

## Fluo Calcium Indicators

### Quick Facts

#### Storage upon receipt:

##### Indicator salts

- Room temperature, 4°C or –20°C
- Desiccate
- Protect from light

##### Dextran conjugate

- –20°C
- Desiccate
- Protect from light

##### Acetoxymethyl (AM) esters (solids)

- –20°C
- Desiccate
- Protect from light

### Introduction

Since being introduced in 1989,<sup>1</sup> fluo-3 imaging has revealed the spatial dynamics of many elementary processes in Ca<sup>2+</sup> signaling.<sup>2–4</sup> Fluo-3 has also been extensively used for flow cytometry;<sup>5</sup> for experiments involving photoactivation of “caged” chelators, second messengers and neurotransmitters;<sup>6,7</sup> and for cell-based pharmacological screening.<sup>8</sup> The most important properties of fluo-3 in these applications are an absorption spectrum compatible with excitation at 488 nm by argon-ion laser sources, and a very large fluorescence intensity increase in response to Ca<sup>2+</sup> binding.

Fluo-4 is an analog of fluo-3 with the two chlorine substituents replaced by fluorines. This fairly minor structural modification results in increased fluorescence excitation at 488 nm and consequently higher signal levels for confocal microscopy, flow cytometry and microplate screening applications. Fluo-5F, fluo-5N and fluo-4FF are analogs of fluo-4 with lower Ca<sup>2+</sup>-binding affinity, making them suitable for detecting intracellular calcium levels in the 1 μM–1 mM range that would saturate the response of fluo-3 and fluo-4. Fluo-4 dextran consists of fluo-4 coupled to a biologically inert dextran carrier (molecular weight = 10,000), providing a new and potentially valuable tool for measuring Ca<sup>2+</sup> transients in presynaptic terminals arising from long axonal projections in heterogeneous fiber tracts.<sup>9</sup>

### Materials

#### Fluo-3, Fluo-4, Fluo-5F, Fluo-5N and Fluo-4FF

Fluo-3, fluo-4, fluo-5F, fluo-5N and fluo-4FF are available as water-soluble salts (F-1240, F-3715, F-14200, F-14221, F-14203, F-23980) and also as cell-permeant acetoxymethyl (AM) esters:

#### Fluo-4 dextran

- Fluo-4 dextran is supplied in solid form in 5 mg units

#### Fluo-3, AM

- F-1241, supplied in solid form in 1 mg units
- F-1242, supplied in sets of 20 vials, each containing 50 μg
- F-14218, supplied as 1 mL of ready-made 1 mM solution in DMSO
- F-14242, for high-throughput screening applications larger quantities are supplied in sets of 40 vials, each containing 1 mg
- F-23915, special FluoroPure™ grade with ≥98% HPLC purity specification, supplied in sets of 10 vials, each containing 50 μg

#### Fluo-4, AM

- F-14201, supplied in sets of 10 vials, each containing 50 μg
- F-14202, for high-throughput screening applications larger quantities are supplied in sets of 5 vials, each containing 1 mg
- F-14217, supplied as 1 mL of ready-made 1 mM solution in DMSO
- F-23917, special FluoroPure™ grade with ≥98% HPLC purity specification, supplied in sets of 10 vials, each containing 50 μg

#### Fluo-5F, AM; Fluo-5N, AM and Fluo-4FF, AM

- Supplied in sets of 10 vials, each containing 50 μg (F-14222, F-14204 and F-23981, respectively).

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 solid 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. DMSO stock solutions of AM esters should be stored protected from light, 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  $\mu\text{M}$  in calcium-free buffer. Transfer the solution to a cuvette and measure the fluorescence intensity using appropriate wavelength settings (excitation at 485 nm, emission at 520 nm). Add calcium to a saturating concentration ( $5 \geq \mu\text{M}$  for fluo-3 and fluo-4;  $\geq 1 \text{ mM}$  for fluo-5F, fluo-5N and fluo-4FF) 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

Fluo-3, fluo-4 and their derivatives all exhibit large fluorescence intensity increases on binding  $\text{Ca}^{2+}$ . Unlike the ultraviolet light-excited indicators fura-2 and indo-1, there is no accompanying spectral shift. The fluorescence intensity increase on  $\text{Ca}^{2+}$  binding is typically  $>100$ -fold.<sup>10</sup> A comparison of physical and spectroscopic properties for fluo-3 and fluo-4 is shown in Table 1. Fluo-5F, fluo-5N and fluo-4FF have very similar properties to fluo-4, with the exception of higher ion dissociation constants —  $K_d(\text{Ca}^{2+})$  is 2.3  $\mu\text{M}$ , 90  $\mu\text{M}$  and 9.7  $\mu\text{M}$ , respectively, measured under the same conditions as the values reported in Table 1. For fluo-4 dextran,  $K_d(\text{Ca}^{2+})$  is significantly higher (typically  $\sim 3 \mu\text{M}$ ; batch-specific values are printed on the product label) than for unconjugated fluo-4, due to the structural modification required for linkage of the indicator to the dextran carrier.

## Applications

### Cell Loading Guidelines

The water-soluble salt forms of  $\text{Ca}^{2+}$  indicators may be loaded into cells by microinjection,<sup>7</sup> addition to patch pipette solutions<sup>11</sup> or using our Influx™ pinocytotic cell-loading reagent (I-14402). Fluo-4 dextran can be dissolved in water at concentrations up to 20% w/v (200  $\mu\text{g}/\mu\text{L}$ ) for microinjection. Coinjection of a reference marker (e.g. Texas Red-labeled 10,000 molecular weight dextran, D-1828) may be necessary for initial identification of labeled cells due to the intrinsically weak fluorescence of fluo-4 in the absence of  $\text{Ca}^{2+}$ .<sup>9</sup> 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>12,13</sup>

1. Dilute an aliquot of DMSO stock solution (1–5 mM) to a final concentration of 1–5  $\mu\text{M}$  in the buffered physiological medium of choice. Avoid amine-containing buffers such as Tris. 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) or 2 g of the solid (P-6867).

2. The organic anion-transport inhibitors probenecid (1–2.5 mM) or sulfapyrazone (0.1–0.25 mM) may be added to the cell

**Table 1.** Comparison of fluo-3 and fluo-4.

Property	fluo-3	fluo-4
$K_d(\text{Ca}^{2+})^*$	325 nM	345 nM
Absorption maximum ( $\text{Ca}^{2+}$ -bound) †	506 nm	494 nm
$\epsilon_{\text{max}}(\text{Ca}^{2+}$ -bound) †	100,000 $\text{cm}^{-1}\text{M}^{-1}$	88,000 $\text{cm}^{-1}\text{M}^{-1}$
$\epsilon_{488 \text{ nm}}(\text{Ca}^{2+}$ -bound) †	43,000 $\text{cm}^{-1}\text{M}^{-1}$	77,000 $\text{cm}^{-1}\text{M}^{-1}$
Emission maximum ( $\text{Ca}^{2+}$ -bound) †	526 nm	516 nm
QY ( $\text{Ca}^{2+}$ -bound) †, ‡	0.15	0.14
$F_{\text{max}}/F_{\text{min}} \S$	$>100$	$>100$

\* Dissociation constant for  $\text{Ca}^{2+}$  determined at 22°C in 100 mM KCl, 10 mM MOPS pH 7.2, 0 to 10 mM CaEGTA.  $K_d = 390 \text{ nM}$  for fluo-3 reported in Molecular Probes' *Handbook of Fluorescent Probes and Research Chemicals, Sixth Edition* (1996). † Value determined at 22°C in 100 mM KCl, 10 mM MOPS pH 7.2 containing 39.8  $\mu\text{M}$  free  $\text{Ca}^{2+}$ . ‡ QY = fluorescence quantum yield. QY = 0.18 for fluo-3 reported in J Biol Chem 264, 8171 (1989). § Fluorescence intensity increase on binding  $\text{Ca}^{2+}$ .

medium to reduce leakage of the de-esterified indicator.<sup>12,14</sup> Stock solutions of sulfinpyrazone and probenecid are necessarily quite alkaline; it is therefore important to readjust the pH of media to which they have been added.

3. 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 fluorescence signals with adequate signal to noise. Subcellular compartmentalization, an inherent problem with the AM ester loading technique, is usually lessened by lowering the incubation temperature.

4. Before fluorescence measurements are commenced, cells should be washed in indicator-free medium (containing an anion transport inhibitor, if applicable) to remove any dye that is non-specifically associated with the cell surface, and then incubated for a further 30 minutes to allow complete de-esterification of intracellular AM esters. Background fluorescence due to indicator leakage can be quenched by addition of anti-fluorescein antibody (A-889) to the external medium just before beginning the experiment.<sup>15</sup>

### Response Calibration

Absorption (fluorescence excitation) and fluorescence emission wavelength maxima are shown in Table 1 (wavelengths for fluo-5F, fluo-5N and fluo-4FF are essentially the same as for fluo-4). For fluorescence microscopy applications, Omega bandpass filter sets XF104 or XF23 and Chroma sets 41028 or 31001 are recommended for detection of fluo-3. Omega sets XF100 or XF23 and Chroma sets 41001 or 31001 are recommended for fluo-4, fluo-5F, fluo-5N and fluo-4FF. 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  $\text{Ca}^{2+}$  concentrations. Calibration solutions based on EGTA  $\text{Ca}^{2+}$  buffering<sup>16,17</sup> are

**Table 2.** Parallel performance comparison of fluo-4 and fluo-3 on Molecular Devices' FLIPR system.

Indicator/Concentration/Time *	Basal Fluorescence †	Stimulated Fluorescence † ‡	Increase §
fluo-3 / 4 $\mu$ M / 60 min	1700	5700	3.4 $\times$
fluo-4 / 4 $\mu$ M / 60 min	4900	21300	4.3 $\times$
fluo-4 / 2 $\mu$ M / 30 min	1200	5400	4.5 $\times$

\* CHO cells stably transfected with rat muscarinic M<sub>1</sub> receptors were loaded with fluo-3 AM and fluo-4 AM according to standard protocols (Molecular Devices Inc.), with variations in indicator concentration in the loading medium and incubation time as shown. † Relative fluorescence intensities after subtraction of microplate background. ‡ Cells were stimulated by addition of the muscarinic agonist carbachol (50  $\mu$ M). § Ratio of stimulated to basal fluorescence intensities.

supplied in a variety of convenient formats in Molecular Probes' Calcium Calibration Buffer Kits. The following equation is used to determine the ion dissociation constant ( $K_d$ ):

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

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  $[Ca^{2+}]_{free}$  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. For example, fluo-3 fluorescence in the nucleoplasm has been found to be twice that in the cytoplasm under conditions of normalized indicator and  $Ca^{2+}$  concentration.<sup>18</sup> In addition, BAPTA-based indicators such as fluo-3 and fluo-4 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).<sup>19</sup> *In situ* response calibrations of intracellular indicators typically yield  $K_d$  values significantly higher than *in vitro* determinations.<sup>10</sup> *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).<sup>15</sup> 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.<sup>12,20</sup>  $F_{min}$  and  $F_{max}$  are then calculated from known numerical relationships to the fluorescence intensity of the  $Mn^{2+}$ -saturated indicator ( $F_{Mn}$ ) (note **B**).

Quantitative  $Ca^{2+}$  measurements using fluo-3, fluo-4 and related indicators are hampered if there are significant  $Ca^{2+}$ -inde-

pendent 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.<sup>21</sup> Co-loading cells with a pair of spectrally contrasted indicators (typically fluo-3 and Fura Red) provides a way to circumvent this deficiency, providing that the relative intracellular concentrations of the two indicators are reasonably consistent from cell to cell.<sup>22,23</sup> 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.<sup>24</sup>

### High-Throughput Screening

Intracellular  $Ca^{2+}$  measurements in 96-well and 384-well microplates are an essential tool for high-throughput pharmacological screening.<sup>8,25,26</sup> Cell samples in microplate wells are loaded with the AM ester form of the indicator using protocols basically similar to those described in *Cell Loading Guidelines*. Parallel comparisons of measurements using fluo-4 and fluo-3 in Molecular Devices' FLIPR™ (Fluorometric Imaging Plate Reader) system show that fluo-4 generates the same fluorescence response to carbachol-stimulated  $Ca^{2+}$  activation in transfected CHO cells using half the loading concentration and half the incubation time. When fluo-4 AM is substituted directly for fluo-3 AM (i.e., identical loading protocols), fluorescence signals are at least doubled (Table 2).

### Notes

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

**[B]** Published applications of this procedure typically assume  $F_{Mn} = F_{max}/5$  and  $F_{max}/F_{min} = 40$  for fluo-3, whereas values of  $F_{max}/F_{min} > 100$  are typically obtained using current production batches (Table 1).<sup>10</sup>

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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
A-1493	A-23187 free acid (calcein) .....	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-6775	Calcium Calibration Buffer Kit #3 *1 µM to 1 mM range (11 x 10 mL)* .....	1 kit
C-3721	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
C-3722	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
F-1241	fluo-3, AM *cell permeant* .....	1 mg
F-1242	fluo-3, AM *cell permeant* *special packaging* .....	20 x 50 µg
F-14218	fluo-3, AM *1 mM solution in DMSO* *cell permeant* .....	1 mL
F-23915	fluo-3, AM *FluoroPure™ grade* *special packaging* .....	10 x 50 µg
F-14242	fluo-3, AM *packaged for high-throughput screening* .....	40 x 1 mg
F-1240	fluo-3, pentaammonium salt *cell impermeant* .....	1 mg
F-3715	fluo-3, pentapotassium salt *cell impermeant* .....	1 mg
F-14201	fluo-4, AM *cell permeant* *special packaging* .....	10 x 50 µg
F-14217	fluo-4, AM *1 mM solution in DMSO* *cell permeant* .....	500 µL
F-23917	fluo-4, AM *FluoroPure™ grade* *special packaging* .....	10 x 50 µg
F-14202	fluo-4, AM *packaged for high-throughput screening* .....	5 x 1 mg
F-14240	fluo-4 dextran, potassium salt, 10,000 MW, anionic .....	5 mg
F-14200	fluo-4, pentapotassium salt *cell impermeant* .....	500 µg
F-23981	fluo-4FF, AM *cell permeant* *special packaging* .....	10 x 50 µg
F-23980	fluo-4FF, pentapotassium salt *cell impermeant* .....	500 µg
F-14222	fluo-5F, AM *cell permeant* *special packaging* .....	10 x 50 µg
F-14221	fluo-5F, pentapotassium salt *cell impermeant* .....	500 µg
F-14204	fluo-5N, AM *cell permeant* *special packaging* .....	10 x 50 µg
F-14203	fluo-5N, pentapotassium salt *cell impermeant* .....	500 µg
I-14402	Influx™ pinocytic 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
T-1210	tetrakis-(2-pyridylmethyl)ethylenediamine (TPEN) .....	100 mg

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