

## Effects of photolabile calcium chelators on fluorescent calcium indicators

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**Abstract** — The fluorescence properties of the calcium indicators Fura-2 and Fluo-3 have been investigated in the presence of the 'caged calcium' photolabile chelators Nitr-5 and DM-nitrophen. The excitation spectra of dilute solutions of these indicators was distorted by the presence of photolabile chelators, owing to differential absorbance of excitation light by the chelators, as well as calcium-dependent fluorescence of the chelators themselves. This distortion was altered on partial photolysis of the chelators, due to changes in their absorbance and fluorescence. At high concentrations of indicators (100  $\mu\text{M}$ ) and photolabile chelators (10 mM), similar to those used experimentally, DM-nitrophen quenched the fluorescence of Fluo-3 at low calcium concentrations. The results suggest that Fura-2 may be used with either chelator, and Fluo-3 with Nitr-5, to measure calcium released on photolysis of the caged compounds, but that careful calibration of the chelator-indicator mixture after the appropriate degree of photolysis is necessary.

Nitr-5 [1] and DM-nitrophen [2] are recently developed 'caged calcium' compounds that permit the experimental generation of rapid, repeatable and quantitative changes in intracellular calcium. These photolabile calcium chelators react to ultraviolet light by undergoing a reduction in calcium binding affinity (Nitr-5) or by photolysis to products that bind calcium only very weakly (DM-nitrophen).

To use these chelators to precisely elevate calcium by desired amounts requires knowing accurately certain key characteristics of the mixtures of these compounds used experimentally [3, 4]. It is first necessary to know the ratio of chelator to calcium that is introduced into cells. If the compounds are injected by pressure into single cells, or by perfusion from patch pipettes, and if the solutions are accurately mixed, then this ratio may be known. Even in the injection solution, knowledge of the composition is limited by the

presence of impurities in some stocks of caged calcium chelators, and by the difficulty of making precise mixtures of very small volume. On the other hand, if cells are loaded by exposure to the acetoxymethyl ester of Nitr-5 (Nitr-5/AM) in the bathing medium [5], no calcium will be loaded concurrently and calcium will be bound to Nitr-5 intracellularly only to the extent that it can be extracted from cytoplasmic buffers, intracellular stores, or leakage into the cell from the medium. Then knowledge of the ratio of free chelator to calcium-bound chelator is unobtainable. Furthermore, even when the ratio of bound-to-free chelator of an injected or perfused solution is known, this ratio may drop under the influence of calcium extrusion or uptake processes, or may rise by binding calcium from other sources in the same way as Nitr-5/AM. Finally, for DM-nitrophen, the elevation in calcium concentration by partial

photolysis of the chelator also depends on the relative concentrations of  $Mg^{2+}$ , ATP and nitrophen in the cells [6] and this is not always accurately known.

If the degree of loading of the intracellular calcium cage is known, the intensity and effectiveness of the light source used for photolysis must also be measured before the change in calcium on exposure to light can be calculated. Flash lamps are difficult to calibrate accurately, and the light intensity will vary as bulbs age and are replaced. Light intensity also depends on such properties as the angle of incident light at air-water interfaces, the degree of absorption by overlying tissues, and absorption of the incident light by cytoplasm, the calcium chelators in the cells, or the cell membrane.

Because of these difficulties, it is usually necessary to measure the calcium change caused by photolysis of photolabile chelators, at least in some experiments, if quantitative use of these tools is important. At present, the fluorescent indicators of calcium, Fura-2, Indo-1 [7] and Fluo-3 [8] are the most popular, widely available, and accurate indicators of intracellular calcium. Fura-2 has the advantage of a shift in its excitation spectrum on binding calcium, allowing the calculation of calcium concentration from the ratio of fluorescence at two excitation wavelengths, independent of dye concentration or sample thickness. Indo-1 displays a shift in emission spectrum on calcium binding, so that it may also be calibrated ratiometrically. But the difficulty of producing precisely registered images at two emission wavelengths [9] limits the usefulness of Indo-1. The fluorescence of Fluo-3 increases up to 40 times on binding calcium but, without a shift in its excitation or emission spectrum, ratiometric calibration is impossible, and its concentration in cells as well as the effective pathlength (which may be limited by the optical depth of field of the objective) must be known accurately. On the other hand, Fura-2 is excited and may be bleached by the same wavelength light used to activate Nitr-5 or DM-nitrophen, while Fluo-3 is excited by light of a much longer wavelength. Similarly, the measurement of calcium in cells containing Nitr-5 or DM-nitrophen using Fura-2 may itself perturb the level of calcium by releasing it from its cage, while excitation of Fluo-3

may proceed without effect on photolabile calcium chelators.

Since each indicator has its own advantages and disadvantages, it is not clear which would be most appropriate for use with calcium cages. In preliminary experiments with Fluo-3 and nitrophen in snail neurons, we found that moderate photolysis of DM-nitrophen was accompanied by a much smaller increase in Fluo-3 fluorescence than expected [B.L. Lancaster and R.S. Zucker, unpublished observations]. We were unsure whether the problem lay with the photolysis of DM-nitrophen or the detection of calcium by Fluo-3. I have therefore examined the properties of both dyes as indicators of calcium released from both Nitr-5 and DM-nitrophen, in order to try to understand the discrepancy in our experiments, to choose the most appropriate indicator for the detection of calcium released from photolabile chelators, and to collect information necessary for the quantitative application of these dyes to the measurement of intracellular calcium released from calcium cages.

## Materials and Methods

Excitation (and in some cases emission) spectra of solutions containing mixtures of a calcium indicator and photolabile calcium chelator were obtained with a modular fluorimeter system (SPEX Fluorolog 2, model F2C, Edison NJ, USA) and SPEX Datamate microcomputer. Spectra of solutions in 1 cm quartz cuvettes were recorded at room temperature in ratio mode using a rhodamine B reference. Correction factors derived by comparing measured and known spectra of reference compounds were applied to the emission photomultiplier output.

Dilute mixtures of indicator dye (1  $\mu M$ ) and calcium chelator (100  $\mu M$ ) were dissolved in 100 mM KCl, 10 mM K-HEPES, pH 7.0. Solutions with zero calcium contained 1 mM  $K_2$ -EGTA. Solutions with excess calcium contained 5 mM  $CaCl_2$ . Solutions with an intermediate or 'low calcium' concentration were buffered with 8 mM  $K_2$ -EGTA and 4 mM  $CaCl_2$ , pH 7.0. The calculated free calcium concentration was 413 nM, using the EGTA binding constants of Martell and Smith [10].

When Nitr-5 or nitrophen was added to these solutions, the free calcium concentration was recalculated by solving simultaneously buffer equations for 8 mM EGTA, 100  $\mu$ M Nitr-5 or DM-nitrophen and 4 mM calcium plus any calcium known to be present in the Nitr-5 or DM-nitrophen stocks used [11]. For Nitr-5 and DM-nitrophen calcium affinities, values of 145 and 5 nM, respectively were used [1, 2].

Solutions containing 100  $\mu$ M of indicator dye and 10 mM of caged calcium chelator, mimicking concentrations used in biological experiments, were placed in 50  $\mu$ m pathlength micro-cuvettes (Vitro Dynamics, Rockaway, NJ, USA). This is similar to typical cell diameters, and is also necessary because of the high absorbance of high concentrations of the compounds, and the resultant screening of fluorescence that would occur in ordinary cuvettes. The micro-cuvettes were 0.5 mm wide and 2 mm long, and after filling with sample solution were placed under light mineral oil in a culture dish whose bottom was replaced with a No. 1 glass cover slip. This prevented evaporation of the sample, which was protected from light and studied within 1 h. For high calcium measurements, the micro-cuvettes contained 80 mM KCl, 10 mM K-HEPES pH 7.0 and 17 mM CaCl<sub>2</sub>, which after adding 10 mM of the sodium salt of photolabile chelator had 7 mM of excess calcium and an ionic strength of about 156 mosm. Calcium-free measurements contained 50 mM K<sub>2</sub>-EGTA at pH 7.0 which, with 10 mM of the sodium salt of the photolabile chelator, should have an ionic strength of 250 mosm. The intermediate-calcium solution contained 50 mM Ca-EGTA and 50 mM K<sub>2</sub>-EGTA at pH 7.0; 10 mM of photolabile chelator was added after loading with calcium (producing 20 mM of NaCl), so this solution had an ionic strength of about 200 mosm. The high ionic strength of this solution might be expected to affect the affinities of the indicator dyes for calcium. However, the indicator and the EGTA  $K_D$ s should be increased by the same factor, with no net effect on fluorescence at intermediate calcium level. Therefore, no ionic strength correction to the estimates of indicator affinities is necessary.

The fluorescence of solutions in these cuvettes was measured using a Zeiss IM35 inverted

microscope attached to the SPEX Fluorolog 2 [12]. Excitation wavelengths were scanned using the SPEX excitation monochromator, with the excitation beam deflected to the sample by dichroic mirror (395 nm for Fura-2, 510 nm for Fluo-3) in the epifluorescence filter set. Emitted light from a 100  $\mu$ m diameter spot was collected by the X63 oil objective (Zeiss Neofluar 1.25 n.a.) and passed through a barrier filter (Zeiss 450 nm long pass for Fura-2, 520-550 nm band pass for Fluo-3) before counting by a photon counter attached to the camera port of the microscope. All spectra were corrected for background fluorescence by subtracting spectra of micro-cuvettes containing buffer solution without any indicator or photolabile chelator.

For these measurements, Fura-2 and Fluo-3 were obtained from Molecular Probes (Eugene, OR, USA) and Nitr-5 and DM-nitrophen were purchased from CalBiochem (La Jolla, CA, USA).

## Results

### *Use of dilute solutions*

Cells are typically loaded with fluorescent calcium indicators at a concentration of about 100  $\mu$ M, providing a reasonably bright fluorescence while interfering little with native calcium buffering mechanisms. Calcium chelators are usually employed at a concentration of about 10 mM, sufficient to make it the dominant buffer with minimal interference from native calcium buffers. But the photolabile calcium chelators Nitr-5 and DM-nitrophen are highly absorbent at 360 nm, the wavelength most effective in photolyzing these compounds [1, 2]. In a standard 1 cm cuvette containing 10 mM of such buffers, with decadic extinction coefficients of about 5000 M<sup>-1</sup>cm<sup>-1</sup>, the Beer-Lambert law indicates that the intensity of incident ultraviolet light will be reduced to 10<sup>-50</sup> at the back surface of the cuvette; integration of the Beer-Lambert law shows that the average light intensity will be less than 1% that at the front surface. To reduce the amount of screening of excitation light, the fluorescence of mixtures of 1  $\mu$ M indicator dye and 100  $\mu$ M photolabile chelator

Table 1 Fluorescence of mixtures of Fura-2 and photolabile calcium chelators

Excited at:	340 nm			380 nm			Fig.
	$F_o$	$F_l$	$F_h$	$F_o$	$F_l$	$F_h$	
Substance	%	%	%	%	%	%	
Fura-2	100	100	100	100	100	100	2
Bleached Fura-2	88	96	97	119	101	109	2
DM-nitrophen	0.3	-	0.5	0.8	-	12	1
Photolysed DM-nitrophen	76	-	11.1	93	-	834	1
Fura-2 + DM-nitrophen	52	49	60	68	72	86	2
Fura-2 + photolysed NP	86	64	58	85	70	196	2
Nitr-5	44	-	17	74	-	340	1
Photolysed Nitr-5	13	-	6.2	6.7	-	157	1
Fura-2 + Nitr-5	95	76	73	83	221	537	2
Fura-2 + photolysed N-5	65	59	63	57	74	382	2

Substance	$R_{min}$	$R_l$	$R_{max}$	$S$	$[Ca_o]$ nM	$K_D$ nM	$R_{max}/R_{min}$
Fura-2	0.802	4.92	32.2	14.5	413	188	40
Bleached Fura-2	0.772	4.60	28.5	12.1	413	213	37
DM-nitrophen	(0.303)	-	(1.16)	(0.696)	-	-	-
Photolysed DM-nitrophen	0.65	-	(0.43)	0.01	-	-	-
Fura-2 + DM-nitrophen	0.680	3.35	22.6	10.5	395	272	33
Fura-2 + photolysed NP	0.808	2.19	9.59	6.33	413	351	12
Nitr-5	0.487	-	1.31	2.38	-	-	-
Photolysed Nitr-5	1.56	-	1.26	0.61	-	-	-
Fura-2 + Nitr-5	0.627	1.69	4.38	3.27	406	314	7.0
Fura-2 + photolysed N-5	0.919	3.90	5.29	2.16	422	91	5.8

The following numbers are expressed as percentages of control (Fura-2 or Fluo-3 alone, unbleached):  $F_o$ , Fluorescence in 0-Ca buffer;  $F_h$ , fluorescence in high-Ca buffer;  $F_l$ , fluorescence in low-Ca buffer.  $[Ca_o]$ , calcium concentration (in nM) in low buffer mixture corrected for effect of Ca-free nitrophen or of Nitr-5 which contained 30% Ca.  $K_D$ , calculated dissociation constant of indicator in nM.  $R$ , 340/380 nm excitation fluorescence ratio for Fura-2 at minimum (0-Ca), maximum (high-Ca) and low (low-Ca) calcium concentrations.  $S$ , fluorescence ratio at 380 nm in 0-Ca to high-Ca. NP is an abbreviation for DM-nitrophen; N-5 is an abbreviation for Nitr-5. Numbers in parentheses are based on very low levels of fluorescence and have little meaning. Dashes (-) mean not measured or not calculated. Fig. refers to number of the figure which illustrates this experiment. Data from this figure, or from average of similar figures

was measured. Now the average light intensity in the cuvette at 360 nm is 60% that at the front surface, which is a tolerable amount of screening. This is also comparable to the amount of screening that would occur in a 50  $\mu$ m diameter cell, where the average intensity of 360 nm light will be reduced to 76%.

From consideration of absorbance alone, we would already expect Nitr-5 and DM-nitrophen to affect Fura-2 fluorescence signals by screening the incident light (but not the emitted light, since the chelators have little absorbance above 500 nm).

Since the absorbance is not identical at 340 and 380 nm, especially after photolysis, this screening effect could affect the ratio of Fura-2 fluorescence excited at these wavelengths, and hence the calibration of this ratio in terms of calcium concentration [7].

#### Fluorescence of photolabile chelators

Another potential source of interference of photolabile calcium chelators with indicator dyes is fluorescence of the chelators themselves. The fluorescence properties of 100  $\mu$ M Nitr-5 or

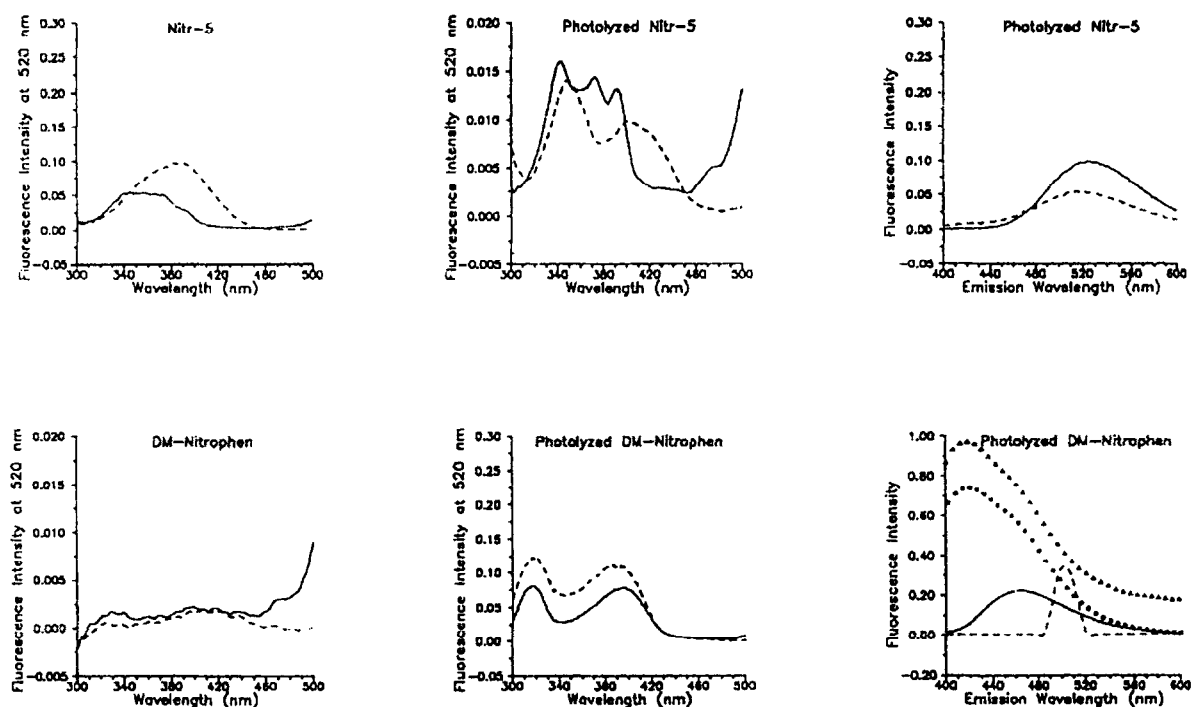


Fig. 1 Excitation spectra of solutions containing 100  $\mu\text{M}$  Nitr-5 or DM-nitrophen before (left) and after (centre) 35% photolysis of the photolabile chelator. Emission was recorded at 520 nm. Emission spectra of the partially photolysed chelators are shown on the right. In the left and centre emission spectra, the solid line is in 0-Ca buffer, the dotted line is in high-Ca buffer and the dashed line (where present) is in low-Ca buffer. The same convention is used in Figures 2–4. The Nitr-5 emission spectrum was excited at the excitation peak: 340 nm in high-Ca and 390 nm in 0-Ca. The 4 DM-nitrophen emission spectra are for the two excitation peaks in the two buffer solutions: 380 nm in high-Ca (solid line) and 0-Ca (dotted line), and 320 nm in high-Ca (circles) and 0-Ca (triangles). In Figures 1–3, the ordinate numbers refer to absolute fluorescence intensity and are normalized to the largest signal recorded. Note changes in scale of y-axes to reveal details of low levels of fluorescence

DM-nitrophen alone were examined and compared with the fluorescence of 1  $\mu\text{M}$  Fura-2 or Fluo-3. Excitation spectra for the photolabile chelators are shown in Figure 1. Fluorescence is measured at 520 nm, close to the fluorescence emission peaks for both Fura-2 (510 nm) and Fluo-3 (530 nm) [7, 8] at which fluorescence is typically measured in biological experiments. Nitr-5 shows an excitation spectrum similar to that of Fura-2 (*see* Fig. 2). Moreover, the shape of the spectrum changes with the binding of calcium. Like Fura-2, the ratio of fluorescence excited at 340 nm to that excited at 380 nm,  $R$ , increases on binding calcium. Furthermore, the amount of fluorescence of 100  $\mu\text{M}$  Nitr-5 is a significant fraction of that of 1  $\mu\text{M}$  Fura-2 (compare Figs 1 and 2). In the absence of calcium, Nitr-5 fluorescence is 44% that of Fura-2

at 340 nm, and 74% at 380 nm (*see* Table 1). At high calcium, the 340 nm fluorescence is only 17% that of Fura-2 at 340 nm, but 340% the Fura-2 fluorescence at 380 nm. Nitr-5 has little fluorescence excited near 500 nm, the usual excitation wavelength for Fluo-3 (*see* Table 2). Thus we should expect Nitr-5 to contribute substantial fluorescence to Fura-2 (but not to Fluo-3) measurements, and significantly influence the conversion of fluorescence data to calcium concentration.

In contrast, DM-nitrophen is far less fluorescent than the indicator dyes (compare Figs 1 and 3 and *see* Tables 1 and 2, and note the change in ordinate scale for DM-nitrophen). This is not likely to contribute significantly to fluorescent signals from either Fura-2 or Fluo-3 measured near 520 nm.

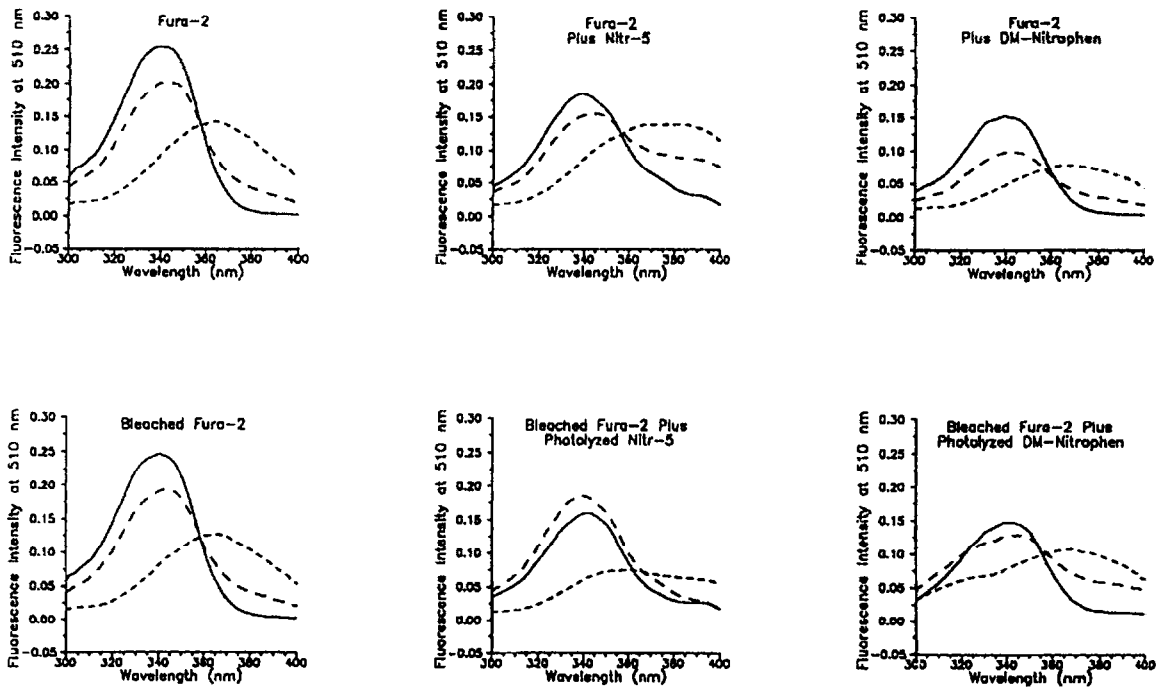


Fig. 2 Excitation spectra of solutions containing 1  $\mu\text{M}$  Fura-2 alone, or with 100  $\mu\text{M}$  Nitr-5 or DM-nitrophen. Emission wavelength, 510 nm. Bleached indicator or photolysed chelator was exposed to sufficient UV light to photolyse approximately 35% of the photolabile chelator

### Emission spectra of photolabile chelators

The emission spectra of Nitr-5 and DM-nitrophen have also been examined (Fig. 1). Nitr-5's spectrum peaks at about 510 nm in zero calcium

solution (excitation at the 390 nm peak), and at 520 nm in high calcium solution (excited at the 340 nm peak). The emission spectra of photolysed DM-nitrophen indicate that it produces two fluorescent photoproducts on photolysis: one has a

Table 2 Fluorescence of mixtures of Fluo-3 and photolabile chelators

Excited at:	505 nm						[Ca <sub>10</sub> ] nM	Figure
	$F_{\text{max}}/F_{\text{min}}$	$F/F_{\text{min}}$	$K_D$	$F_0$ %	$F_1$ %	$F_h$ %		
Substance								
Fluo-3	25.5	11.5	550	100	100	100	413	3
Bleached Fluo-3	27.2	11.9	580	94	97	100	413	3
DM-nitrophen	(51)	—	—	0.5	—	0.6	—	1
Photolysed DM-nitrophen	(20)	—	—	1.2	—	1.0	—	1
Fluo-3 + DM-nitrophen	30.4	8.7	1110	100	76	120	395	3
Fluo-3 + photolysed NP	23.1	8.9	742	94	73	85	413	3
Nitr-5	12.4	—	—	3.2	—	0.9	—	1
Photolysed Nitr-5	12.4	—	—	4.0	—	1.9	—	1
Fluo-3 + Nitr-5	25.5	10.5	641	98	93	102	306	3
Fluo-3 + photolysed N-5	29.8	9.9	944	96	57	112	422	3

Abbreviations as in Table 1, except  $F_{\text{max}}$ , fluorescence in high-Ca buffer;  $F_0$ , fluorescence in low-Ca buffer;  $F_{\text{min}}$ , fluorescence in 0-Ca buffer

maximum emission at 500 nm and is excited at 380 nm. This product shifts its emission peak to about 465 nm in the presence of excess calcium. The other has a maximum emission at 420 nm and is excited at 380 nm, independent of calcium concentration. The emission spectra of Nitr-5 and DM-nitrophen both have significant energy near 520 nm, so it is not possible to select an emission wavelength that distinguishes Fura-2 or Fluo-3 fluorescence from that of Nitr-5 or the DM-nitrophen photoproducts.

### Effects of photolysis

Since experiments using photolabile calcium chelators involve their photolysis, the fluorescence properties of Nitr-5 and DM-nitrophen were examined after partial photolysis (about 35% conversion). The excitation spectra, still measured at 520 nm, are shown in Figure 1. Nitr-5's fluorescence drops to about 30% (note the change in ordinate scale), therefore decreasing its expected interference with Fura-2 by its own fluorescence.

However, photolysed Nitr-5 is 2–4.5 times as absorbant as unphotolysed Nitr-5, depending on the level of calcium [1]. Thus, photolysis of Nitr-5 will increase the screening of the excitation light, and this will interfere with Fura-2 signals.

DM-nitrophen behaved quite differently upon photolysis. The photoproducts were 100x more fluorescent than the unphotolysed chelator at 340 nm, and 20x more fluorescent at 380 nm (*see* Fig. 1 and Table 1). Thus after partial photolysis, we would expect DM-nitrophen to contribute significant new fluorescence to Fura-2 signals used to calculate free calcium levels. The absorbance of this compound drops somewhat at 380 nm on photolysis [2], thus diminishing slightly its screening effect on Fura-2 at this wavelength.

The light used to photolyse Nitr-5 or DM-nitrophen could significantly alter the calcium-sensitivity of calcium indicators by bleaching [13]. This was checked by comparing the excitation spectra of Fura-2 (Fig. 2) and Fluo-3 (Fig. 3) before and after exposure to the same light used to photolyse about 35% of photolabile calcium

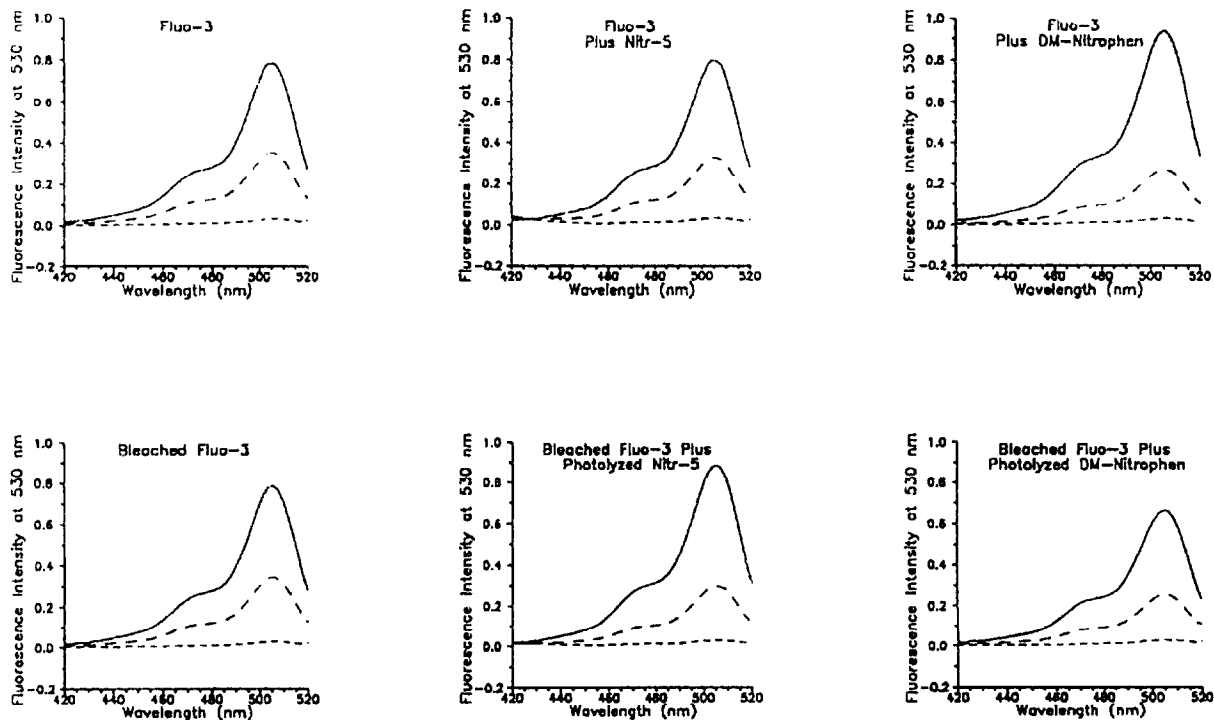


Fig. 3 Excitation spectra of solutions containing 1  $\mu$ M Fluo-3 alone, or with 100  $\mu$ M Nitr-5 or DM-nitrophen. Emission wavelength 530 nm

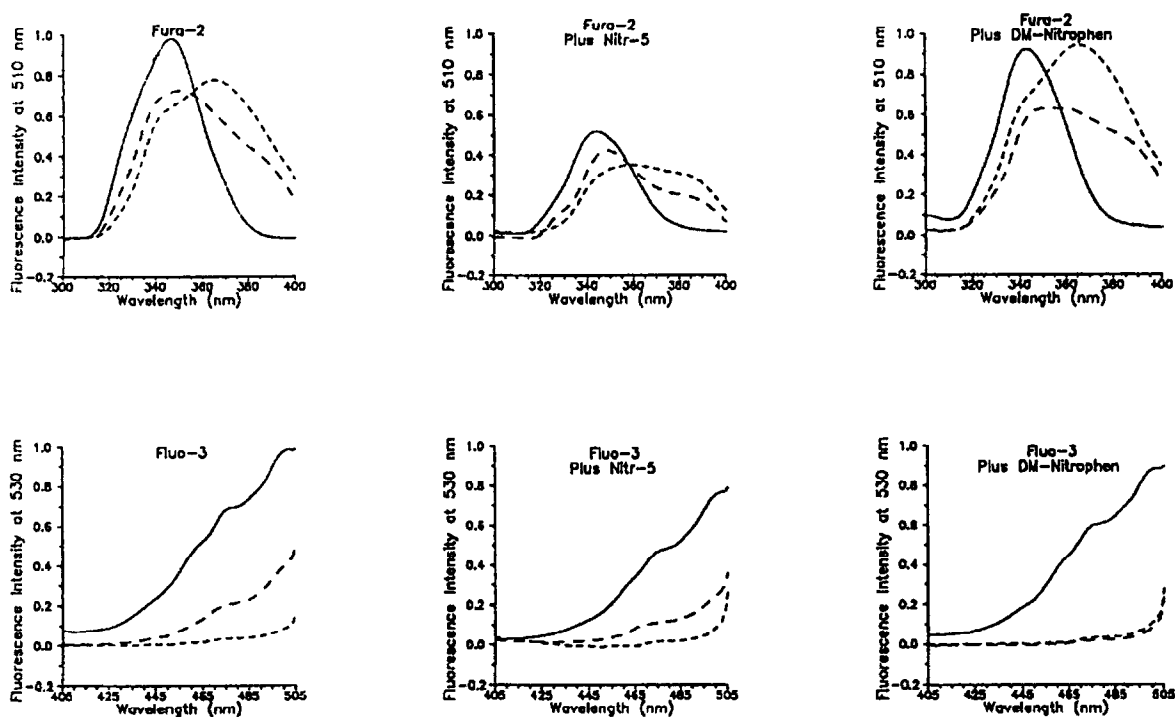


Fig. 4 Excitation spectra of solutions in micro-cuvettes containing 100  $\mu\text{M}$  Fura-2 or Fluo-3 alone, or with 10 mM Nitr-5 or DM-nitrophen

chelators. Bleaching of both dyes was minimal, with little effect on the fluorescence levels used to calibrate the indicators for calcium measurement.

#### *Fluorescence of dilute indicator-chelator mixtures*

Figure 2 and Table 1 compare spectra of 1  $\mu\text{M}$  Fura-2 alone with spectra of Fura-2 plus 100  $\mu\text{M}$  of Nitr-5. Several effects may be observed. Fluorescence is reduced to similar extents at 340 nm and 380 nm in calcium-free solution. The reduction is less than expected from the absorbance of Nitr-5, due to the addition of its own fluorescence. When calcium is present, the extra fluorescence of Nitr-5 at 380 nm exceeds that of Fura-2, greatly reducing the 340/380 excitation fluorescence ratio and increasing the apparent  $K_D$  of Fura-2. On photolysis, the fluorescence of Nitr-5 is greatly reduced, leading to less distortion in the 340/380 ratio from this source. But the absorbance is dramatically increased, especially at 380 nm in low calcium, and at both 340 nm and 380 nm in high

calcium. This alters the distortion of the Fura-2 fluorescence and now the apparent  $K_D$  of Fura-2 is less than in the absence of Nitr-5.

Figure 2 and Table 1 also illustrate effects of DM-nitrophen on Fura-2. The fluorescence is reduced at 340 nm, less so at 380 nm. This is due to the absorbance of DM-nitrophen which is larger at 340 nm, especially in low calcium. This reduces the 340/380 excitation fluorescence ratio, but to somewhat different degrees at different calcium levels, leading to a change in the apparent affinity of Fura-2 for calcium. Photolysis of DM-nitrophen brings in the fluorescence of its photoproducts, which at high calcium exceeds that of Fura-2 at 380 nm. This reduces the 340/380 ratio even further at high calcium, and causes a further reduction in the apparent calcium affinity of the mixture. Thus, both Nitr-5 and DM-nitrophen alter somewhat the fluorescent properties of Fura-2 but with proper calibration, the mixture can still be used as a detector of calcium concentration.

The fluorescence of Fluo-3 (at 1  $\mu\text{M}$ ) is little



**Table 3** Fluorescence of concentrated mixtures of Fura-2 and photolabile calcium chelators

Excited at: Substance	340 nm			380 nm			Figure
	$F_o$ %	$F_l$ %	$F_h$ %	$F_o$ %	$F_l$ %	$F_h$ %	
Fura-2	100	100	100	100	100	100	4
Fura-2/DM-nitrophen	110	88	74	124	115	101	4
Fura-2/Nitr-5	41	48	56	48	45	89	4

Substance	$R_{min}$	$R_{lo}$	$R_{max}$	$S$	$[Ca_{lo}]$ nM	$K_D$ nM	$R_{max}/R_{min}$
Fura-2	0.887	1.50	16.6	12.0	413	848	18.7
Fura-2/DM-nitrophen	0.793	0.99	12.1	14.7	413	1580	15.3
Fura-2/Nitr-5	0.771	1.59	10.4	6.41	413	693	13.5

Abbreviations as in Table 1

affected by the addition of 100  $\mu$ M Nitr-5 or DM-nitrophen. One unexpected interaction observed is a reduction in fluorescence at intermediate levels of calcium, when DM-nitrophen or photolysed Nitr-5 is present. This effect is much greater at higher dye and chelator concentrations (*see* below). Another is an excess of fluorescence at high calcium levels. These effects combine to reduce somewhat the apparent affinity of the indicators for calcium. Nevertheless, the mixture can again be calibrated to measure calcium concentration.

#### Fluorescence of concentrated indicator-chelator mixtures

Because none of these measurements revealed the strong DM-nitrophen interaction with Fluo-3 observed in biological experiments (*see* above), the

measurements were repeated using micro-cuvettes containing concentrations similar to those used experimentally – namely 100  $\mu$ M indicator and 10 mM chelator. Results are illustrated in Figure 4 and Tables 3 and 4. The final solutions used for these measurements were made by mixing 1  $\mu$ l of 50 mM caged calcium chelator, 1  $\mu$ l of 500  $\mu$ M calcium indicator, and 3  $\mu$ l of buffer solutions with 167% the concentrations of the salts indicated in Materials and Methods. This led to inevitable errors of up to 30% in the final concentrations of constituents, so that differences in the absolute levels of fluorescence are not significant. The spectra are also distorted by the transmission characteristics of the microscope optics attached to the SPEX Fluorolog, and so look somewhat different from those scanned directly from standard cuvettes. Finally, background subtraction of fluorescence from solutions without indicator or photolabile chelator was difficult,

**Table 4** Fluorescence of concentrated mixtures of Fluo-3 and photolabile calcium chelators

Excited at: Substance	505 nm			$F_o$ %	$F_l$ %	$F_h$ %	$[Ca_{lo}]$ nM	Fig.
	$F_{max}/F_{min}$	$F_l/F_{min}$	$K_D$					
Fluo-3	14.9	5.49	865	100	100	100	413	4
Fluo-3/DM-nitrophen	12.3	17.7	xxxx	110	14	90	413	4
Fluo-3/Nitr-5	14.7	4.22	1340	77	59	76	413	4

Abbreviations as in Table 2: xxxx means undefined

because these often emitted nearly as much light as calcium-bound Fura-2 at longer wavelengths, or as calcium-free Fluo-3.

For Fura-2 the effects of the photolabile chelators generally resemble those measured more accurately in dilute solution. The 340/380 excitation fluorescence ratio is depressed, especially at intermediate and high calcium, and the apparent affinity is reduced. A high buffer concentration was needed to offset the effects of the photolabile chelators on free calcium. The rather larger reduction in apparent affinity in the presence of DM-nitrophen is somewhat anomalous, but was observed in three separate repetitions of the measurements on fresh solutions.

For Fluo-3 in the presence of 10 mM Nitr-5, the results again resemble those observed in dilute solution, with the main effect being a reduction in apparent calcium affinity of the indicator. DM-nitrophen however, had rather surprising effects on Fluo-3 fluorescence at high concentrations. The fluorescence in intermediate (413 nM) calcium was sharply reduced, to less than that in either zero or high calcium solutions. This anomalous behaviour, observed several times in micro-cuvettes, was also seen in snail neurons perfused with 10 mM DM-nitrophen and 100  $\mu$ M Fluo-3 and exposed to sufficient UV to raise calcium to the micromolar range.

The Fluo-3/DM-nitrophen mixture exhibited normal fluorescence at millimolar calcium concentration (Fig. 4 and Table 4). To determine at what calcium concentration 100  $\mu$ M Fluo-3 begins to fluoresce in the presence of 10 mM DM-nitrophen, an intermediate calcium buffer was prepared using 50 mM CaHEEDTA and 50 mM K<sub>2</sub>HEEDTA (Sigma Chemical, St Louis, MO, USA) instead of EGTA. At pH 7.0, this provides a free calcium concentration of 5.17  $\mu$ M, as calculated from the hydrogen and calcium binding constants of Martell and Smith [10]. This solution displayed 78% as much fluorescence as that with 7 mM excess calcium, indicating a  $K_D$  of 1.43  $\mu$ M for 100  $\mu$ M Fluo-3 mixed with 10 mM DM-nitrophen, similar to that seen in the presence of Nitr-5 (Table 4). Therefore, DM-nitrophen does not have a particular effect on the affinity of Fluo-3 for calcium; rather it seems to specifically inhibit

Fluo-3's fluorescence at calcium levels around 1  $\mu$ M or less.

## Discussion

Mixtures of calcium chelators and fluorescent calcium indicators have somewhat different fluorescent properties from those of the indicators alone. The main effects are differential absorbance of the excitation light at different wavelengths by the caged calcium chelators, and additional fluorescence added by the chelators or their photoproducts. In addition, Fluo-3 fluorescence at low calcium levels is greatly reduced, probably by quenching in the presence of high concentrations of DM-nitrophen, making this indicator unsuitable for use with this chelator. In general, mixtures of Fura-2 with Nitr-5 or DM-nitrophen, and Fluo-3 with Nitr-5 may be re-calibrated as calcium indicators in the presence of photolabile chelators. Since the calibrations are disrupted by similar degrees for these three mixtures, there is little to favor one indicator over the other for use with Nitr-5. Fura-2 has the disadvantage that it is bleached by the same light used to photolyse caged calcium, and that the light used to excite it photolyses the chelators. However, light sufficient to photolyse 35% of the chelators causes only minimal Fura-2 bleaching, and the light intensity used to excite Fura-2 can be reduced to a level such that very little chelator photolysis occurs. This can be checked by repeatedly measuring calcium and looking for a change in fluorescence indicating a rise in free calcium concentration. Thus, these intrinsic disadvantages of Fura-2 are easily overcome. Another apparent disadvantage of Fura-2 is that its narrow-band fluorescence intensity is somewhat weaker than that of Fluo-3, about 30% (compare Figs 2 and 3). However, the broad-band fluorescence brightness of Fura-2 is similar to that of Fluo-3, because the emission spectrum of Fura-2 is about 3 times as broad as that of Fluo-3 [1, 8]. On the other hand, Fura-2 has the major advantage that it is a ratiometric indicator, and can give a good measure of calcium concentration without accurate knowledge of indicator concentration or sample thickness. Because of these advantages, Fura-2

would seem to be the preferable indicator for quantitative use with Nitr-5, as well as the only appropriate one to use with high concentrations of DM-nitrophen.

These results suggest ways in which measurements of calcium in the presence of photolabile chelators can be improved. One is to reduce somewhat, to perhaps 2–5 mM, the concentration of the photolabile chelator, so as to reduce the effects of its absorbance and fluorescence. This may increase the susceptibility of the chelator's regulation of calcium to cellular calcium removal processes, however. Another possibility is to increase the concentration of the indicator to as much as 1 mM, since calcium is already under the control of an extraneous buffer. This should reduce effects due to the fluorescence of the caged calcium chelators.

This study adds interactions with calcium chelators to the list of factors that can influence the behaviour of fluorescent calcium indicators. Others include ionic strength, viscosity, photobleaching and non-cytoplasmic sequestration of acetoxymethyl esters [13–17]. The precise calibration factors for converting fluorescence to  $[Ca^{2+}]$  will also depend on the wavelengths and bandwidths chosen for excitation and emission, as well as the bandpass characteristics of all of the elements in the optical path. Perhaps the best approach to these problems is to calibrate the calcium indicators in the cells to be studied with the same substances present in calibration solutions as during experiments [18]. In the case of photolabile chelators, calibration should also be done with different amounts of chelator photolysis, corresponding to those induced experimentally. In vivo calibration is readily done if cells can be perfused with patch pipettes, and is also possible with micro-injection of solutions into single cells. Calcium-free measurements can be obtained using pH-adjusted EGTA with no calcium added to the photodynamic chelator. High calcium measurements require calcium in excess of photolabile chelators, and pH buffering. Intermediate calcium measurements require strong pH-adjusted EGTA calcium buffering, with photolabile chelator added with sufficient calcium to have little effect on the free calcium as set by EGTA. It is necessary to use EGTA rather than

BAPTA, because BAPTA has significant fluorescence at 520 nm, especially when excited at 390 nm (data not shown).

It is possible that some of the indicator- photolabile chelator interactions reported here are due to impurities in either the indicators or the caged calcium compounds, and would not be seen with absolutely pure compounds. In any case these interactions characterize mixtures made from substances obtained from commercial sources.

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