Zn^{2+} , Pb^{2+}) with substantially higher affinity than Ca^{2+} . Perturbations to calcium measurements caused by presence of these ions can be controlled using the heavy metal-selective chelator TPEN (T-1210).¹⁵ *In situ* response calibrations of intracellular indicators typically yield K_d values significantly higher than *in vitro* determinations.² *In situ* calibrations are performed by exposing loaded cells to controlled Ca^{2+} buffers in the presence of ionophores such as A-23187 (A-1493), 4-bromo A-23187 (B-1494) or ionomycin (I-24222).¹⁶ An alternative method is to saturate the intracellular indicator with Mn^{2+} by adding 2 mM Mn^{2+} to the extracellular medium in the presence of ionophore.⁸ F_{min} and F_{max} are then calculated from their known numerical relationships to the fluorescence intensity of the Mn^{2+} -saturated indicator (F_{Mn}) (note **B**).

Quantitative Ca^{2+} measurements using rhod-2 and X-rhod-1 are hampered if there are significant Ca^{2+} -independent fluorescence intensity fluctuations from cell to cell due to variations in the intracellular indicator concentration. The absence of a spectral shift upon Ca^{2+} binding precludes elimination of these fluctuations by the use of ratiometric detection techniques.¹⁷ Variations in Ca^{2+} as a function of time can be calibrated relative to the basal (pre-stimulus) Ca^{2+} concentration determined from independent measurements.¹⁸

Notes

[A] Adherent cultures do not need to be lifted for loading.

[B] Published values for rhod-2 (determined *in vitro*) are $F_{\text{Mn}}/F_{\text{max}} = 0.24$ and $F_{\text{max}}/F_{\text{min}} = 15$.⁸ Values of $F_{\text{max}}/F_{\text{min}} > 100$ are typically obtained in quality control analysis at Molecular Probes.

References

1. J Physiol 507, 405 (1998); 2. Am J Physiol 274, H728 (1998); 3. J Neurosci 17, 1701 (1997); 4. Am J Physiol 266, C1291 (1994); 5. Neuron 13, 837 (1994); 6. J Biol Chem 264, 8171 (1989); 7. J Cell Biol 137, 633 (1997); 8. J Cell Biol 136, 833 (1997); 9. Cell 82, 415 (1995); 10. J Physiol 433, 207 (1991); 11. Methods Cell Biol 40, 155 (1994); 12. *Cell Biology: A Laboratory Handbook, 2nd Edition*, J.E. Celis, Ed., Volume 3, pp 363–374, Academic Press (1998); 13. Methods Cell Biol 40, 3 (1994); 14. Methods Enzymol 172, 230 (1989); 15. Biophys J 71, 1048 (1996); 16. Cell Calcium 21, 233 (1997); 17. Methods Cell Biol 30, 157 (1989); 18. Biophys J 67, 1942 (1994).

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

Cat #	Product Name	Unit Size
A-1493	A-23187 free acid (calcimycin)	10 mg
B-1494	4-bromo A-23187, free acid	1 mg
C-3723	Calcium Calibration Buffer Concentrate Kit *zero and 100 mM CaEGTA (2 x 5 mL)*	1 kit
C-3008	Calcium Calibration Buffer Kit #1 *zero and 10 mM CaEGTA (2 x 50 mL)*	1 kit
C-3009	Calcium Calibration Buffer Kit #2 *zero to 10 mM CaEGTA (11 x 10 mL)*	1 kit
C-3721	Calcium Calibration Buffer Kit with Magnesium #1 *zero and 10 mM CaEGTA with 1 mM Mg ²⁺ (2 x 50 mL)*	1 kit
C-3722	Calcium Calibration Buffer Kit with Magnesium #2 *zero to 10 mM CaEGTA with 1 mM Mg ²⁺ (11 x 10 mL)*	1 kit
I-14402	Influx™ pinocytotic cell-loading reagent *makes 10 x 5 mL*	1 set
I-24222	ionomycin, calcium salt	1 mg
P-6867	Pluronic® F-127 *low UV absorbance*	2 g
P-3000	Pluronic® F-127 *20% solution in DMSO*	1 mL
P-6866	Pluronic® F-127 *sterile 10% solution in water*	30 mL
R-1244	rhod-2, AM *cell permeant*	1 mg
R-1245	rhod-2, AM *cell permeant* *special packaging*	20 x 50 µg
R-14220	rhod-2, tripotassium salt *cell impermeant*	1 mg
R-14208	rhod-5N, AM *cell permeant* *special packaging*	10 x 50 µg
R-14207	rhod-5N, tripotassium salt *cell impermeant*	500 µg
R-23983	rhod-FF, AM *cell permeant* *special packaging*	10 x 50 µg
R-23982	rhod-FF, tripotassium salt *cell impermeant*	500 µg
T-1210	tetrakis-(2-pyridylmethyl)ethylenediamine (TPEN)	100 mg
X-14210	X-rhod-1, AM *cell permeant* *special packaging*	10 x 50 µg
X-14209	X-rhod-1, tripotassium salt *cell impermeant*	500 µg
X-23985	X-rhod-5F, AM *cell permeant* *special packaging*	10 x 50 µg
X-23984	X-rhod-5F, tripotassium salt *cell impermeant*	500 µg
X-14212	X-rhod-5N, AM *cell permeant* *special packaging*	10 x 50 µg
X-14211	X-rhod-5N, tripotassium salt *cell impermeant*	500 µg
X-23987	X-rhod-FF, AM *cell permeant* *special packaging*	10 x 50 µg
X-23986	X-rhod-FF, tripotassium salt *cell impermeant*	500 µg

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