BCECF

Introduction

BCECF (2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein), introduced by Roger Tsien and co-workers in 1982,[1] is the most widely used fluorescent indicator for intracellular pH. Several characteristics contribute to this widespread utility:

- The pK_a of 7.0 is ideally matched to the normal range of cytoplasmic pH (~6.8–7.4).
- The fluorescence excitation profile is pH-dependent (Figure 1), allowing the implementation of ratiometric measurement techniques (see *Intracellular pH Calibration*).
- The absorption maximum of the base form of BCECF is very close to the 488-nm argon-ion laser line, making it ideally suited for flow cytometry [2] and confocal microscopy [3] applications.
- BCECF has 4–5 negative charges at pH 7–8, aiding intracellular retention.
- The acetoxymethyl (AM) ester derivative is membrane-permeant, allowing noninvasive bulk loading of cell suspensions.
- BCECF AM is nonfluorescent. Its conversion to fluorescent BCECF via the action of intracellular esterases can be used as an indicator of cell viability.

Some characteristics of BCECF are less than optimal. Firstly, the fluorescence excitation isosbestic point is quite far from the excitation maximum (Figure 1), giving poor signal-to-noise characteristics in ratio imaging microscopy.[4,5] Secondly, pH-dependent changes in the fluorescence emission spectral profile are very small, so that although dual emission ratio measurements are possible,[2] they are not often performed.

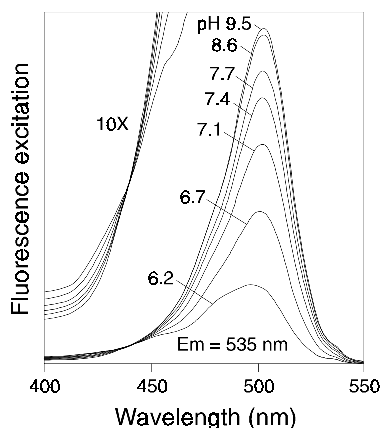

Figure 1. *The pH-dependent fluorescence excitation spectra of BCECF. The 10X enlargements of the region below 470 nm clearly illustrate the excitation isosbestic point at ~439 nm.*

Our bibliography on BCECF AM, listing hundreds of published applications, is continually updated and is available at our Web site (www.probes.com). Although applications in mammalian cells [6-8] are predominant, BCECF has also been employed for pH measurements in perfused tissues,[9-11] intercellular spaces,[12,13] plant cells,[14,15] bacteria [16,17] and yeast.[18,19] BCECF AM has also been employed in assays for various functional properties of cells including viability and cytotoxicity,[20-24] apoptosis,[25] adhesion,[26-31] multidrug resistance [32-34] and chemotaxis.[27]

Molecular Probes also offers microinjectable BCECF dextrans (D-1878), which exhibit excellent intracellular retention, reduced compartmentalization and a much lower degree of phototoxicity compared to BCECF AM.[35] Our 10,000 MW BCECF dextran (D-1878) was used with patch-pipette techniques to measure nuclear and cytosolic pH in frog neurons.[36]

Materials

BCECF AM is supplied in 1 mg units (B-1150), in 20 × 50 µg units (B-1170), and in 1 mL units of 1 mg/mL (~1.6 mM) solution in *anhydrous* dimethylsulfoxide (DMSO) (B-3051). Stock solutions should be prepared by dissolving the solid material in *anhydrous* DMSO at 1–10 mM. Both solid material and DMSO stock solutions should be stored desiccated at -20°C. With the 1 mg solid units (B-1150), it is often convenient to dissolve the entire contents in DMSO and divide this stock solution into small aliquots prior to storage. Dilute working solutions in aqueous media (see *Loading in Mammalian Cells*) should be used immediately and should not be stored. BCECF AM is normally colorless and nonfluorescent (although faint color and fluorescence are tolerable). Solutions exhibiting strong fluorescence and coloration (indicated by absorbance at >400 nm) probably contain a significant amount of hydrolyzed material and should be discarded. Stock solutions of BCECF AM should be stable for at least 6 months if prepared and stored as directed above.

BCECF (B-1151) free acid may be stored at room temperature, protected from light until required for use. Stock solutions may be prepared in aqueous buffers with pH >6. BCECF dextran (D-1878) should be stored in solid form at -20°C, protected from light. Most aqueous buffers are suitable for preparation of stock solutions of these conjugates.

Molecular Structures

Several different molecular species can be obtained in synthetic preparations of BCECF AM (Figure 2). BCECF AM prepared by Molecular Probes is predominately a mixture of Forms II and III (Figure 2). Our quality control specifications require that the summation of the different BCECF AM structural forms

constitutes >90% of the total product (determined by HPLC). Because the molecular weights of the species are different, the effective molecular weight for each individual production lot is subject to minor variations. Lot-specific molecular weight values, based on HPLC and NMR composition analysis, are reported on the product's label. All three forms of BCECF AM shown in Figure 2 appear to be converted to the same product — BCECF acid (B-1151) — by intracellular esterase hydrolysis.

Applications

Loading in Mammalian Cells

Most mammalian cells can be loaded without permeabilization by incubation with dilute aqueous dispersions of cell-permeant BCECF AM. Once within the cell, nonspecific esterases hydrolyze the nonfluorescent AM ester precursor, yielding the fluorescent, pH-sensitive indicator. The low leakage rate of the polyanionic indicator and the small intracellular volume results in the final intracellular concentration being much higher than the external incubation concentration. In general, BCECF loaded via the AM ester method appears less susceptible to intracellular compartmentalization than calcium indicators such as fura-2.[38] A variety of physical evidence has been assembled indicating that BCECF remains free and dissociated within the cytoplasm.[4,39] The following protocol is recommended as an approximate guide to the loading conditions for BCECF AM:

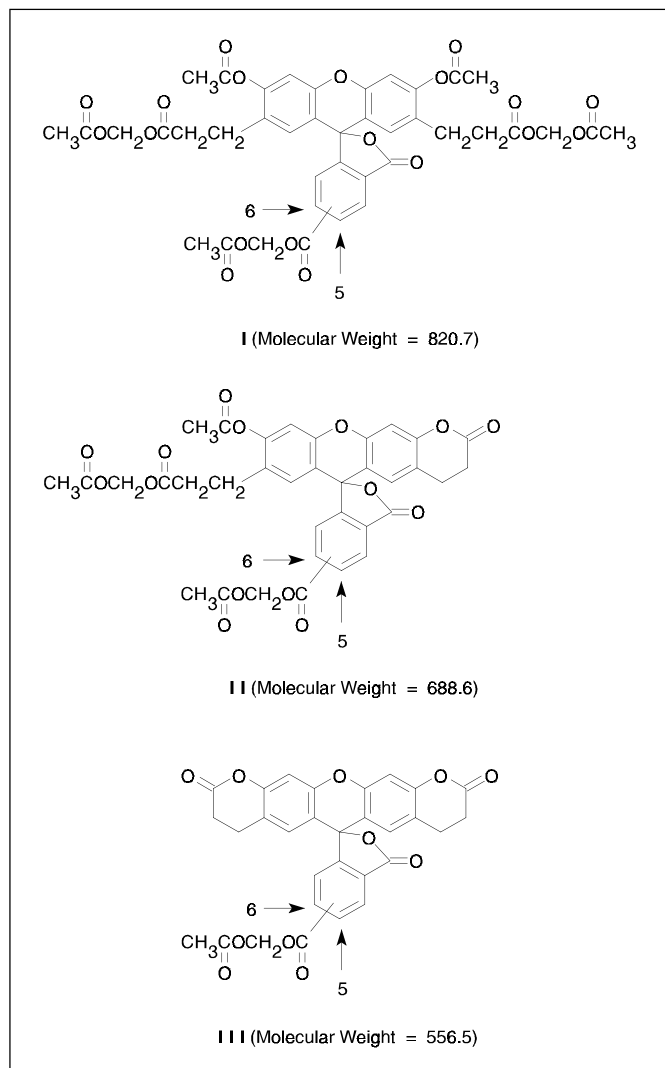

Figure 2. Molecular structures of BCECF, AM.

1. Prepare viable cells in suspension (~10^6 cells/mL) (note **A**).

2. Dilute an aliquot of 1 mM AM ester stock solution (see *Materials*) 100- to 500-fold into a physiological saline buffer such as Hanks' buffered salt solution (HBSS). In general, the minimum concentration of AM ester necessary to obtain an adequate signal should be used (incubation concentrations as low as 0.1 µM may be sufficient) to minimize accumulation of the by-products of AM ester hydrolysis (formaldehyde and acetic acid). The loading medium should be free of amino acids or buffers containing primary or secondary amines, since aliphatic amines may cleave the AM esters and prevent loading. The presence of serum, which may contain endogenous esterase activity, should also be avoided until after loading is complete.

3. Add one volume of aqueous AM ester dispersion to one volume of cell suspension. Incubate for 15–60 minutes at 4°C to 37°C.

4. Wash the cells twice with fresh culture medium.

Application to Tissue Samples

Generally similar BCECF AM loading protocols to that described in *Loading in Mammalian Cells* have been successfully applied to a variety of tissue samples including rat arteries;[40,41] rat salivary glands and pancreas;[9] rabbit kidney collecting tubules[8] and rabbit gastric glands.[11] In most of these experiments, the tissue sample is mounted in a perfusion chamber[38] and BCECF AM (~1–5 µM) is added to the perfusate for 5–60 minutes, followed by extensive washing with unmodified perfusate. For pH measurements in intercellular spaces, loading can be performed by direct injection of a brief pulse of 0.2–0.5 mM BCECF acid (B-1151).[12,13] BCECF acid (50–100 µM) can be loaded into isolated cells or tissue slices by diffusion from a patch pipette for correlated fluorescence imaging and electrophysiological recording.[41,42]

Loading in Other Cell Types

Bacteria: Both gram-positive and gram-negative bacteria have been loaded with BCECF by subjecting a dense cell suspension to a brief acid shock (50 mM HCl for 5 minutes) in the presence of 0.5 mM BCECF acid (B-1151).[16,17] The indicator appears to be well retained in *Lactococcus lactis* incubated on ice; however energization with lactose stimulates rapid efflux.[17]

Yeast and Fungi: Attempts to load the yeast *Saccharomyces cerevisiae* by incubation with 10 µM BCECF AM do not yield particularly good uptake of the indicator, apparently because of inefficient AM ester hydrolysis by the yeast intracellular esterases.[18,19] Carboxy SNARF-1 AM acetate reportedly shows much better uptake in this case.[19] Loading of the fungus *Neurospora crassa* with BCECF AM reportedly results in accumulation of the indicator in vacuoles instead of an even cytoplasmic distribution.[43]

Plants: Loading of suspended protoplasts (2×10^6/mL) from the crabgrass *Digitaria sanguinalis* by incubation with low concentrations of BCECF AM (10 nM) produces an even cytosolic distribution of the indicator.[14] Higher incubation concentrations

(3 µM) used to load maize root hair cells result in vacuolar accumulation.[15]

Intracellular pH Calibration

The pH-dependent spectral shifts exhibited by BCECF (Figure 1) allow calibration of the pH response in terms of the **ratio** of fluorescence intensities measured at two different excitation wavelengths (equation 1).[44] A number of fluorescence measurement artifacts are eliminated in formulation of the ratio, including photobleaching, leakage and nonuniform loading of the indicator, cell thickness and instrument stability.[4,5]

$$[H+] = K_a \frac{(R-R_A)}{(R_B-R)} \times \frac{F_{A(\lambda 2)}}{F_{B(\lambda 2)}} \quad (1)$$

where R is the ratio $F_{(\lambda 1)}/F_{(\lambda 2)}$ of fluorescence intensities (F) measured at two wavelengths λ1 and λ2 and the subscripts A and B represent the limiting values at the acidic and basic endpoints of the titration, respectively. Note that background fluorescence corrections should be subtracted **before** calculation of R. A typical BCECF calibration would use a dual-excitation ratio with λ1 = 490 nm and λ2 = 440 nm and fixed emission at 535 nm. Dual-excitation filter sets for fluorescence microscopy applications are available from Omega Optical Inc. (www.omegafilters.com, set XF16) and Chroma Technology Corp. (www.chroma.com, set 71001).

For confocal microscopy, helium–cadmium laser output at 442 nm has been used for excitation in combination with the 488 nm argon-ion laser line.[3,45] Note that selection of λ2 at the pH-independent isosbestic point (~439 nm for BCECF) eliminates the normalization factor $F_{A(\lambda 2)}/F_{B(\lambda 2)}$ from equation (1). Although the emission spectral profile of BCECF is much less pH-dependent than that of the excitation spectrum, 525/640 nm fluorescence emission ratios (excited at 488 nm) are occasionally used for flow cytometric pH measurements.[2,25]

The logarithmic form of equation (1) is:

$$pH = pK_a + \log\left[\frac{(R- R_A)}{(R_B - R)} \times \frac{F_{A(\lambda 2)}}{F_{B(\lambda 2)}}\right] \quad (2)$$

In this form, the data should yield a linear plot with a slope of 1 and an intercept equal to the pK_a. Calibrating the fluorescence response of BCECF to pH-controlled buffers *in vitro* yields a pK_a of 7.0.[1,44] However, since the response may be somewhat different when the dye is loaded in cells,[38] *in situ* calibration is generally advisable for each experimental system. *In situ* calibration can be performed by using the ionophore nigericin (N-1495) at a concentration of 10–50 µM in the presence of 100–150 mM potassium to equilibrate the intracellular pH with the controlled extracellular medium.[37,46] A number of alternative *in situ* calibration methods for BCECF have been described in the literature.[47,48]

Cell Function Assays

Cytotoxicity assays using BCECF AM are based on the generation of fluorescence by intracellular esterase action and its retention correlated with membrane integrity as indicators of cell viability. Cells are loaded with BCECF AM following protocols similar to that described in *Loading in Mammalian Cells*, and cytotoxicity is expressed in terms of either the fluorescence remaining associated with the cells or the fluorescence released into the supernatant, relative to the initial cellular fluorescence prior to the cytotoxic treatment.[20,22,24] Cytotoxicity assays using BCECF are suitable for implementation on high-throughput fluorescence microplate readers and provide rapid and convenient alternatives to ^{51}Cr release assays.[22] Cell adhesion assays using BCECF AM employ similar principles. In a typical example,[30] CHO cells were seeded in microplate wells, to which were then added murine R1.1 cells loaded by incubation with 2 µM BCECF AM at 37°C for 30 minutes. After incubation for 20 minutes, the plates were washed and the number of R1.1 cells bound was determined by proportionality to the remaining BCECF fluorescence measured using a fluorescence microplate reader.

Notes

[A] Conditions for loading adherent cells are basically similar to those given for cells in suspension. Adherent cells in culture dishes may be immersed in AM ester loading solution in physiological saline (step 2). To load cells grown on coverslips, simply transfer the coverslips to a dish containing BCECF AM loading medium after gently pouring off the maintenance medium. Adherent cultures do not need to be lifted for loading.

References

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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
B-1151	2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein (BCECF acid) *mixed isomers*	1 mg
B-1150	2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein, acetoxymethyl ester (BCECF, AM)	1 mg
B-1170	2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein, acetoxymethyl ester (BCECF, AM) *special packaging*	20 x 50 µg
B-3051	2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein, acetoxymethyl ester (BCECF, AM) *1 mg/mL solution in dry DMSO*	1 mL
D-1878	dextran, BCECF, 10,000 MW, anionic	10 mg
N-1495	nigericin, free acid	10 mg

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