Fluo Calcium Indicators

Quick Facts

Storage upon receipt:

- ≤ –20°C
- Desiccate
- Protect from light

Introduction

Since being introduced in 1989,[1] fluo-3 imaging has revealed the spatial dynamics of many elementary processes in Ca^{2+} signaling.[2-4] Fluo-3 has also been extensively used for flow cytometry;[5] for experiments involving photoactivation of "caged" chelators, second messengers, and neurotransmitters;[6,7] and for cell-based pharmacological screening.[8] 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^{2+} 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^{2+}-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. The Fluo-4 dextrans consist of fluo-4 coupled to a biologically inert dextran carrier (molecular weight = 10,000), providing a new and potentially valuable tool for measuring Ca^{2+} transients in presynaptic terminals arising from long axonal projections in heterogeneous fiber tracts.[9] Molecular Probes offers two reactive forms of Fluo-4, an iodoacetamide and a cadaverine. The fluo-4 iodoacetamide can react with sulfhydryl groups to form unique fluorescent Ca^{2+}-sensitive probes, including proteins, peptides, and thiol-modified surfaces. The amine-containing Fluo-4 cadaverine can react with aldehydes, ketones, and activated esters.

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, dextrans, or cadaverine; a thiol-reactive iodoacetamide; and also as cell-permeant acetoxymethyl (AM) esters, as detailed below:

Fluo products as 1 mg salts
- F1240, F3715

Fluo products as 500 mg salts
- F14200, F14221, F14203, F23980

Fluo-4 dextrans
- The Fluo-4 dextrans are supplied in solid form in 5 mg units (F14240, F36250)

Fluo-4 cadaverine
- The Fluo-4 cadaverine is supplied in 500 µg units (F36201)

Fluo-4 iodoacetamide
- The Fluo-4 iodoacetamide is supplied in 500 µg units (F36200)

Fluo-3, AM
- F1241, supplied in solid form in 1 mg units.
- F1242, supplied in sets of 20 vials, each containing 50 µg.
- F14218, supplied as 1 mL of ready-made 1 mM solution in DMSO.
- F14242, large quantities available for high-throughput screening. Supplied in sets of 40 vials, each containing 1 mg.
- F23915, special FluoroPure™ grade with ≥98% HPLC purity specification, supplied in sets of 10 vials, each containing 50 µg.

Fluo-4, AM
- F14201, supplied in sets of 10 vials, each containing 50 µg.
- F14202, large quantities available for high-throughput screening. Supplied in sets of 5 vials, each containing 1 mg.
- F14217, supplied as 500 µL of ready-made 1 mM solution in DMSO.
- F23917, 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 (F14222, F14204, and F23981, respectively).

After receipt, these products 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 di-methylsulfoxide (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 µM 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≥ µM for fluo-3 and fluo-4; ≥1 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 Ca^{2+}. Unlike the ultra-violet light–excited indicators fura-2 and indo-1, there is no accompanying spectral shift. The fluorescence intensity increase on Ca^{2+} binding is typically >100-fold.[10] A comparison of physical andspectroscopic 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 (Ca^{2+}) is 2.3 µM, 90 µM, and 9.7 µM, respectively, measured under the same conditions as the values reported in Table 1. For the fluo-4 dextrans, the K_d (Ca^{2+}) is somewhat higher than the free indicator; the high affinity version has a K_d (Ca^{2+}) of ~600 nM and the low affinity version has a K_d (Ca^{2+}) of ~3 µM (batch specific values are printed on the label).

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

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

* Dissociation constant for Ca^{2+} determined at 22°C in 100 mM KCl, 10 mM MOPS, pH 7.2, 0 to 10 mM CaEGTA. K_d = 390 nM for fluo-3 reported in Molecular Probes' The Handbook: A Guide to Fluorescent Probes and Labeling Technologies, Tenth Edition (2005). † Value determined at 22°C in 100 mM KCl, 10 mM MOPS, pH 7.2 containing 39.8 µM free 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 Ca^{2+} in a solution assay.

Applications

Cell Loading Guidelines

The water-soluble salt forms of Ca^{2+} indicators may be loaded into cells by microinjection,[7] addition to patch pipette solutions,[11] or using our Influx™ pinocytotic cell-loading reagent (I14402). Fluo-4 dextran can be dissolved in water at concentrations up to 20% w/v (200 µg/µL) for microinjection. Coinjection of a reference marker (e.g., Texas Red–labeled 10,000 molecular weight dextran, D1828) may be necessary for initial identification of labeled cells due to the intrinsically weak fluorescence of fluo-4 in the absence of Ca^{2+}.[9] 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.[12,13]

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 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 (P6866) or 2 g of the solid (P6867).

2. The organic anion-transport inhibitors probenecid (1–2.5 mM) or sulfinpyrazone (0.1–0.25 mM) may be added to the cell medium to reduce leakage of the de-esterified indicator.[12,14] 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 nonspecifically 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 (A889) to the external medium just before beginning the experiment.[15]

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

Indicator/ Concentration/ Time*	Basal Fluorescence †	Stimulated Fluorescence † ‡	Fold-increase §
Fluo-3 / 4 µM / 60 min	1700	5700	3.4
Fluo-4 / 4 µM / 60 min	4900	21300	4.3
Fluo-4 / 2 µM / 30 min	1200	5400	4.5

* CHO cells stably transfected with rat muscarinic M_1 receptors were loaded with fluo-3 AM and fluo-4 AM according to protocols supplied by 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 µM). § Ratio of stimulated to basal fluorescence intensities.

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). 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. Calibration solutions based on EGTA Ca^{2+} buffering[16,17] are 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):

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

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.[18] 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 (T1210).[19] *In situ* response calibrations of intracellular indicators typically yield K_d values significantly higher than *in vitro* determinations.[10] *In situ* calibrations are performed by exposing loaded cells to controlled Ca^{2+} buffers in the presence of ionophores such as A-23187 (A1493), 4-bromo A-23187 (B1494), or ionomycin (I24222).[15] 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.[12,20] 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+}-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.[21] 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.[22,23] 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.[24]

High-Throughput Screening

Intracellular Ca^{2+} measurements in 96-well and 384-well microplates are an essential tool for high-throughput pharmacological screening.[8,25,26] 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).

Thiol Reactions

Reaction conditions for the thiol-reactive fluo-4 iodoacetamide can be obtained from the product information sheet, *Thiol-Reactive Probes,* mp00003. Copies of this publication are available from our Web site (probes.invitrogen.com), Customer Service or Technical Service.

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).[10]

References

1. J Biol Chem 264, 8171 (1989); **2.** J Physiol 499, 307 (1997); **3.** Am J Physiol 270, C148 (1996); **4.** Cell 80, 259 (1995); **5.** Methods Cell Biol 41, 149 (1994); **6.** Biophys J 74, 523 (1998); **7.** J Neurosci 17, 1701 (1997); **8.** *Calcium Signaling Protocols (Methods in Molecular Biology, Volume 114)*, D. Lambert, Ed., pp 125–133, Humana Press (1999); **9.** Neuron 27, 25 (2000); **10.** Biophys J 65, 865 (1993); **11.** *Imaging Neurons: A Laboratory Manual*, R. Yuste, F. Lanni and A. Konnerth, Eds, pp 35.1–35.10, Cold Spring Harbor Laboratory Press (2000); **12.** Methods Cell Biol 40, 155 (1994); **13.** *Cell Biology: A Laboratory Handbook, 2nd Edition*, J.E. Celis, Ed., Volume 3, pp 363–374, Academic Press (1998); **14.** Cell Calcium 11, 57 (1990); **15.** J Biol Chem 266, 22975 (1991); **16.** Methods Cell Biol 40, 3 (1994); **17.** Methods Enzymol 172, 230 (1989); **18.** Cell Calcium 21, 275 (1997); **19.** Biophys J 71, 1048 (1996); **20.** J Immunol Methods 127, 197 (1990); **21.** Methods Cell Biol 30, 157 (1989); **22.** Cell Calcium 14, 359 (1993); **23.** Cell Calcium 18, 377 (1996); **24.** Biophys J 67, 1942 (1994); **25.** Anal Biochem 270, 242 (1999); **26.** Genet Eng News 19, 44 (1999).

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^{2+} (2 x 50 mL)*	1 kit
C3722	Calcium Calibration Buffer Kit with Magnesium #2 *zero to 10 mM CaEGTA with 1 mM Mg^{2+} (11 x 10 mL)*	1 kit
C3723	Calcium Calibration Buffer Concentrate Kit *zero and 100 mM CaEGTA (2 x 5 mL)*	1 kit
C6775	Calcium Calibration Buffer Kit #3 *1 µM to 1 mM range (11 x 10 mL)*	1 kit
F1240	fluo-3, pentaammonium salt *cell impermeant*	1 mg
F1241	fluo-3, AM *cell permeant*	1 mg
F1242	fluo-3, AM *cell permeant* *special packaging*	20 x 50 µg
F14200	fluo-4, pentapotassium salt *cell impermeant*	500 µg
F14201	fluo-4, AM *cell permeant* *special packaging*	10 x 50 µg
F14202	fluo-4, AM *packaged for high-throughput screening*	5 x 1 mg
F14203	fluo-5N, pentapotassium salt *cell impermeant*	500 µg
F14204	fluo-5N, AM *cell permeant* *special packaging*	10 x 50 µg
F14217	fluo-4, AM *1 mM solution in DMSO* *cell permeant*	500 µL
F14218	fluo-3, AM *1 mM solution in DMSO* *cell permeant*	1 mL
F14221	fluo-5F, pentapotassium salt *cell impermeant*	500 µg
F14222	fluo-5F, AM *cell permeant* *special packaging*	10 x 50 µg
F14240	fluo-4 dextran, potassium salt, 10,000 MW, anionic (low-affinity version)	5 mg
F14242	fluo-3, AM *packaged for high-throughput screening*	40 x 1 mg
F23915	fluo-3, AM *FluoroPure™ grade* *special packaging*	10 x 50 µg
F23917	fluo-4, AM *FluoroPure™ grade* *special packaging*	10 x 50 µg
F23980	fluo-4FF, pentapotassium salt *cell impermeant*	500 µg
F23981	fluo-4FF, AM *cell permeant* *special packaging*	10 x 50 µg
F36200	fluo-4 iodoacetamide, pentapotassium salt	500 µg
F36201	fluo-4 cadaverine, pentapotassium salt	500 µg
F36250	fluo-4 dextran, potassium salt, 10,000 MW, anionic (high-affinity version)	5 mg
F3715	fluo-3, pentapotassium salt *cell impermeant*	1 mg
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
T1210	tetrakis-(2-pyridylmethyl)ethylenediamine (TPEN)	100 mg

Contact Information

Further information on Molecular Probes products, including product bibliographies, is available from your local distributor or directly from Molecular Probes. Customers in Europe, Africa and the Middle East should contact our office in Paisley, United Kingdom. All others should contact our Technical Service Department in Eugene, Oregon.

Please visit our website — **probes.invitrogen.com** — for the most up-to-date information.

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