

20.1 Introduction to Ca^{2+} Measurements with Fluorescent Indicators

Fluorescent probes that show a spectral response upon binding Ca^{2+} have enabled researchers to investigate changes in intracellular free Ca^{2+} concentrations using fluorescence microscopy, flow cytometry and fluorescence spectroscopy.^{1–6} These fluorescent indicators, most of which are derivatives of the Ca^{2+} chelators EGTA, APTRA and BAPTA,⁷ have evolved largely through the efforts of Roger Tsien and his colleagues and, more recently, through those of scientists at Molecular Probes.

Selection Criteria for Fluorescent Ca^{2+} Indicators

Molecular Probes offers the widest available selection of fluorescent Ca^{2+} indicators for detecting changes in intracellular Ca^{2+} over the range of <50 nM to >50 μM (Table 20.1). We and our distributors are the primary suppliers of fura-2, indo-1, fluo-3 (Figure 20.1) and rhod-2, and we exclusively offer a number of other indicators for intracellular Ca^{2+} . Our fura-4F, fura-5F, fura-6F and fura-FF indicators provide increased response sensitivity to intracellular Ca^{2+} concentration in the 0.5–5 μM range compared to fura-2. The fluo-3, fluo-4, Oregon Green 488 BAPTA, Calcium Green, X-rhod-1 and Fura Red indicators and their variants allow Ca^{2+} detection over a wide concentration range and offer increased brightness and reduced phototoxicity. We also offer indicators that are conjugated to high- or low-molecular weight dextrans for improved cellular retention and less compartmentalization, as well as lipophilic Ca^{2+} indicators for possible use in studying near-membrane Ca^{2+} (Section 20.4). Molecular Probes strives to provide the highest-purity indicators available anywhere. The AM ester forms of most of our indicators are typically at least 95% pure by HPLC analysis, although purity often exceeds 98%. Furthermore, the AM esters of many of the Ca^{2+} and Mg^{2+} indicators are available in special packaging for more convenient handling and for reduced risk of deterioration during storage. Fluo-3 AM and fluo-4 AM, both of which are extensively used for high-throughput screening for new drug candidates, are available specially packaged at a discounted price (F-14242, F-14202; Section 20.3).

A number of factors should be considered when selecting a fluorescent Ca^{2+} indicator, some of which are summarized in Table 20.1 and include the following:

- **Indicator form** (salt, AM ester or dextran conjugate), which influences the cell-loading method and affects the indicator's intracellular distribution and retention. The salt and dextran forms are typically loaded by microinjection, electroporation, infusion from a patch-pipette or by using our Influx pinocytotic cell-loading reagent (I-14402, Section 20.8). 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. For a discussion of ratiometric methods and AM ester loading, see the technical note Loading and Calibration of Intracellular Ion Indicators.
- **Measurement mode**, which is dictated by whether qualitative or quantitative ion concentration data are required. 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.
- **Dissociation constant** (K_d), which must be compatible with the Ca^{2+} concentration range of interest. Indicators have a detectable response in the concentration range from approximately $0.1 \times K_d$ to $10 \times K_d$. For ratiometric indicators, the Ca^{2+} response range is also somewhat dependent on the measurement wavelengths used.^{8,9} The K_d of Ca^{2+} indicators is dependent on many factors, including pH, temperature,^{10–12} ionic strength, viscosity, protein binding and the presence of Mg^{2+} and other ions. Consequently, K_d values for intracellular indicators are usually significantly higher than corresponding values measured in cell-free solutions (Table 20.2).

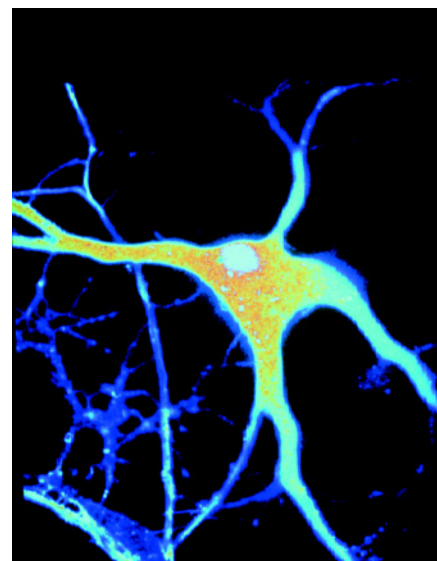


Figure 20.1 A pyramidal neuron from rat hippocampus was first exposed to Alzheimer's β -amyloid peptide and then to the excitatory amino acid glutamate. Confocal laser-scanning microscopy imaging using the intracellular Ca^{2+} indicator fluo-3 (F-1241, F-1242, F-14218, F-14242, F-23915) shows that β -amyloid peptide destabilizes the neuron's calcium homeostasis and increases its vulnerability to excitotoxicity. Image contributed by Mark P. Mattson, Sanders-Brown Center on Aging, University of Kentucky.

Ion Indicators from Molecular Probes

Molecular Probes is the world's principal source of fluorescent ion indicators for Ca^{2+} , Mg^{2+} , Zn^{2+} , metals, Na^+ , K^+ pH and several anions, including Cl^- . Many of these indicators have been developed internally and are available exclusively from Molecular Probes and its distributors. As part of our ISO certification process, we have established specifications for all of our products and perform regular recertifications for our products. Our Fluoro-Pure-grade reagents (see the list and specifications in Section 20.3) include guaranteed high purity versions of fura-2 AM, fluo-3 AM and fluo-4 AM. Generous discounts are available on our indicators for high-throughput screening applications (please contact custom@probes.com for further information).

TECHNICAL NOTE

Loading and Calibration of Intracellular Ion Indicators

There are two major prerequisites for measuring intracellular ion concentrations using fluorescent indicators:

Loading: The indicator must be localized in the region (most commonly the cytosol but sometimes the mitochondria) where the ion concentration is to be measured.

Calibration: The fluorescence of the indicator must be quantitatively related to the concentration of the free ion.

This technical note focuses on these prerequisites. Further information on the practical aspects of ion measurements using fluorescent indicators can be found in several reviews.^{1–3}

Loading

Cell loading methods can be divided into two groups. Bulk loading procedures are applicable to large populations of cells and include:

- Acetoxymethyl (AM) ester loading^{5,9}
- Acid loading^{10,11} (particularly applicable to plant cells)
- ATP-induced permeabilization¹²
- Cationic liposome delivery¹³
- Electroporation¹⁴
- Hypoosmotic shock
- Our Influx pinocytic cell-loading reagent (I-14402, Section 20.8)

Procedures such as microinjection¹⁵ and infusion from patch-pipettes^{16,17} must be carried out one cell at a time. Reviews of some of these techniques have been published;^{18,19} see also Table 14.1.

The AM Ester Loading Technique

The noninvasive and technically straightforward AM ester technique is by far the most popular method for loading fluorescent ion indicators (Figure 1). The carboxylate groups of indicators for Ca^{2+} and other cations and the phenolic hydroxyl groups of pH indicators are derivatized as acetoxymethyl or acetate esters, respectively, rendering the indicator permeant to membranes and insensitive to ions. Once inside the cell, these derivatized indicators are hydrolyzed by ubiquitous intracellular esterases, releasing the ion-sensitive polyanionic indicator.

In practice, a 1–10 mM stock solution of the ester probe in anhydrous dimethylsulfoxide (DMSO) is prepared and divided into appropriately sized aliquots that can be stored desiccated at -20°C . This procedure will curtail the spontaneous ester hydrolysis that can occur in moist environments. Before loading, the DMSO stock solution should be diluted at least 1:200 in serum-free culture medium to a final concentration of about 1–10 μM . The nonionic and nondenaturing detergent Pluronic F-127 (P-3000, P-6866, P-6867; Section 20.8) is frequently added to help disperse the indicator in the loading medium.⁵ After incubation at $20\text{--}37^{\circ}\text{C}$ for 15–60 minutes, the cells should be washed two to three times with fresh serum-free culture medium (serum may contain esterase activity). The loading medium should also be free of amino acids or buffers containing primary or secondary amines because aliphatic amines may cleave the AM esters and prevent loading. The overall loading efficiency is typically 10–40%, depending on the molecular structure of the indicator, the type of cells and the incubation conditions.

Problems with AM Ester Loading

Compartmentalization: For calibration purposes (see below), it is usually assumed that fluorescent indicators are homogeneously distributed in the cytosol and equally responsive to variations of intracellular ion concentration. However, AM esters and their hydrolysis products are capable of accumulating in any membrane-enclosed structure within the cell. In addition, indicators in poly-anionic form may be sequestered within organelles via active transport processes.²⁰ Compartmentalization is usually more pronounced at higher loading temperatures and is particularly acute in plant and fungal cells.^{21,22} The extent of compartmentalization can be assessed by image analysis, as well as fluorometrically using membrane permeabilization reagents, such as Triton X-100.⁵

Incomplete AM ester hydrolysis: Residual unhydrolyzed AM esters may be present extracellularly due to incomplete removal by washing. Inside the cell, low levels of intracellular esterase activity, which can vary considerably from one cell type to another, may produce only partial AM ester hydrolysis.^{23–25} Because even partially hydrolyzed AM esters are Ca^{2+} -insensitive, detection of their fluorescence as part of the total signal leads to an underestimation of the Ca^{2+} concentration.^{26,27} Fluorescence quenching by Mn^{2+} , which only binds to completely de-esterified indicators, can be used to quantitate these effects. Note that although some indicators are fluorescent in the AM ester form, others are not (Table 20.1).

Leakage: Extrusion of anionic indicators from cells by organic ion transporters can be reduced by cooling the sample or by applying inhibitors such as probenecid and sulfipyrazone.²⁰ AM esters are extruded by the P-glycoprotein multidrug transporter²⁸ (Section 15.6). Ratiometric measurements (see below) help to minimize the impact of indicator leakage on experimental data.

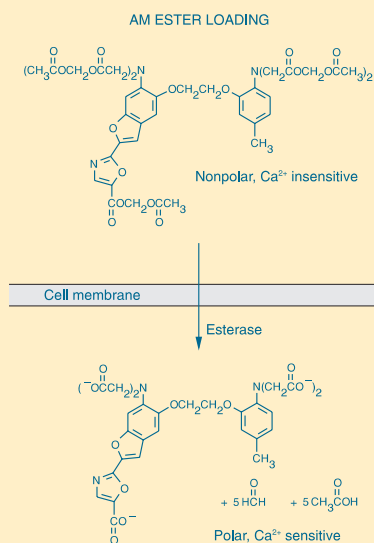


Figure 1 Schematic diagram of the processes involved in loading cells using membrane-permeant acetoxymethyl (AM) ester derivatives of fluorescent indicators, in this case fura-2. Note the generation of potentially toxic by-products (formaldehyde and acetic acid).

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Calibration

Ion Dissociation Constants

The dissociation constant (K_d) is the key conversion parameter linking fluorescence signals to ion concentrations. For pH indicators, K_d is conventionally expressed as its negative log (pK_a). The concentration range over which an indicator produces an observable response is approximately $0.1 \times K_d$ to $10 \times K_d$. For ratiometric measurements, the response range also depends on wavelength-dependent parameters.^{29,30} For BAPTA-based Ca^{2+} indicators in particular, the K_d is very sensitive to a number of environmental factors, including temperature, pH, ionic strength and interactions of the indicator with proteins.^{31–34} Examination of published data shows that values of K_d determined *in situ* within cells can be up to fivefold higher than values determined *in vitro*^{31,35–37} (Table 20.2), underscoring the importance of performing calibrations to determine K_d directly in the system under study.

Calibration Methodology

Calibration procedures basically consist of recording fluorescence signals corresponding to a series of precisely manipulated ion concentrations. The resulting sigmoidal titration curve is either linearized by means of a Hill plot or analyzed directly by nonlinear regression to yield K_d . For *in vitro* calibrations of Ca^{2+} indicators, EGTA buffering is widely used to produce defined Ca^{2+} concentrations that can be calculated from the K_d of the Ca^{2+} -EGTA complex.^{5,7,38} This technique is used in Molecular Probes' Calcium Calibration Buffer Kits (Section 20.8). *In situ* calibrations of intracellular indicators generally utilize an ionophore to equilibrate the controlled external ion concentration with the ion concentration within the cell.^{4,39} Commonly used ionophores include:

- A-23187 (A-1493), 4-bromo A-23187 (B-1494) or ionomycin (I-24222) for Ca^{2+} and Mg^{2+} (see Section 20.8)
- Pyrithione (P-24193, Section 20.8) for Zn^{2+}
- Nigericin (N-1495; Section 21.2, Section 22.2) for H^+ and Cl^-
- Gramicidin (G-6888, Section 22.1) for Na^+
- Valinomycin (V-1644, Section 22.1) for K^+

Ratiometric Calibration

Indicators that show an excitation or emission spectral shift upon ion binding can be calibrated using a ratio of the fluorescence intensities measured at two different wavelengths, resulting in the cancellation of artifactual variations in the fluorescence signal that might otherwise be misinterpreted as changes in ion concentration (Figure 2). Note that background levels must be subtracted from the component fluorescence intensities before calculation of the ratio. Examples of indicators exhibiting ion-dependent spectral shifts can be found in Figure 20.3, Figure 20.4, Figure 21.11 and Figure 21.3. The ratio of two intensities with opposite ion-sensitive responses (for example, 340 nm/380 nm in Figure 20.3) gives the largest possible dynamic range of ratio signals for a particular indicator. Alternatively, the ratio of an ion-sensitive intensity to an ion-insensitive intensity (measured at a spectral isosbestic point; e.g., 360 nm in Figure 20.3) can be used (Figure 2). Ratiometric measurements reduce or eliminate variations of several determining factors in the measured fluorescence intensity, including indicator concentration, excitation pathlength, excitation intensity and detection efficiency.^{40,41} Artifacts

that are eliminated include photobleaching and leakage of the indicator, variable cell thickness, and nonuniform indicator distribution within cells (due to compartmentalization) or among populations of cells (due to loading efficacy variations).

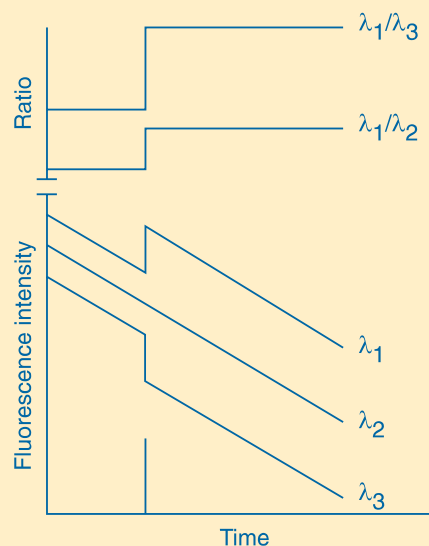


Figure 2 Simulated data demonstrating the practical importance of ratiometric fluorescence techniques. The figure represents an ion indicator that exhibits a fluorescence intensity increase in response to ion binding at wavelength λ_1 and a corresponding decrease at λ_3 . Fluorescence measured at an isosbestic point (λ_2) is independent of ion concentration. The intracellular indicator concentration diminishes rapidly due to photobleaching, leakage (assuming the extracellular indicator is not detectable) or some other process. The change of intracellular ion concentration due to a stimulus applied at the time indicated by the arrow is unambiguously identified by recording the fluorescence intensity ratios λ_1/λ_3 or λ_1/λ_2 .

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37. *Biophys J* 65, 865 (1993);
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Table 20.1 Summary of fluorescent Ca²⁺ indicators available from Molecular Probes.

Ca ²⁺ Indicator	Salt *	AM Ester †	Dextran ‡	Mode §	K _d (nM) **	Notes
Bis-fura	B-6810			Ex 340/380	370	1
BTC	B-6790	B-6791		Ex 400/480	7000	2
Calcium Green-1	C-3010	C-3011, C-3012	C-6765, C-3713, C-3714	Em 530	190	3,4
Calcium Green-2	C-3730	C-3732		Em 535	550	3,5
Calcium Green-5N	C-3737	C-3739		Em 530	14,000	3
Calcium Orange	C-3013	C-3015		Em 575	185	2
Calcium Crimson		C-3018		Em 615	185	2
Fluo-3	F-1240, F-3715	F-1241, F-1242, F-14218, F-14242, F-23915		Em 525	390	3,4
Fluo-4	F-14200	F-14201, F-14202, F-14217, F-23917	F-14240	Em 520	345	3, 6
Fluo-5F	F-14221	F-14222		Em 520	2300	3
Fluo-4FF	F-23980	F-23981		Em 520	9700	3
Fluo-5N	F-14203	F-14204		Em 520	90,000	3
Fura-2	F-1200, F-6799	F-1201, F-1221, F-1225, F-14185	F-3029	Ex 340/380	145	2
Fura-5F	F-14176	F-14177		Ex 340/380	400	2
Fura-4F	F-14174	F-14175		Ex 340/380	770	2
Fura-6F	F-14178	F-14179		Ex 340/380	5300	2
Fura-FF	F-14180	F-14181		Ex 340/380	5500	2
Fura Red	F-14219	F-3020, F-3021		Ex 420/480	140	2, 7
Indo-1	I-1202	I-1203, I-1223, I-1226	I-3032	Em 405/485	230	2
Indo-5F	I-23912	I-23913		Em 405/485	470	2
Mag-fluo-4	M-14205	M-14206		Em 520	22,000	3
Mag-fura-2	M-1290	M-1291, M-1292		Ex 340/380	25,000	2
Mag-fura-5	M-3103	M-3105		Ex 340/380	28,000	2
Mag-indo-1	M-1293	M-1295		Em 405/485	35,000	2, 8
Magnesium Green	M-3733	M-3735		Em 530	6000	3
Oregon Green 488 BAPTA-1	O-6806	O-6807	O-6798, O-6797	Em 520	170	3
Oregon Green 488 BAPTA-2	O-6808	O-6809		Em 520	580	3,9
Oregon Green 488 BAPTA-6F	O-23990	O-23991		Em 520	3000	3
Oregon Green 488 BAPTA-5N	O-6812	O-6813		Em 520	20,000	3
Quin-2	Q-23918	Q-1289		Em 495	60	2, 10
Rhod-2	R-14220	R-1244, R-1245		Em 580	570	3
Rhod-FF	R-23982	R-23983		Em 580	19,000	3
Rhod-5N	R-14207	R-14208		Em 580	320,000	3
X-rhod-1	X-14209	X-14210		Em 600	700	3
X-rhod-5F	X-23984	X-23985		Em 600	1600	3
X-rhod-FF	X-23986	X-23987		Em 600	17,000	3

* Catalog number for the cell-impermeant salt. † Catalog number(s) for the cell-permeant AM ester. ‡ Catalog number(s) for the dextran conjugates. § Measurement wavelengths, in nm, where Ex = Fluorescence excitation and Em = Fluorescence emission. Indicators for which a pair of wavelengths are listed have dual-wavelength, ratio-measurement capability. ** Ca²⁺ dissociation constant, measured at Molecular Probes *in vitro* at 22°C in 100 mM KCl, 10 mM MOPS, pH 7.2, unless otherwise noted. K_d values depend on temperature, ionic strength, pH and other factors, and are usually higher *in vivo*. Because indicator dextrans are intrinsically polydisperse and have variable degrees of substitution these values may vary; lot-specific K_d values are printed on the vial in most cases.

1. Similar Ca²⁺-dependent fluorescence response to fura-2 but ~75% greater molar absorptivity. **2.** The AM ester form is fluorescent (a major potential source of error in Ca²⁺ measurements). **3.** The AM ester form is nonfluorescent. **4.** Calcium Green-1 is more fluorescent than fluo-3 in both Ca²⁺-bound and Ca²⁺-free forms. The magnitude of the Ca²⁺-dependent fluorescence increase is greater for fluo-3; see Figure 20.22. **5.** Larger Ca²⁺-dependent fluorescence increase than Calcium Green-1. **6.** The K_d value for fluo-4 dextran (~3100 nM) is much higher than that of the free dye. **7.** Can also be used in combination with fluo-3 for dual-wavelength ratio measurements, Ex = 488 nm, Em = 530/670 nm (Cell Calcium 18, 377 (1995); Cytometry 17, 135 (1994); Cell Calcium 14, 359 (1993)). **8.** K_d determined in 100 mM KCl, 40 mM HEPES, pH 7.0 at 22°C (Biochem Biophys Res Commun 177, 184 (1991)). **9.** Larger Ca²⁺-dependent fluorescence increase than Oregon Green 488 BAPTA-1. **10.** K_d determined in 120 mM KCl, 20 mM NaCl, pH 7.05 at 37°C (Methods Enzymol 172, 230 (1989)).

Table 20.2 Comparison of *in vitro* and *in situ* K_d values for various Ca^{2+} indicators.

Indicator	K_d <i>in vitro</i> (nM) *	K_d <i>in situ</i> (nM) †	Cell/Tissue Type
Calcium Green-1	190	930	HeLa cells ¹
fluo-3	390	2570	Frog skeletal muscle ²
fluo-4	345	1000	HeLa cells ¹
fura-2	145	371	U373-MG astrocytoma cell ³
fura-2	145	350	Rabbit gastric gland ⁴
indo-1	230	844	Rabbit cardiac myocyte ⁵
Oregon Green 488 BAPTA-1	170	430	HeLa cells ¹
rhod-2	570	720	Mouse heart ⁶

* Values determined at 22°C in 100 mM KCl, 10 mM MOPS, pH 7.2, 0–10 mM CaEGTA. † Values determined in the cellular environments listed in the adjacent column.

1. Cell Calcium 28, 213 (2000); 2. Biophys J 65, 865 (1993); 3. Cell Calcium 21, 233 (1997); 4. Methods Enzymol 192, 38 (1990); 5. Biophys J 68, 1453 (1995); 6. Cell Calcium 29, 217 (2001).

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. We also offer several compounds and buffers for measuring and manipulating intracellular and extracellular Ca^{2+} . These products, which are discussed in Section 20.8, include caged- Ca^{2+} reagents and caged chelators (NP-EGTA, DMNP-EDTA and diazo-2), as well as Calcium Calibration Buffer Kits, BAPTA-derived buffers, ion-selective chelating polymers (Calcium Sponge) and the important Ca^{2+} ionophores ionomycin, A-23187 and its nonfluorescent analog, 4-bromo A-23187. Reagents for probing Ca^{2+} regulation and second messenger activity are described in more detail in Chapter 18. Our reagents for the study of Ca^{2+} channels are described in Section 16.3.

Reference Guides for Using Fluorescent Ca^{2+} Indicators

In order to meet the needs of researchers new to this technology, Molecular Probes offers selected books that provide surveys of fluorescent ion indicators and techniques for using them.

- **Calcium Signaling Protocols (Methods in Molecular Biology, Volume 114)** (C-14945), edited by Lambert, provides optimized protocols for routine fluorometric Ca^{2+} measurements, as well as for confocal microscopy, subcellular Ca^{2+} imaging, Ca^{2+} channel activity determinations and detection of Ca^{2+} release from intracellular stores.
- **Fluorescent and Luminescent Probes for Biological Activity: A Practical Guide to Technology for Quantitative Real-Time Analysis, Second Edition** (F-14944), edited by Mason, is a comprehensive survey of optical probe techniques, including fluorescent ion indicators.⁶

Other reviews of these indicators include those by O'Malley, Burbach and Adams,¹³ Williams, Bowser and Petrou,¹⁴ Johnson,¹⁵ Scheenen, Hofer and Pozzan,¹⁶ Takahashi and co-workers,³ Silver,⁵ and Kao.⁴ Several earlier reviews on ion indicators also contain useful technical information.^{17–20}

Product List — 20.1 Introduction to Ca^{2+} Measurements with Fluorescent Indicators

Cat #	Product Name	Unit Size
C-14945	Calcium Signaling Protocols (Methods in Molecular Biology Volume 114). David Lambert, ed. Humana Press (1999); 376 pages, hard cover	each
F-14944	Fluorescent and Luminescent Probes for Biological Activity. A Practical Guide to Technology for Quantitative Real-Time Analysis, Second Ed. W.T. Mason, ed. Academic Press (1999); 647 pp, comb bound	each

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