

Cyclic AMP Fluorosensor[®] (FICRhR)

C-6660 Cyclic AMP Fluorosensor[®] (FICRhR)

Quick Facts

Storage upon receipt:

- -20°C
- Avoid freeze-thaw cycles
- Protect from light

Note: For immediate use, the product may be thawed and stored at 4°C, protected from light.

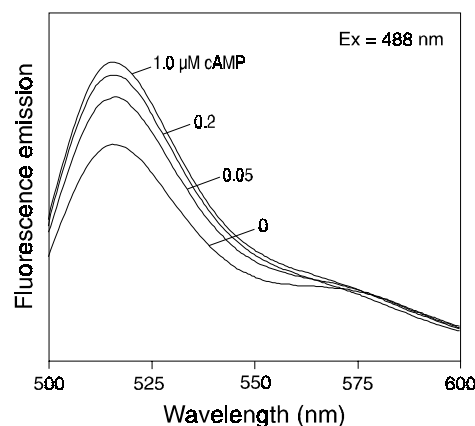


Figure 2. Emission spectra of Cyclic AMP Fluorosensor at indicated concentrations of cAMP.

Introduction

In collaboration with Atto Instruments, Inc., Molecular Probes is pleased to offer Cyclic AMP Fluorosensor[®] (FICRhR), the first fluorescent probe available for the non-destructive measurement of cyclic AMP (cAMP) in living cells by digital video imaging, confocal microscopy or microphotometry. Originally developed by Dr. Roger Tsien and co-workers,^{1,2} the probe consists of cAMP-dependent protein kinase A (PKA) in which murine recombinant catalytic (C) and type II regulatory (R) subunits are labeled with fluorescein and rhodamine, respectively. In the

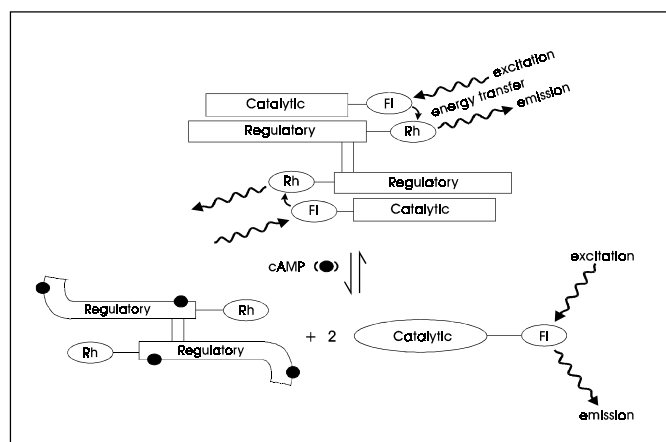


Figure 1. Schematic depiction of the structure and response mechanism of Cyclic AMP Fluorosensor (FICRhR). Fluorescence resonance energy transfer from the fluorescein-labeled (Fl) catalytic subunit to the rhodamine-labeled (Rh) regulatory subunit is eliminated upon cyclic AMP-dependent dissociation of the holoenzyme. The resulting increase of the 488 nm-excited fluorescein (520 nm) to rhodamine (580 nm) emission ratio can be used to measure intracellular cyclic AMP. Adapted from reference 1.

holoenzyme configuration (C_2R_2) the proximity of the fluorescein and rhodamine labels allows fluorescence resonance energy transfer (FRET) to occur, such that excitation at 488 nm results in sensitized rhodamine emission at about 580 nm, as well as directly excited fluorescein emission at about 520 nm (Figure 1).

As with native PKA, the two subunits reversibly dissociate upon binding cAMP (4 cAMP per C_2R_2), resulting in separation of the fluorophores such that FRET no longer occurs. Consequently, C-bound fluorescein emission increases and R-bound rhodamine emission decreases (Figure 2). The ratio of emission intensities measured at about 520 nm and 580 nm with excitation at 488 nm can therefore be quantitatively related to the concentration of cAMP.

Because Cyclic AMP Fluorosensor is a protein complex with an aggregate molecular weight of 172 kD, it must be pressure microinjected into the cytoplasm for intracellular cAMP measurements. Cyclic AMP Fluorosensor exhibits cAMP binding affinity and kinase activity similar to those of the unlabeled protein kinase.^{1,2} Therefore, it is capable of triggering the same downstream signaling upon binding cAMP as the native enzyme. Published applications of Cyclic AMP Fluorosensor by Tsien and co-workers include identification of the signaling pathway for cAMP activation of neuronal nicotinic acetylcholine receptors³ and comparison of Ca^{2+} (measured using fura-2) and cAMP as controlling factors for intracellular vesicle motility.⁴ The latter type of investigation is facilitated by the availability of high-speed digital imaging instrumentation for simultaneous imaging of cAMP and Ca^{2+} in the same cell.⁵ The fluorescent labeling of the kinase's C-subunit allows the translocation of this subunit to the nucleus to be tracked following cAMP-dependent dissociation from the R-subunit.^{6,7} Using FICRhR, translocation can be

correlated with subsequent transcription factor phosphorylation in cAMP-dependent gene regulation processes.⁸

Materials

Cyclic AMP Fluorosensor (C-6660) is provided in 10 μ L units at an average concentration of 20 μ M (lot-to-lot variation \pm 5 μ M) in 25 mM potassium phosphate, 1 mM EDTA, 0.5 mM β -mercaptoethanol, 2.5% glycerol, pH 7.3. The product has been filtered through an 0.22 μ m membrane filter and is ready for direct microinjection into living cells (see *Microinjection into Cells*). The products are shipped frozen on dry ice and should be kept at -20°C , protected from light, for prolonged storage. For immediate use, the products may be thawed and stored at 4°C , protected from light. **Repeated freezing and thawing should be avoided.** If stored properly, Cyclic AMP Fluorosensor should be stable and responsive to cAMP (Figure 2) for at least 3 months.

Experimental Application

Microinjection into Cells

1.1 Preparation of Cells. Cultured cells can be grown on 25 mm round glass coverslips and then inserted in a special Attofluor[®] cell chamber (available from Molecular Probes, A-7816) which firmly holds the coverslip and provides a positive sealing action to prevent leakage of solution onto the microscope. Microinjection is then performed in the medium of choice, making sure that the medium contains sufficient buffer to maintain the pH while the cells are being microinjected.

1.2 Injection Apparatus. Microinjection apparatus capable of delivering a starting injection pressure (P_i) of about 100–150 hPa and a capillary holding pressure of about 50–75 hPa is recommended. Both these parameters need to be adjusted depending upon the pipet tip diameter, the concentration of Cyclic AMP Fluorosensor to be injected and the resistance of the cell. The Eppendorf Transjector 5246 and Micromanipulator 5171 system works quite well.

1.3 Pipet Tips. The microinjection pipet tip opening should be adjusted depending upon the size and the sensitivity to the injection procedure of the cell type being studied. For general-purpose microinjection of cells >20 μ m in diameter, a pipet tip opening of about 0.5 μ m is usually appropriate. Although thinner tips would be preferable, concentrated Cyclic AMP Fluorosensor tends to clog them more easily.² Thin-walled 1.0 mm borosilicate glass capillaries incorporating a microfilament for back-filling (such as TW 100F capillaries from World Precision Instruments, Inc.) are suitable for preparing microinjection needles. A Flaming/Brown horizontal-type pipet puller, such as Model P-97 from Sutter Instrument Co. fabricates reproducible micropipets and offers the possibility of a variety of pulling programs to better fit the individual cell type injection needs. Alternatively, prefabricated micropipets (Femtotips, Eppendorf No. 5242 952.008, opening diameter = 0.5 μ m \pm 0.2 μ m) can be used. Microloaders (Eppendorf No. 5242 956.003) are needed to load Femtotips, while standard polypropylene 0.5–10 μ L microtips are suitable for back-loading custom-pulled glass pipets.

1.4 Microinjection Parameters. With either custom-made or prefabricated pipets, <0.5 μ L of the 20 μ M Cyclic AMP Fluorosensor stock solution supplied is generally required to load the micropipet tip (note A). With optimal microinjection parameters, this amount of Cyclic AMP Fluorosensor is sufficient, on average, to inject more than 100 cells. Injection of the stock solution will produce a final approximate intracellular concentration of 0.2–2 μ M assuming an injection volume equal to 1–10% of the total cell volume.² A minimal but continuous flow of the stock solution from the tip of the microinjection needle is usually optimal, and will minimize clogging of the pipet (note B). Photobleaching of Cyclic AMP Fluorosensor during microinjection can be prevented by performing the procedure in red light. This can be accomplished by inserting a >600 nm longpass filter in the microscope transmitted light illumination pathway. The microinjection process can be readily observed using a fluorescence microscope (e.g. Zeiss Axiovert[®] 135 microscope with phase contrast optics and a 32X 0.4 NA Zeiss LD Acrostigmat objective). After cells are microinjected it is important to wash them with fresh medium. This reduces background fluorescence by removing any Cyclic AMP Fluorosensor that has leaked into the medium, either from the pipet or from cells that were injured during microinjection and subsequently died.

Microscopy and Imaging Configuration

2.1 Excitation and Emission Filters. Fluorescence ratio imaging using Cyclic AMP Fluorosensor requires excitation at 488 nm and measurement of the 520/580 nm fluorescence emission intensity ratio (note C). Spectral characteristics of suitable optical filters are shown in Figure 3 in relation to emission spectra of Cyclic AMP Fluorosensor with and without cAMP.

2.2 Digital Fluorescence Imaging Configuration. The Carl Zeiss[®] Attofluor RatioVision[®] hardware and software has been successfully used for simultaneous dual emission and dual excitation imaging of cAMP and Ca^{2+} using Cyclic AMP Fluorosensor and fura-2 in a variety of cultured cells.⁵ The system consists of a Zeiss Axiovert 135 microscope (Fluar 40X or 100X 1.3 NA, oil immersion objective) with either the standard Attofluor RatioVision hardware and software (consisting of an excitation wavelength filter selector with 10 nm bandpass filters centered at 334, 380, 460 and 488 nm) or an Attofluor RatioArc[®]

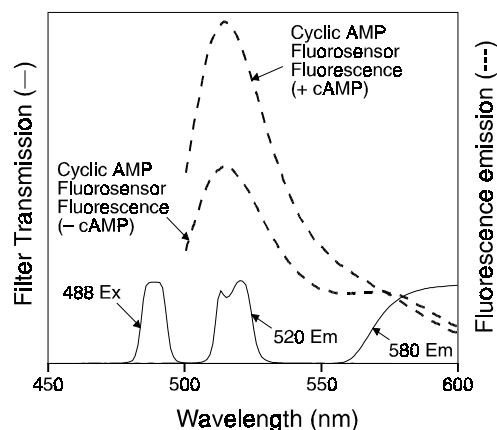


Figure 3. Composite spectral curves of excitation and emission filters used for imaging Cyclic AMP Fluorosensor (—) superimposed on emission spectra of the probe with and without cAMP (---).

excitor, which allows for the electronic control of excitation intensity and real-time 30 ratio/second dual wavelength excitation rates (note C). Illumination is provided by a 100W mercury arc lamp. A Zeiss FT510 dichroic reflector is used to separate the excitation light, which is below 510 nm from the emission light at >515 nm. Emission filters for 520 nm and 580 nm (>570 nm longpass) are inserted in the respective Attofluor ICCD emission cameras.

2.3 Dual Emission Camera Alignment. Emission ICCD cameras need to be rigorously aligned to ensure accuracy of dual emission ratio imaging results. Dual ICCD cameras mounted on the Zeiss Axiovert 135 inverted microscope within the Attofluor RatioVision digital imaging workstation allow for an absolute alignment (within 1 pixel of one another) of the 520 and 580 nm emission images. Dual emission ICCD cameras are aligned before each days experiments; the cameras should be given an ~20 min warm-up period before this procedure is undertaken. Prepare a mixture of 0.1–0.2 mM carboxyfluorescein (C-1904) and the same concentration of carboxytetramethylrhodamine (C-300) in water (1:1 molar ratio of dyes) to mimic the fluorescence emission of the fluorescein- and rhodamine-labeled Cyclic AMP Fluorosensor. A 5–10 μ L drop of this fluorescent mixture is placed in the center of a glass coverslip inserted in the Attofluor Cell Chamber, positioned on the microscope stage and analyzed with a Fluor 40X 1.3 NA oil immersion objective or with the objective to be used during the experiment. While simultaneously observing the fluorescence emission ratio using the Attofluor RatioVision system and the image plane reticle, finely adjust the camera alignment micrometers so that the reticles from the 520 nm and 580 nm images are superimposed.

2.4 Calibration. Because methods for controlling intracellular free cAMP concentrations at intermediate levels between zero and saturating have yet to be devised, complete cAMP response calibration of the Cyclic AMP Fluorosensor is currently only possible *in vitro*.² Microfluorometric *in vitro* calibration methods are preferred, allowing the use of micromolar Cyclic AMP Fluorosensor concentrations without consuming large amounts of valuable material (note D). A small aliquot of Cyclic AMP Fluorosensor (~1–5 μ M) is either sandwiched as a thin film between two coverslips with 0 or 1 mM added cAMP or confined inside a microdialysis capillary (9 kD cutoff) and exposed to various cAMP concentrations.^{2,6} In both cases, Cyclic AMP Fluorosensor emission response is measured directly on the microscope stage using the same instrument configuration to be used for experimental measurements (steps 2.1–2.3). The free cAMP concentration is obtained from the total cAMP concentration by subtracting a correction for bound cAMP, calculated from the percentage cAMP saturation of the Cyclic AMP Fluorosensor and the sensor concentration, assuming 4 moles of cAMP bound per mole of dissociated holoenzyme. Note that the usual Hill formula used for analysis of fura-2 and indo-1 fluorescence ratio data⁹ must be modified to take account of cooperativity in

the binding of cAMP to Cyclic AMP Fluorosensor.² The apparent cAMP dissociation constant of Cyclic AMP Fluorosensor determined using these methods is typically about 0.1 μ M.^{1,2} For *in vivo* calibration, a representation of the zero intracellular free cAMP ratio is obtained by coinjecting ~1 mM R_p -adenosine cyclic 3',5'-phosphorothioate (a commercially available cAMP antagonist) with Cyclic AMP Fluorosensor. Saturating intracellular cAMP levels can be obtained by incubation of cells for 15 minutes with 10 μ M dibutyryl cAMP AM, a cell-permeant cAMP derivative.¹⁰

Notes

[A] Bacterial contamination of the stock solution of Cyclic AMP Fluorosensor could occur when dipping many non-sterile microinjection pipet tips into the stock solution. To restrict possible contamination, the stock can be aliquoted into small portions to be used separately.

[B] If increasing difficulty in microinjection occurs, centrifugation at 16,000 \times g at 4°C for 20 minutes generally gives a satisfactory improvement.

[C] The fluorescein labels incorporated in Cyclic AMP Fluorosensor are much more susceptible to photobleaching than the rhodamine labels. Consequently, fluorescein photobleaching may result in artifactual changes in the 520/580 nm emission ratio. Photobleaching is a function of the frequency, duration and intensity of illumination. Therefore the maximum rate of data acquisition will depend upon the amount of photobleaching that is tolerable during an experiment.

[D] The labeled holoenzyme may slowly dissociate at concentrations below 10 nM in the **absence** of cAMP. Therefore, calibration procedures using mL volumes of highly diluted Cyclic AMP Fluorosensor in standard 10 mm \times 10 mm fluorescence cuvettes are generally not recommended.

Suppliers of Related Equipment and Materials

- High speed digital imaging and photometry systems
Atto Instruments, Inc., Rockville, MD
phone: (301) 340-7320; fax: (301) 340-9775.
Carl Zeiss, Inc., Thornwood, NY
phone: (800) 233-2343; fax: (914) 681-7446.
- Microinjection apparatus and supplies
Eppendorf Scientific, Inc., Madison, WI
phone: (800) 421-9988; fax: (608) 231-1339.
Sutter Instrument Co., Novato, CA
phone: (415) 883-0128; fax: (415) 883-0572.
World Precision Instruments, Inc., Sarasota, FL
phone: (813) 371-1003; fax: (813) 377-5428.

References

1. Nature 349, 694 (1991);
2. Adams, S.R. *et al.*, in *Fluorescent and Luminescent Probes for Biological Activity*, pp.133–149, W. T. Mason and G. Relf, Eds., Academic Press, London, (1993);
3. J Neurosci 14, 3540 (1994);
4. J Cell Biol 117, 57 (1992);
5. Proc Natl Acad Sci USA 93, 4577 (1996);
6. Science 260, 222 (1993);
7. Mol Biol Cell 4, 993 (1993);
8. Mol Cell Biol 13, 4852 (1993);
9. J Biol Chem 260, 3440 (1985);
10. J Biol Chem 268, 6316 (1993).

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

Cat #	Product Name	Unit Size
C-6660	Cyclic AMP Fluorosensor [®] (FICRhR) *20±5 µM*	10 µL

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 Leiden, the Netherlands. All others should contact our Technical Assistance Department in Eugene, Oregon.

Please visit our Web site — www.probes.com — for the most up-to-date information

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