

## Rhod Calcium Indicators

### Quick Facts

#### Storage upon receipt:

- $\leq -20^{\circ}\text{C}$
- Desiccate
- Protect from light

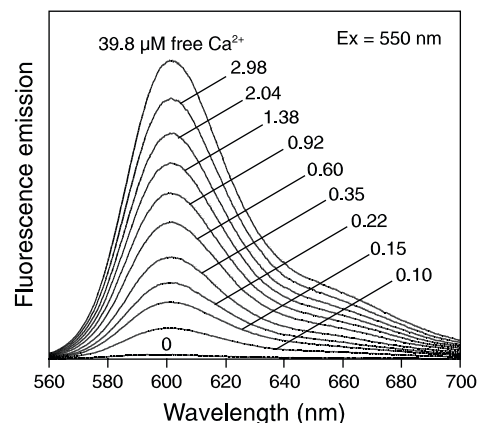
### Introduction

Long-wavelength calcium indicators are valuable for experiments in cells and tissues with high levels of autofluorescence,<sup>1,2</sup> and also for detecting calcium release generated by photoreceptors and photoactivatable chelators.<sup>3-5</sup> Rhod-2, with fluorescence excitation and emission maxima at 552 nm and 581 nm, respectively, was first introduced in 1989.<sup>6</sup> More recently, variants with longer-wavelength excitation and emission (X-rhod-1) and lower  $\text{Ca}^{2+}$ -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 into 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  $\text{Ca}^{2+}$ ;<sup>7-9</sup> however, the finding of localized subcellular accumulation on which the data interpretation is based is not universally supported.<sup>2</sup> The rhod dextrans consist of rhod indicators coupled to a dextran carrier (MW = 10,000), these conjugates will not leak out of cells and therefore are suitable for longer-term calcium measurements.

### Materials

#### Contents

Rhod-2, rhod-5N, X-rhod-1, X-rhod-5F, X-rhod-FF and X-rhod-5N are available as cell-permeant acetoxymethyl (AM) esters and as water-soluble salts. Rhod-FF is only available as a cell-permeant AM ester. Rhod-2, AM is supplied either in 1 mg (R1244) units or in a set of 20 vials containing 50  $\mu\text{g}$  each (R1245). 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  $\mu\text{g}$  each (R14208, R23983, X14210, X23985, X23987 and X14212, respectively). The rhod dextrans are supplied in solid form in 5 mg units.



**Figure 1.** Fluorescence emission spectra of X-rhod-1 in solutions containing zero to 39.8  $\mu\text{M}$  free  $\text{Ca}^{2+}$ .

### Storage

After receipt, the indicator salts should be stored desiccated and protected from light at  $\leq -20^{\circ}\text{C}$ . The acetoxymethyl (AM) esters should be stored desiccated and protected from light at  $\leq -20^{\circ}\text{C}$ ; 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 ( $\leq -20^{\circ}\text{C}$ ) 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  $\mu\text{M}$  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 ( $\geq 5 \mu\text{M}$  for rhod-2 and X-rhod-1;  $\geq 1 \text{ mM}$  for rhod-FF, X-rhod-5F and X-rhod-FF;  $\geq 10 \text{ mM}$  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.

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

Product Name	Ex/Em Ca <sup>2+</sup> -free*	Ex/Em Ca <sup>2+</sup> -bound*	e <sub>max</sub> †	K <sub>d</sub> (Ca <sup>2+</sup> ) ‡
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 Ca<sup>2+</sup>-bound indicator, in cm<sup>-1</sup>M<sup>-1</sup>.

‡ Dissociation constants (K<sub>d</sub>) determined at 22°C in 100 mM KCl, 10 mM MOPS, pH 7.2. K<sub>d</sub> values depend on pH, temperature, ionic strength and other factors and are usually significantly higher in cellular environments.

## Properties

Rhod-2, X-rhod-1 and their derivatives all exhibit large fluorescence intensity increases upon binding Ca<sup>2+</sup> (Figure 1). The fluorescence intensity increase upon Ca<sup>2+</sup> binding is typically >100-fold. Unlike the UV-excitable indicators fura-2 and indo-1, there is no accompanying spectral shift. A summary of physical and spectroscopic properties is shown in Table 1. The high and low affinity versions of the rhod dextrans have a K<sub>d</sub> of (Ca<sup>2+</sup>) of ~750 nM and 5.2 μM, respectively. Both conjugates have Ex/Em maxima of 530/576 nm when bound to Ca<sup>2+</sup>.

## Applications

### Cell Loading Guidelines

The water-soluble salt forms of Ca<sup>2+</sup> indicators may be loaded into cells by microinjection,<sup>10</sup> infusion from a patch-pipette or using our Influx™ pinocytotic cell-loading reagent (I14402). The rhod dextrans can be dissolved in water at concentrations up to 20% (w/v). 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.<sup>11,12</sup> Chemical reduction of rhod-2, AM with sodium borohydride prior to loading has been found to enhance mitochondrial localization of the indicator.<sup>9</sup> A separate technical bulletin describing this procedure is available (Product Information Sheet X1244).

**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 (P3000MP) before dilution in to 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 (P6866) and 2 g solid units (P6867).

**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.<sup>9</sup>

### 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](http://www.omegafilters.com)). Chroma filters are supplied by Chroma Technology Corp. ([www.chroma.com](http://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<sup>2+</sup> concentrations. Solutions based on EGTA Ca<sup>2+</sup> buffering<sup>13,14</sup> 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<sup>2+</sup> concentration range from about 10 μM to 10 mM. The following equation is used to determine the ion dissociation constant (K<sub>d</sub>):

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

where F<sub>min</sub> is the fluorescence intensity of the indicator in the absence of calcium, F<sub>max</sub> is the fluorescence of the calcium-saturated indicator and F is the fluorescence at intermediate calcium levels. When K<sub>d</sub> is known, the same equation is used to obtain [Ca<sup>2+</sup>]<sub>free</sub> 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<sup>2+</sup>, Zn<sup>2+</sup>, Pb<sup>2+</sup>) with substantially higher affinity than Ca<sup>2+</sup>. Perturbations to calcium measurements caused by presence of these ions can be controlled using the heavy metal-selective chelator TPEN (T1210).<sup>15</sup> *In situ* response calibrations of intracellular indicators typically yield K<sub>d</sub> values significantly higher than *in vitro* determinations.<sup>2</sup> *In situ* calibrations are performed by

exposing loaded cells to controlled Ca<sup>2+</sup> buffers in the presence of ionophores such as A23187 (A1493), 4-bromo A23187 (B1494) or ionomycin (I24222).<sup>16</sup> An alternative method is to saturate the intracellular indicator with Mn<sup>2+</sup> by adding 2 mM Mn<sup>2+</sup> to the extracellular medium in the presence of ionophore.<sup>8</sup> F<sub>min</sub> and F<sub>max</sub> are then calculated from their known numerical relationships to the fluorescence intensity of the Mn<sup>2+</sup>-saturated indicator (F<sub>Mn</sub>) (note **B**).

Quantitative Ca<sup>2+</sup> measurements using rhod-2 and X-rhod-1 are hampered if there are significant Ca<sup>2+</sup>-independent fluorescence intensity fluctuations from cell to cell due to variations in the intracellular indicator concentration. The absence of a spectral shift upon Ca<sup>2+</sup> binding precludes

elimination of these fluctuations by the use of ratiometric detection techniques.<sup>17</sup> Variations in Ca<sup>2+</sup> as a function of time can be calibrated relative to the basal (pre-stimulus) Ca<sup>2+</sup> concentration determined from independent measurements.<sup>18</sup>

## Notes

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

[B] Published values for rhod-2 (determined *in vitro*) are F<sub>Mn</sub>/F<sub>max</sub> = 0.24 and F<sub>max</sub>/F<sub>min</sub> = 15.<sup>8</sup> Values of F<sub>max</sub>/F<sub>min</sub> >100 are typically obtained in quality control analysis at Molecular Probes.

## References

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

Cat #	Product Name	Unit Size
A1493	A-23187 free acid (calcimycin).....	10 mg
B1494	4-bromo A-23187, free acid.....	1 mg
C3008MP	Calcium Calibration Buffer Kit #1 *zero and 10 mM CaEGTA (2 x 50 mL)*.....	1 kit
C3009	Calcium Calibration Buffer Kit #2 *zero to 10 mM CaEGTA (11 x 10 mL)*.....	1 kit
C3721	Calcium Calibration Buffer Kit with Magnesium #1 *zero and 10 mM CaEGTA with 1 mM Mg <sup>2+</sup> (2 x 50 mL)*.....	1 kit
C3722	Calcium Calibration Buffer Kit with Magnesium #2 *zero to 10 mM CaEGTA with 1 mM Mg <sup>2+</sup> (11 x 10 mL)*.....	1 kit
C3723	Calcium Calibration Buffer Concentrate Kit *zero and 100 mM CaEGTA (2 x 5 mL)*.....	1 kit
I14402	Influx™ pinocytic cell-loading reagent *makes 10 x 5 mL*.....	1 set
I24222	ionomycin, calcium salt.....	1 mg
P3000MP	Pluronic® F-127 *20% solution in DMSO*.....	1 mL
P6866	Pluronic® F-127 *10% solution in water* *0.2 µm filtered*.....	30 mL
P6867	Pluronic® F-127 *low UV absorbance*.....	2 g
R1244	rhod-2, AM *cell permeant*.....	1 mg
R1245MP	rhod-2, AM *cell permeant* *special packaging*.....	20 x 50 µg
R14207	rhod-5N, tripotassium salt *cell impermeant*.....	500 µg
R14208	rhod-5N, AM *cell permeant* *special packaging*.....	10 x 50 µg
R14220	rhod-2, tripotassium salt *cell impermeant*.....	1 mg
R23983	rhod-FF, AM *cell permeant* *special packaging*.....	10 x 50 µg
R34676	rhod dextran, potassium salt, 10,000 MW, anionic (high-affinity version).....	5 mg
R34677	rhod dextran, potassium salt, 10,000 MW, anionic (low-affinity version).....	5 mg
T1210	tetrakis-(2-pyridylmethyl)ethylenediamine (TPEN).....	100 mg
X14209	X-rhod-1, tripotassium salt *cell impermeant*.....	500 µg
X14210	X-rhod-1, AM *cell permeant* *special packaging*.....	10 x 50 µg
X14212	X-rhod-5N, AM *cell permeant* *special packaging*.....	10 x 50 µg
X23984	X-rhod-5F, tripotassium salt *cell impermeant*.....	500 µg
X23985	X-rhod-5F, AM *cell permeant* *special packaging*.....	10 x 50 µg
X23987	X-rhod-FF, AM *cell permeant* *special packaging*.....	10 x 50 µg

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