continued from previous page

Calibration

Ion Dissociation Constants

The dissociation constant (K_d) is the key conversion parameter linking fluorescence signals to ion concentrations, assuming that the indicator is operating as an equilibrium sensor. This conventional assumption requires that the concentration of the indicator is close to the K_d value. Because intracellular indicator concentrations can easily reach 10–100 µM, even if the externally applied concentration is only 1–10 µM, this assumption is not always valid.[37] For pH indicators, K_d is conventionally expressed as its negative log (pK_a). The concentration range over which an indicator produces an observable response is approximately 0.1 × K_d to 10 × K_d. For ratiometric measurements, the response range also depends on wavelength-dependent parameters.[38,39] For BAPTA-based Ca^{2+} indicators in particular, the K_d is very sensitive to a number of environmental factors, including temperature, pH, ionic strength and interactions of the indicator with proteins.[40–43] Examination of published data shows that values of K_d determined *in situ* within cells can be up to 5-fold higher than values determined *in vitro*[3,40,44–46] (Table 19.2), underscoring the importance of performing calibrations to determine the K_d directly in the system under study.

Calibration Methodology

Calibration procedures basically consist of recording fluorescence signals corresponding to a series of precisely manipulated ion concentrations. The resulting sigmoidal titration curve is either linearized by means of a Hill plot or analyzed directly by nonlinear regression to yield K_d. For *in vitro* calibrations of Ca^{2+} indicators, EGTA buffering is widely used to produce defined Ca^{2+} concentrations that can be calculated from the K_d of the Ca^{2+}-EGTA complex.[24,47,48] This technique is used in the Calcium Calibration Buffer Kits (Section 19.8). *In situ* calibrations of intracellular indicators generally utilize an ionophore to equilibrate the controlled external ion concentration with the ion concentration within the cell.[4,49] Commonly used ionophores include:

- A-23187 (A1493), 4-bromo A-23187 (B1494) or ionomycin (I24222) for Ca^{2+} and Mg^{2+} (Section 19.8)
- Nigericin (N1495; Section 20.2, Section 21.2) for H^+ and Cl^-
- Gramicidin (G6888, Section 21.1) for Na^+
- Valinomycin (V1644, Section 21.1) for K^+

Ratiometric Calibration

Indicators that show an excitation or emission spectral shift upon ion binding can be calibrated using a ratio of the fluorescence intensities measured at two different wavelengths, resulting in the cancellation of artifactual variations in the fluorescence signal that might otherwise be misinterpreted as changes in ion concentration (Figure 2). Note that background levels must be subtracted from the component fluorescence intensities *before* calculation of the ratio. Examples of indicators exhibiting ion-dependent spectral shifts include the Ca^{2+} indicators fura-2 (Figure 3) and indo-1 (Section 19.2), and the pH indicators BCECF and SNARF®-1 (Section 20.2). The ratio of two intensities with opposite ion-sensitive responses (for example, 340 nm/380 nm in Figure 3) gives the largest possible dynamic range of ratio signals for a particular indicator. Alternatively, the ratio of an ion-sensitive intensity to an ion-insensitive intensity (measured at a spectral isosbestic point, e.g., 360 nm in Figure 3) can be used (Figure 2). Ratiometric measurements reduce or eliminate variations of several determining factors in the measured fluorescence intensity, including indicator concentration, excitation path length, excitation intensity and detection efficiency.[50,51] Artifacts that are eliminated include photobleaching and leakage of the indicator, variable cell thickness, and nonuniform indicator distribution within cells (due to compartmentalization) or among populations of cells (due to loading efficacy variations).

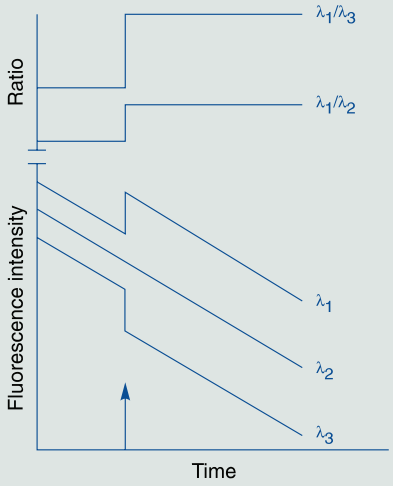


Figure 2 Simulated data demonstrating the practical importance of ratiometric fluorescence techniques. This figure represents an ion indicator that exhibits a fluorescence intensity increase in response to ion binding at wavelength λ_1 and a corresponding decrease at λ_3. Fluorescence measured at an isosbestic point (λ_2) is independent of ion concentration. The intracellular indicator concentration diminishes rapidly due to photobleaching, leakage (assuming the extracellular indicator is not detectable) or some other process. The change of intracellular ion concentration due to a stimulus applied at the time indicated by the arrow is unambiguously identified by recording the fluorescence intensity ratios λ_1/λ_3 or λ_1/λ_2.

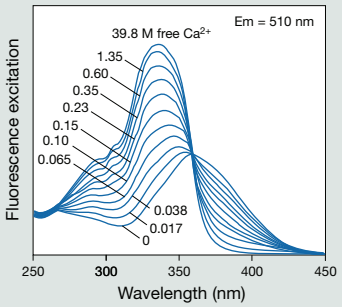
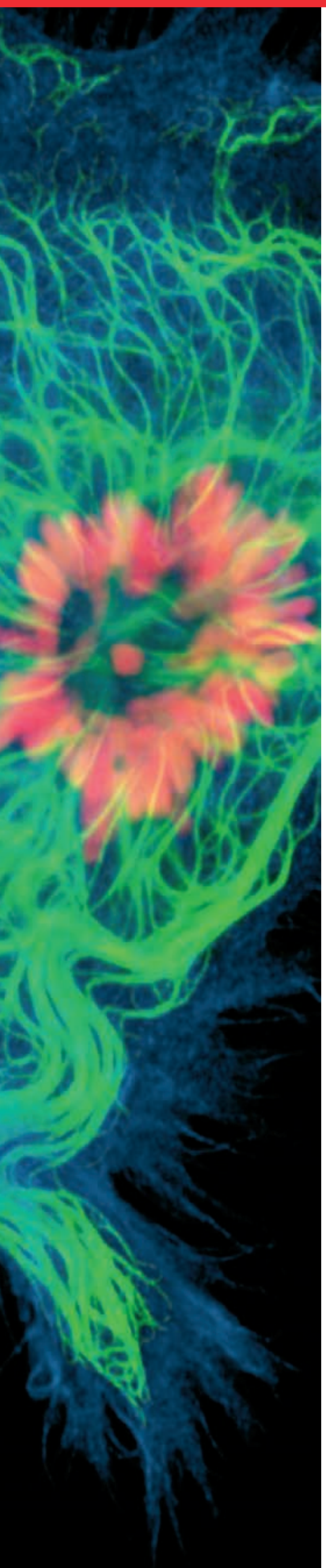


Figure 3 Fluorescence excitation spectra of fura-2 (F1200, F6799) in solutions containing 0–39.8 µM free Ca^{2+}

1. Methods Cell Biol (2007) 81:415; **2**. Methods (2008) 46:143; **3**. Cell Calcium (2000) 28:213; **4**. Methods Mol Biol (2006) 312:229; **5**. Methods Mol Biol (2009) 489:93; **6**. Nat Protoc (2006) 1:380; **7**. Nature (1981) 290:527; **8**. J Biol Chem (1987) 262:8884; **9**. Nat Protoc (2009) 4:862; **10**. Neuron (2007) 53:789; **11**. J Neurophysiol (2005) 93:1793; **12**. Cytometry (1997) 28:316; **13**. Biotechniques (2002) 33:358; **14**. Bioconjug Chem (2009) 20:249; **15**. Org Biomol Chem (2008) 6:4516; **16**. Nano Lett (2004) 4:2019; **17**. Plant J (2006) 46:327; **18**. J Neurosci Methods (2009) 184:332; **19**. J Neurosci Methods (2005) 141:41; **20**. Methods (2003) 30:79; **21**. Biochem J (1994) 302:5; **22**. CSH Protoc (2009) 2009:pdb.prot5201; **23**. Proc Natl Acad Sci U S A (2001) 98:4295; **24**. Methods Cell Biol (1994) 40:155; **25**. Cell Calcium (1990) 11:57; **26**. J Exp Biol (1994) 196:419; **27**. J Microsc (1992) 166:57; **28**. Cell Calcium (1990) 11:63; **29**. Am J Physiol (1988) 255:C304; **30**. Anal Biochem (1988) 169:159; **31**. Biophys J (1994) 67:476; **32**. Biophys J (1993) 65:561; **33**. J Microsc (2007) 226:74; **34**. J Neurosci Methods (2008) 167:140; **35**. J Biol Chem (1993) 268:21493; **36**. Anal Biochem (2009) 390:212; **37**. Mol Pharmacol (2002) 62:618; **38**. Cell Calcium (1991) 12:29; **39**. Cell Calcium (1998) 24:17; **40**. Biophys J (1994) 67:1646; **41**. Biophys J (1992) 63:89; **42**. Biochem Biophys Res Commun (1991) 180:209; **43**. Biochem Biophys Res Commun (1991) 177:184; **44**. Cell Calcium (2003) 34:1; **45**. Biophys J (1995) 68:1453; **46**. Biophys J (1993) 65:865; **47**. Methods Enzymol (1989) 172:230; **48**. Cell Calcium (1991) 12:279; **49**. Cell Calcium (1997) 21:233; **50**. Methods Cell Biol (1989) 30:157; **51**. J Biol Chem (1985) 260:3440.

invitrogen



Molecular Probes™ Handbook

A Guide to Fluorescent Probes and Labeling Technologies

11th Edition (2010)

CHAPTER 20

pH Indicators

Molecular Probes Resources
Molecular Probes Handbook (online version)
Comprehensive guide to fluorescent probes and labeling technologies
thermofisher.com/handbook

Molecular Probes Fluorescence SpectraViewer
Identify compatible sets of fluorescent dyes and cell structure probes
thermofisher.com/spectraviewer

BioProbes Journal of Cell Biology Applications
Award-winning magazine highlighting cell biology products and applications
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APTER 20
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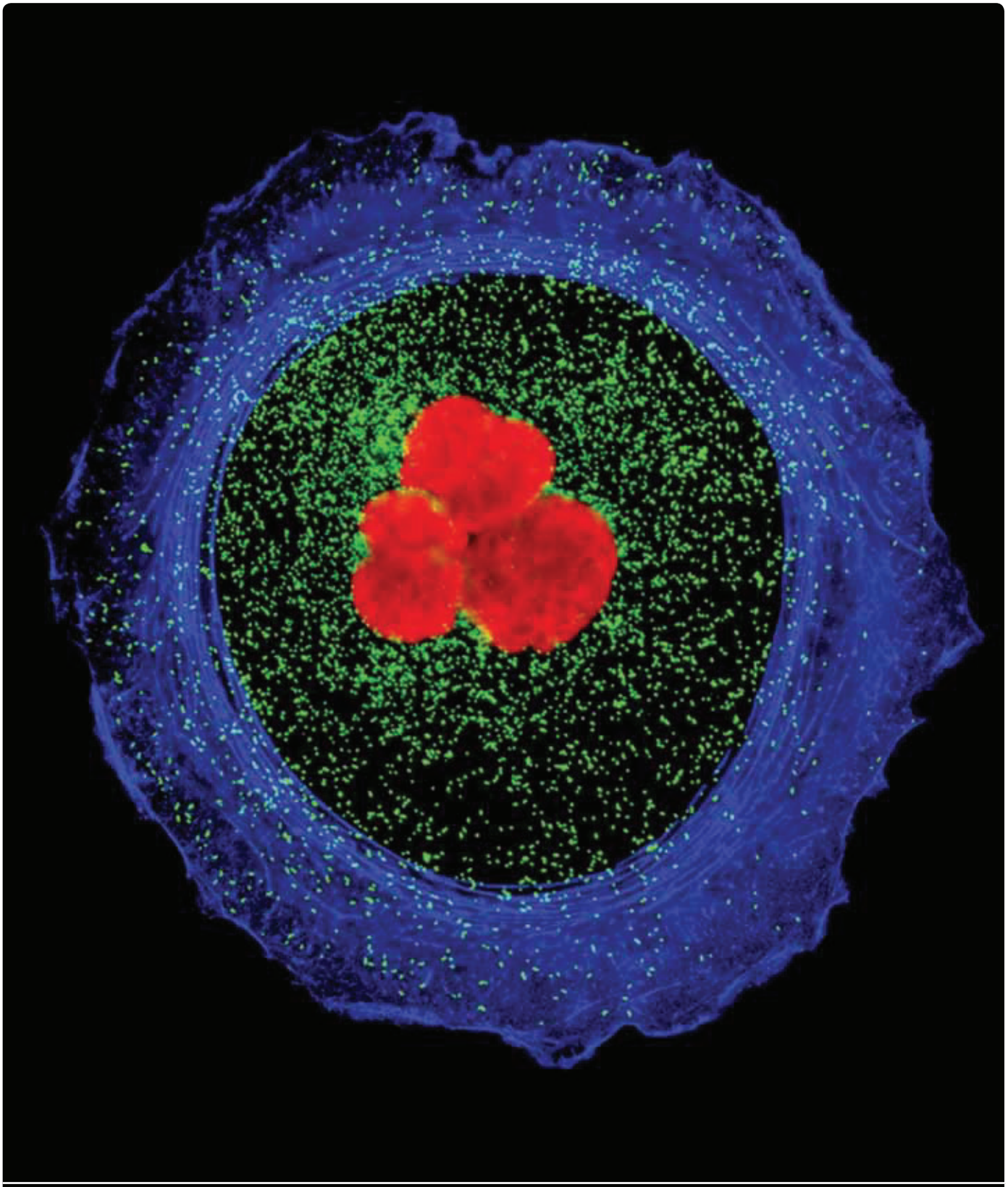

Alexa Fluor® 568 goat anti–mouse IgG, Alexa Fluor® 488 goat anti-rabbit IgG and Alexa Fluor® 350 phalloidin.

20.1 Overview of pH Indicators

The ability of dyes—notably litmus, phenolphthalein and phenol red—to change their color in response to a pH change has found widespread application in research and industry. Fluorescent dyes, however, provide the increased sensitivity required for optical pH measurements *inside* live cells. They also offer much greater spatial sampling capability when compared with microelectrode techniques.[1] These advantages have spurred the development of improved fluorescent dyes that can sense pH changes within physiological ranges. Of course, many of the same fluorescent pH indicators can also be used as pH sensors in cell-free media.

To quantitatively measure pH, it is essential to match the indicator's pK_a to the pH of the experimental system. Consequently, the following two sections of this chapter are divided into pH indicators for use in environments with near-neutral pH (Section 20.2) and pH indicators for use in relatively acidic environments (Section 20.3). Intracellular pH is generally between ~6.8 and 7.4 in the cytosol and ~4.5 and 6.0 in the cell's acidic organelles. Unlike intracellular free Ca^{2+} concentrations, which can rapidly change by perhaps 100-fold, the pH inside a cell varies by only fractions of a pH unit, and such changes may be quite slow.

We offer a variety of fluorescent pH indicators, pH indicator conjugates and other reagents for pH measurements in biological systems. Among these are several probes with unique optical responses and specialized localization characteristics:

- Visible light–excitable SNARF® pH indicators enable researchers to determine intracellular pH in the physiological range using dual-emission or dual-excitation ratiometric techniques (Section 20.2), thus providing important tools for confocal laser-scanning microscopy and flow cytometry.
- pHrodo™ dye and LysoSensor™ probes, for use in acidic environments such as lysosomes (Section 20.3).
- Fluorescent pH indicators coupled to dextrans are extremely well retained in cells, do not bind to cellular proteins and have a reduced tendency to compartmentalize[2] (Section 20.4).

Families of Molecular Probes® pH indicators are listed in Table 20.1 in approximate order of decreasing pK_a value.

Table 20.1 Molecular Probes® pH indicator families, in order of decreasing pK_a.

Parent Fluorophore	pH Range	Typical Measurement
SNARF® indicators	6.0–8.0	Emission ratio 580/640 nm
HPTS (pyranine)	7.0–8.0	Excitation ratio 450/405 nm
BCECF	6.5–7.5	Excitation ratio 490/440 nm
Fluoresceins and carboxyfluoresceins	6.0–7.2	Excitation ratio 490/450 nm
LysoSensor™ Green DND-189	4.5–6.0	Single emission 520 nm
Oregon Green® dyes	4.2–5.7	Excitation ratio 510/450 nm or excitation ratio 490/440 nm
LysoSensor™ Yellow/Blue DND-160	3.5–6.0	Emission ratio 450/510 nm
pHrodo™ dye	See below *	Single emission 585 nm

* pHrodo™ succinimidyl ester exhibits a complex pH titration profile. Decreasing pH (from pH 9 to pH 2) produces a continuous (but nonlinear) fluorescence increase. This pH response profile typically changes upon conjugation of the dye to proteins and other biomolecules.

REFERENCES

1. Chem Rev (2010) 110:2709; **2**. Methods Cell Biol (1989) 29:59.

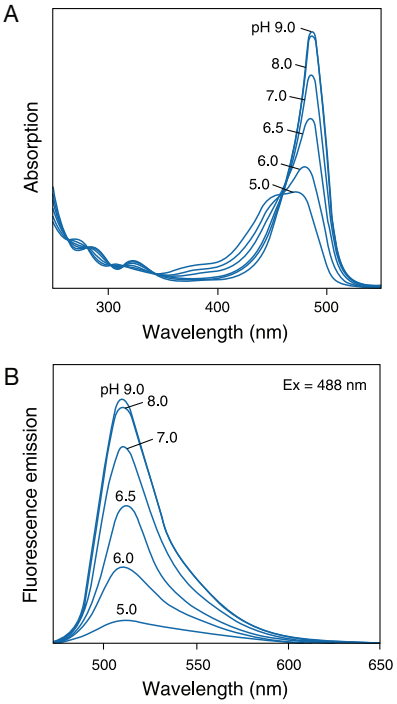

Figure 20.2.2 The pH-dependent spectra of fluorescein (F1300): **A**) absorption spectra, **B**) emission spectra.

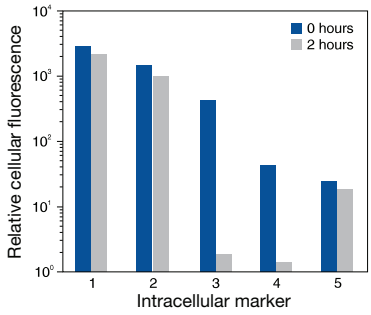

Figure 20.2.3 Loading and retention characteristics of intracellular marker dyes. Cells of a human lymphoid line (GePa) were loaded with the following cell-permeant acetoxymethyl ester (AM) or acetate derivatives of fluorescein: **1**) calcein AM (C1430, C3099, C3100MP), **2**) BCECF AM (B1150), **3**) fluorescein diacetate (FDA, F1303), **4**) carboxyfluorescein diacetate (CFDA, C1354) and **5**) CellTracker™ Green CMFDA (5-chloromethylfluorescein diacetate, C2925, C7025). Cells were incubated in 4 µM staining solutions in Dulbecco's modified eagle medium containing 10% fetal bovine serum (DMEM+) at 37°C. After incubation for 30 minutes, cell samples were immediately analyzed by flow cytometry to determine the average fluorescence per cell at time zero (0 hours). Retained cell samples were subsequently washed twice by centrifugation, resuspended in DMEM+, maintained at 37°C for 2 hours and then analyzed by flow cytometry. The decrease in the average fluorescence intensity per cell in these samples relative to the time zero samples indicates the extent of intracellular dye leakage during the 2-hour incubation period.

20.2 Probes Useful at Near-Neutral pH

Fluorescein and Fluorescein Derivatives

Fluorescein and many of its derivatives exhibit multiple, pH-dependent ionic equilibria.[1–5] Both the phenol and carboxylic acid functional groups of fluorescein are almost totally ionized in aqueous solutions above pH 9 (Figure 20.2.1). Acidification of the fluorescein dianion first protonates the phenol (pK_a ~6.4) to yield the fluorescein monoanion, then the carboxylic acid (pK_a <5) to produce the neutral species of fluorescein. Further acidification generates a fluorescein cation (pK_a ~2.1).

Only the monoanion and dianion of fluorescein are fluorescent, with quantum yields of 0.37 and 0.93, respectively, although excitation of either the neutral or cationic species is reported to produce emission from the anion with effective quantum yields of 0.31 and 0.18, respectively.[2] A further equilibrium involves formation of a colorless, nonfluorescent lactone (Figure 20.2.1). The lactone is not formed in aqueous solution above pH 5 but may be the dominant form of neutral fluorescein in solvents such as acetone. The pH-dependent absorption spectra of fluorescein (Figure 20.2.2) clearly show the blue shift and decreased absorptivity indicative of the formation of protonated species. However, the fluorescence emission spectrum of most fluorescein derivatives, even in acidic solution, is dominated by the dianion, with only small contributions from the monoanion. Consequently, the wavelength and shape of the emission spectra resulting from excitation close to the dianion absorption peak at 490 nm are relatively independent of pH, but the fluorescence intensity is dramatically reduced at acidic pH (Figure 20.2.2).

We offer a broad variety of fluorescein-derived reagents and fluoresceinated probes that can serve as sensitive fluorescent pH indicators in a wide range of applications. Chemical substitutions of fluorescein may shift absorption and fluorescence maxima and change the pK_a of the dye; however, the effects of acidification on the spectral characteristics illustrated in Figure 20.2.2 are generally maintained in all fluorescein derivatives.

Fluorescein and Its Diacetate

The cell-permeant fluorescein diacetate (FDA, F1303) is still occasionally used to measure intracellular pH,[6] as well as to study cell adhesion[7] or, in combination with propidium iodide (P1304MP, P3566, P21493; Section 8.1), to determine cell viability.[8,9] However, fluorescein (F1300), which is formed by intracellular hydrolysis of FDA, rapidly leaks from cells (Figure 20.2.3). Thus, other cell-permeant dyes such as the acetoxymethyl (AM) esters of BCECF and calcein are now preferred for intracellular pH measurements and cell viability assays (Section 15.2).

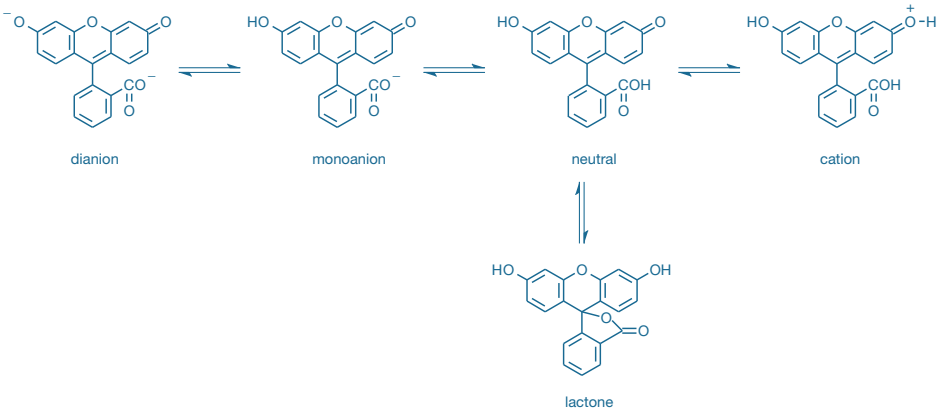

Figure 20.2.1 Ionization equilibria of fluorescein.

Carboxyfluorescein and Its Cell-Permeant Esters

Fluorescein's high leakage rate out of cells makes it very difficult to quantitate intracellular pH, because the decrease in the cell's fluorescence due to dye leakage cannot be easily distinguished from that due to acidification. The use of carboxyfluorescein diacetate (CFDA, C195) for intracellular pH measurements partially addresses this problem.[10,11] CFDA is moderately permeant to most cell membranes and, upon hydrolysis by intracellular nonspecific esterases, forms carboxyfluorescein (5(6)-FAM, C194, C1904), which has a pH-dependent spectral response very similar to that of fluorescein. As compared with fluorescein, carboxyfluorescein contains an extra negative charge and is therefore better retained in cells[12] (Figure 20.2.3). The mixed-isomer preparation of CFDA (C195) is usually adequate for intracellular pH measurements because the single isomers of carboxyfluorescein exhibit essentially identical pH-dependent spectra with a pK_a ~6.5. For experiments requiring a pure isomer, the single-isomer preparations of carboxyfluorescein (C1359, C1360; Section 1.5) and CFDA (C1361, C1362; Section 15.2) are available. In addition, we offer the AM ester of CFDA (5-CFDA, AM, C1354), which is electrically neutral and facilitates cell loading. Upon hydrolysis by intracellular esterases, this AM ester also yields carboxyfluorescein.[13–15]

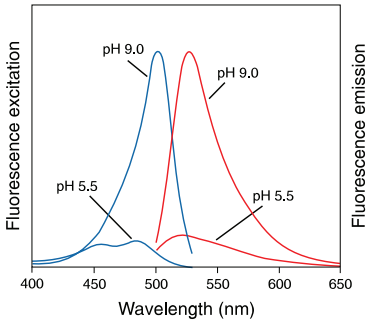

Figure 20.2.5 Fluorescence excitation (detected at 535 nm) and emission (excited at 490 nm) spectra of BCECF in pH 9.0 and pH 5.5 buffers.

BCECF and Its AM Ester

Although carboxyfluorescein is better retained in cells than is fluorescein, its pK_a of ~6.5 is lower than the cytosolic pH of most cells (pH ~6.8–7.4). Consequently, its fluorescence change is less than optimal for detecting small pH changes above pH 7. Since its introduction by Roger Tsien in 1982,[16,17] the polar fluorescein derivative BCECF (B1151) and its membrane-permeant AM ester (B1150, B1170, B3051) have become the most widely used fluorescent indicators for estimating intracellular pH. Also, a flow cytometric assay has been developed that uses BCECF to estimate the concentration of intracellular K^+.[18] BCECF's four to five negative charges at pH 7–8 improve its retention in cells (Figure 20.2.3), and its pK_a of 6.98 is ideal for typical intracellular pH measurements.

As with fluorescein and carboxyfluorescein, absorption of the phenolate anion (basic) form of BCECF is red-shifted and has increased molar absorptivity relative to the protonated (acidic) form (Figure 20.2.4); there is little pH-dependent shift in the fluorescence emission spectrum of BCECF upon excitation at 505 nm. BCECF is typically used as a dual-excitation ratiometric pH indicator. Signal errors caused by variations in concentration, path length, leakage and photobleaching are greatly reduced with ratiometric methods (Loading and Calibration of Intracellular Ion Indicators—Note 19.1). Intracellular pH measurements with BCECF are made by determining the pH-dependent ratio of emission intensity (detected at 535 nm) when the dye is excited at ~490 nm versus the emission intensity when excited at its isosbestic point of ~440 nm (Figure 20.2.4, Figure 20.2.5). Because BCECF's absorption at 440 nm is quite weak, increasing the denominator wavelength to ~450 nm provides improved signal-to-noise characteristics for ratio imaging applications.[19–21] As with other intracellular pH indicators, *in situ* calibration of BCECF's fluorescence response is usually accomplished using 10–50 µM nigericin (N1495) in the presence of 100–150 mM K^+ to equilibrate internal and external pH.[22,23] Alternative calibration methods have also been reported.[24–26]

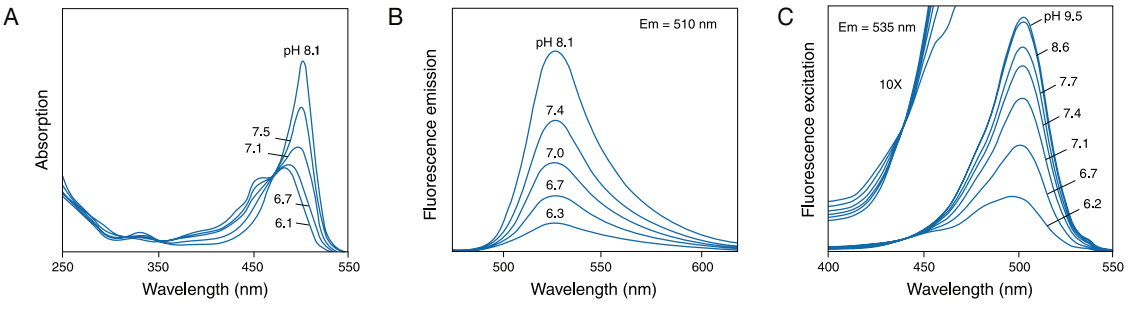

Figure 20.2.4 The pH-dependent spectra of BCECF (B1151): **A**) absorption spectra, **B**) emission spectra and **C**) excitation spectra. The fluorescence excitation spectra on the left in panel C have been enlarged 10X to reveal BCECF's 439 nm isosbestic point. Note that the isosbestic point of the excitation spectra of BCECF is different from that of the absorption spectra (compare panels A and **C**).

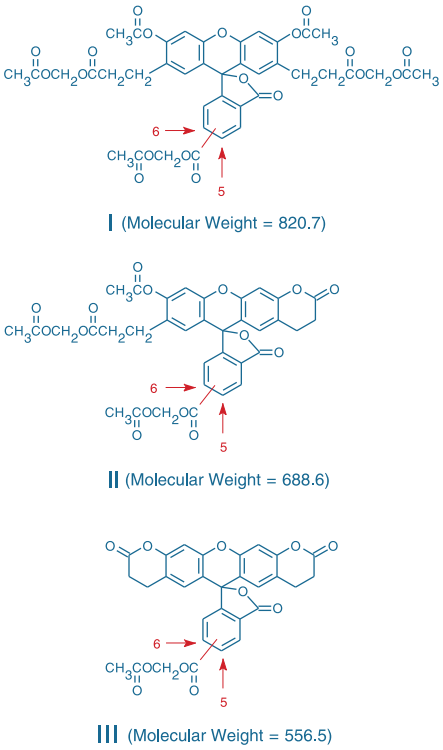

Figure 20.2.6 Structures of the AM esters of BCECF (B1150, B1170, B3051).

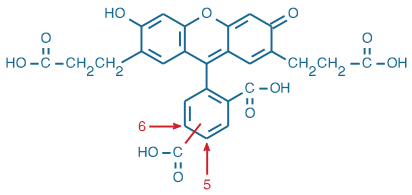

Figure 20.2.7 2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein (BCECF acid, B1151).

Loading of live cells for measurement of intracellular pH is readily accomplished by incubating cell suspensions or adherent cells in a 1–10 µM solution of the AM ester of BCECF. At least three different molecular species can be obtained in synthetic preparations of the AM ester of BCECF; however, all three forms shown in Figure 20.2.6 appear to be converted to the same product—BCECF acid (B1151, Figure 20.2.7)—by intracellular esterase hydrolysis. Although we can readily prepare the pure tri(acetoxymethyl) ester form (Form I in Figure 20.2.6), some researchers have found that cell loading with a mixture of the lactone Forms II and III is more efficient. Consequently, we produce BCECF AM predominantly as a mixture of Forms II and III with a typical percentage composition ratio of 45:55, as determined by HPLC, NMR and mass spectrometry. The AM ester of BCECF is available in a single 1 mg vial (B1150), specially packaged as a set of 20 vials that each contains 50 µg (B1170) and as a 1 mg/mL solution (~1.6 mM) in anhydrous dimethylsulfoxide (DMSO) (B3051). We highly recommend purchasing the set of 20 vials in order to reduce the potential for product deterioration caused by exposure to moisture.

Our bibliography for BCECF AM lists more than 1200 journal citations, including references for the use of BCECF AM to investigate:

- Cl^-/HCO_3^- exchange [27–30]
- K^+/H^+ exchange [31,32]
- Na^+/H^+ exchange [33–35]
- Na^+/Ca^{2+} exchange [36]
- NH_4^+ transport [37,38]
- Lactate transport and metabolism [39–41]
- Apoptosis [42–44] (Section 15.5)
- Phagocytosis [45–47] (Section 16.1)
- Regulation of pancreatic insulin secretion [48]
- Voltage-activated H^+ conductance in neurons [49]

The cell-impermeant BCECF acid (B1151) is useful for pH measurements in intercellular spaces of epithelial cell monolayers,[50] interstitial spaces of normal and neoplastic tissue [51,52] and isolated cell fractions.[53] BCECF has also been employed for two-photon fluorescence lifetime imaging of the skin stratum corneum to detect aqueous acid pockets within the lipid-rich extracellular matrix.[54] The free acid of BCECF can be loaded into cells by microinjection [26] or electroporation or by using our Influx™ pinocytic cell-loading reagent (I14402, Section 19.8). It has also been loaded into bacterial cells by brief incubation at pH ~2.[55,56] In addition to the cell-permeant BCECF AM and cell-impermeant BCECF acid, we offer dextran conjugates of BCECF (D1878, D1880; Section 20.4).

Fluorescein Sulfonic Acid and Its Diacetate

The fluorescein-5-(and 6-)sulfonic acid (F1130, Figure 20.2.8) is much more polar than carboxyfluorescein. Consequently, once inside cells or liposomes, it is relatively well retained. Some cells can be loaded directly with 5-sulfofluorescein diacetate [57–60] (SFDA, S1129). Direct ratiometric measurement of the pH in the trans-Golgi of live human fibroblasts was achieved by simultaneously microinjecting liposomes loaded with both fluorescein sulfonic acid and sulforhodamine 101 [61] (S359, Section 14.3). Fluorescein-5-(and 6-)sulfonic acid is more commonly used to measure barrier permeability of membranes [62,63] (Section 14.3).

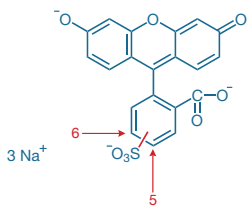

Figure 20.2.8 Fluorescein-5-(and-6)-sulfonic acid, trisodium salt (F1130).

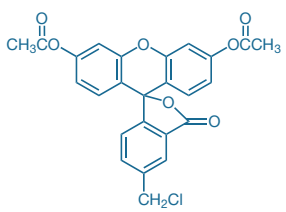

Figure 20.2.9 CellTracker™ Green CMFDA (5-chloromethylfluorescein diacetate, C2925).

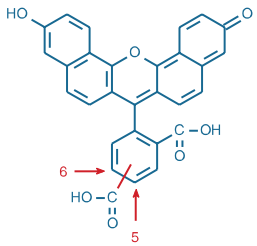

Figure 20.2.10 5-(and-6)-carboxynaphthofluorescein (C652).

Chemically Reactive Fluorescein Diacetates

One means for overcoming the cell leakage problem common to the above pH indicators, including BCECF, is to trap the indicator inside the cell via conjugation to intracellular constituents. CellTracker™ Green CMFDA (C2925, C7025; Figure 20.2.9) and chloromethyl SNARF®-1 (C6826) incorporate a thiol-reactive chloromethyl moiety that reacts with intracellular thiols, including glutathione and proteins, to yield well-retained products (Figure 20.2.3). Cleavage of the acetate groups of the CMFDA conjugate by intracellular esterases yields a conjugate that retains the pH-dependent spectral properties of fluorescein. Because of its superior retention as compared with SNARF® AM and BCECF AM, CellTracker™ Green CMFDA was employed to monitor the intracellular pH response to osmotic stress in CHO, HEK 293 and Caco-2 cells.[64] Similarly, the amine-reactive succinimidyl ester of CFDA (CFSE, C1157) can be used for long-term pH studies of live cells, producing a conjugate with the pH-sensitive properties of carboxyfluorescein.[65]

Carboxynaphthofluorescein

Carboxynaphthofluorescein (C652, Figure 20.2.10) has pH-dependent red fluorescence (excitation/emission maxima ~598/668 nm at pH >9) with a relatively high pK_a of ~7.6. The long-wavelength pH-dependent spectra of carboxynaphthofluorescein have been exploited in the construction of fiber-optic pH sensors.[66,67] This long-wavelength pH indicator is also available in membrane-permeant diacetate form (C13196) for passive intracellular loading and as an amine-reactive succinimidyl ester (C653, Section 20.4) for preparing pH-sensitive conjugates.

SNARF® pH Indicator

The seminaphthorhodafluors (SNARF® dyes) are visible light–excitable fluorescent pH indicators.[68] The SNARF® indicators have both dual-emission and dual-excitation properties, making them particularly useful for confocal laser-scanning microscopy[69–72] (Figure 20.2.11), flow cytometry[21,73–75] and microplate reader–based measurements.[76] The dual-emission properties of the SNARF® indicators make them preferred probes for use in fiber-optic pH sensors.[77–79] These pH indicators can be excited by the 488 or 514 nm spectral lines of the argon-ion laser and are sensitive to pH values within the physiological range. Dextran conjugates of the SNARF® dyes are described in Section 20.4.

Carboxy SNARF®-1 Dye and Its Cell-Permeant Ester

The carboxy SNARF®-1 dye (C1270, Figure 20.2.12), which is easily loaded into cells as its cell-permeant AM ester acetate (C1271, C1272), has a pK_a of about 7.5 at room temperature and between 7.3 and 7.4 at 37°C. Thus, carboxy SNARF®-1 is useful for measuring pH changes

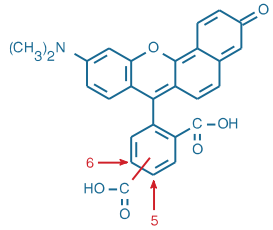

Figure 20.2.12 5-(and-6)-carboxy SNARF®-1 (C1270).

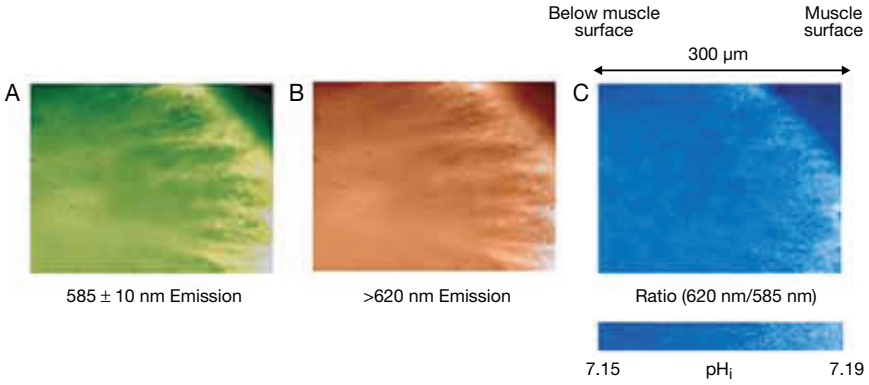

Figure 20.2.11 Confocal fluorescence images of rabbit papillary muscle loaded by perfusion with carboxy SNARF®-1 AM acetate (C1271, C1272). The first two images (A and B) were acquired through 585 ± 10 nm bandpass and >620 nm longpass emission filters, respectively. The 620 nm/585 nm fluorescence ratio image in the third image (C) is more uniform than the component images A and B due to cancellation of intensity variations resulting from heterogeneous uptake of the fluorescent indicator. Images contributed by Barbara Muller-Borer and John Lemasters, University of North Carolina, and reprinted with permission from Am J Physiol (1998) 275:H1937.

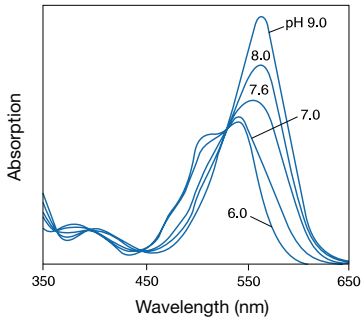

Figure 20.2.13 The pH-dependent absorption spectra of carboxy SNARF®-1 (C1270).

between pH 7 and pH 8. Like fluorescein and BCECF, the absorption spectrum of the carboxy SNARF®-1 pH indicator undergoes a shift to longer wavelengths upon deprotonation of its phenolic substituent (Figure 20.2.13). In contrast to the fluorescein-based indicators, however, carboxy SNARF®-1 also exhibits a significant pH-dependent emission shift from yellow-orange to deep-red fluorescence as conditions become more basic (Figure 20.2.14, Figure 20.2.15). This pH dependence allows the ratio of the fluorescence intensities from the dye at two emission wavelengths—typically 580 nm and 640 nm—to be used for quantitative determinations of pH (Loading and Calibration of Intracellular Ion Indicators—Note 19.1) (Figure 20.2.11). For practical purposes, it is often desirable to bias the detection of carboxy SNARF®-1 fluorescence towards the less fluorescent acidic form by using an excitation wavelength between 488 nm and the excitation isosbestic point at ~530 nm, yielding balanced signals for the two emission ratio components (Figure 20.2.14, Figure 20.2.16). When excited at 488 nm, carboxy SNARF®-1 exhibits an emission isosbestic point of ~610 nm and a lower fluorescent signal than obtained with 514 nm excitation.[72] Alternatively, when excited by the 568 nm spectral line of the Ar-Kr laser found in some confocal laser-scanning microscopes, carboxy SNARF®-1 exhibits a fluorescence increase at 640 nm as the pH increases and an emission isosbestic point at 585 nm.[72] As with other ion indicators, intracellular environments may cause significant changes to both the spectral properties and pK_a of carboxy SNARF®-1,[80–83] and the indicator should always be calibrated in the system under study.

The spectra of carboxy SNARF®-1 are well resolved from those of fura-2[84,85] and indo-1[86] (Section 19.2), as well as those of the fluo-3,[85,87,88] fluo-4, Calcium Green™ and Oregon Green® 488 BAPTA Ca^{2+} indicators (Section 19.3), permitting simultaneous measurements of intracellular pH and Ca^{2+} (Figure 20.2.17). Carboxy SNARF®-1 has also been used in combination with the Na^+ indicator SBFI (S1262, S1263, S1264; Section 21.1) to simultaneously detect pH and Na^+ changes.[89] The relatively long-wavelength excitation and emission characteristics of carboxy SNARF®-1 facilitate studies in autofluorescent cells[90] and permit experiments that employ ultraviolet light–photoactivated caged probes[91] (Section 5.3). Incubation of cells for several hours after loading with carboxy SNARF®-1 AM ester acetate results in compartmentally selective retention of the dye, allowing *in situ* measurements of mitochondrial pH[92] (Figure 20.2.18).

SNARF®-4F and SNARF®-5F Dyes and Their Cell-Permeant Esters

Although the carboxy SNARF®-1 indicator possesses excellent spectral properties, its pK_a of ~7.5 may be too high for measurements of intracellular pH in some cells. For quantitative measurements of pH changes in the typical cytosolic range (pH ~6.8–7.4), we now recommend SNARF®-5F carboxylic acid (Figure 20.2.19), which has a pK_a value of ~7.2, as the indicator with the optimal spectral properties for estimating cytosolic pH (Figure 20.2.20). SNARF®-4F carboxylic acid (Figure 20.2.21) has a somewhat more acidic pH sensitivity maximum (pK_a ~6.4) but retains its dual-emission spectral properties (Figure 20.2.22). SNARF®-4F has been used for pH imaging in kidney tissues using two-photon excitation (780 nm) microscopy; the pH-dependent emission shift response was observed to be essentially the same as seen with one-photon

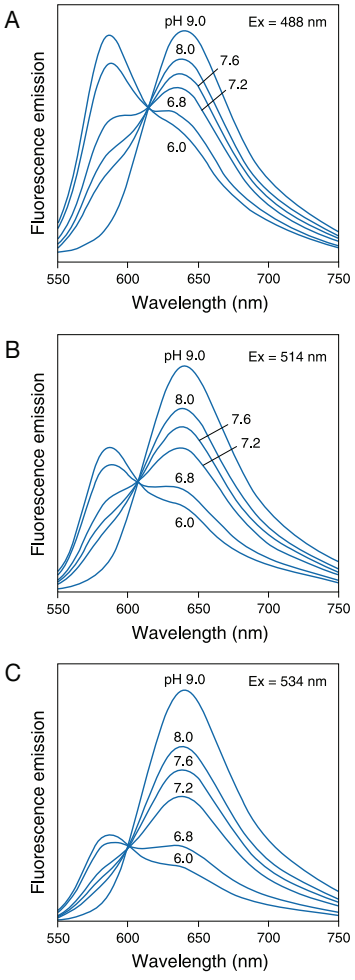

Figure 20.2.14 The pH-dependent emission spectra of carboxy SNARF®-1 (C1270) when excited at **A**) 488 nm, **B**) 514 nm and **C**) 534 nm.

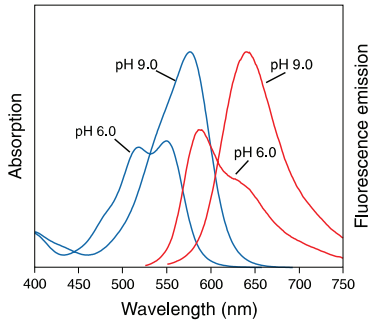

Figure 20.2.15 Absorption and fluorescence emission (excited at 514 nm) spectra of carboxy SNARF®-1 in pH 9.0 and pH 6.0 buffers.

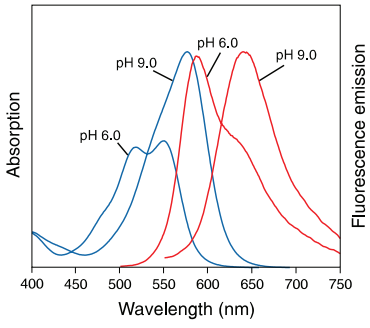

Figure 20.2.16 Absorption and fluorescence emission (excited at 488 nm) spectra of carboxy SNARF®-1 in pH 9.0 and pH 6.0 buffers.

excitation.[93] This study also reported nigericin calibrations that yielded different pK_a values (6.8 versus 7.4) in the kidney cortex and kidney ileum, respectively, emphasizing the importance of performing *in situ* calibrations. Both SNARF®-4F and SNARF®-5F[94] allow dual-excitation and dual-emission ratiometric pH measurements, making them compatible with the same instrument configurations used for carboxy SNARF®-1 in ratio imaging and flow cytometry applications. SNARF®-4F and SNARF®-5F are available as free carboxylic acids (S23920, S23922) and as cell-permeant AM ester acetate derivatives (S23921, S23923).

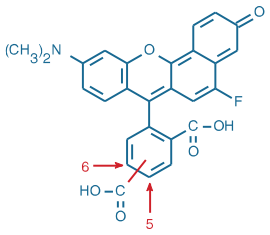

Figure 20.2.19 SNARF®-5F 5-(and-6)-carboxylic acid (S23922).

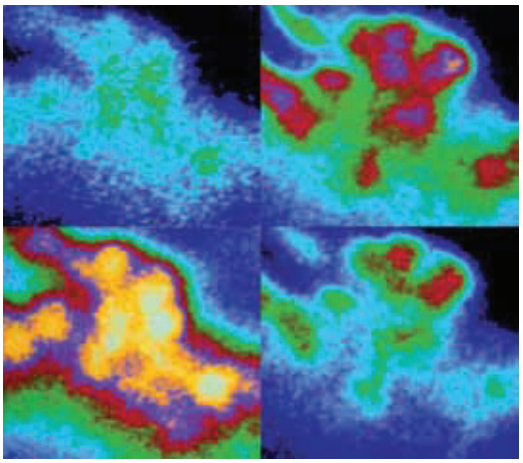

Figure 20.2.17 Rat pituitary intermediate lobe melanotropes labeled with the indo-1 AM (I1203, I1223, I1226) and carboxy SNARF®-1, AM, acetate (C1271, C1272) indicators. Pseudocolored fluorescence from the dual-emission Ca^{2+} indicator indo-1 is shown at 405 and 475 nm (left panels). Pseudocolored fluorescence from the dual-emission pH indicator carboxy SNARF®-1 is shown at 575 and 640 nm (right panels). Image contributed by Stephen J. Morris, University of Missouri-Kansas City, and Diane M. Beatty, Molecular Probes, Inc.

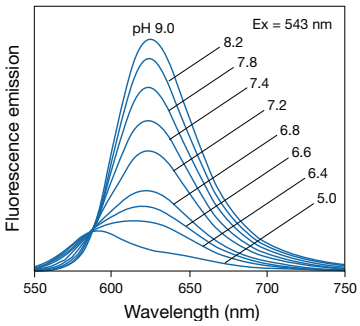

Figure 20.2.20 Fluorescence emission spectra of SNARF®-5F 5-(and 6-)carboxylic acid (S23922) as a function of pH.

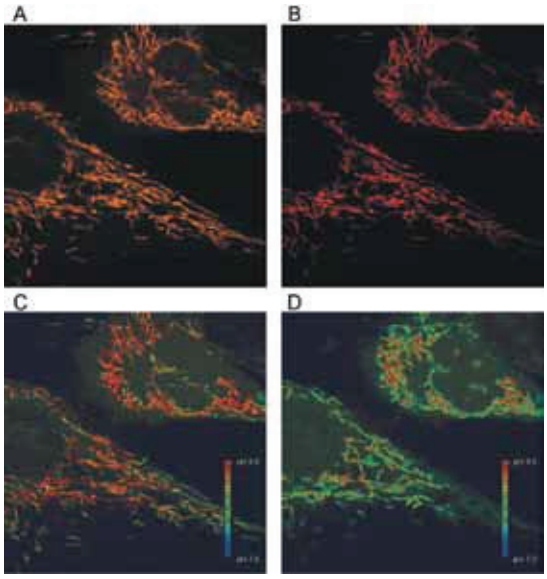

Figure 20.2.18 Selective loading of carboxy SNARF®-1 into mitochondria. BHK cells were loaded with 10 µM carboxy SNARF®-1, AM, acetate (C1271, C1272) for 10 minutes, followed by incubation for 4 hours at room temperature. **A**) Confocal image (488 nm excitation) of mitochondrial-selective loading of carboxy SNARF®-1 visualized through a 560–600 nm bandpass filter. **B**) Confocal image of the same cells as in A, but using a 605 nm dichroic mirror and a 610 nm longpass filter. **C**) Ratio image (A and **B**) of mitochondria in cells pseudocolored to represent different pH levels. **D**) Change in mitochondrial pH following the addition of 10 µM carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), resulting in a decrease (acidification) of mitochondrial pH. Image contributed by Brian Herman, University of Texas Health Science Center, San Antonio, and reprinted with permission from Biotechniques 30, 804 (2001).

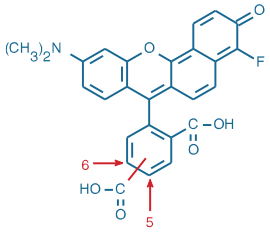

Figure 20.2.21 SNARF®-4F 5-(and-6)-carboxylic acid (S23920).

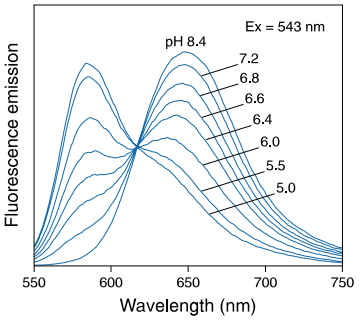

Figure 20.2.22 Fluorescence emission spectra of SNARF®-4F 5-(and 6-)carboxylic acid (S23920) showing the pH-dependent spectral shift that is characteristic of this and other SNARF® pH indicators.

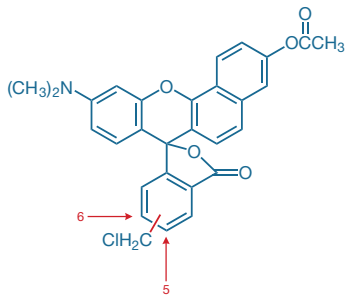

Figure 20.2.23 5-(and-6)-chloromethyl SNARF®-1, acetate (C6826).

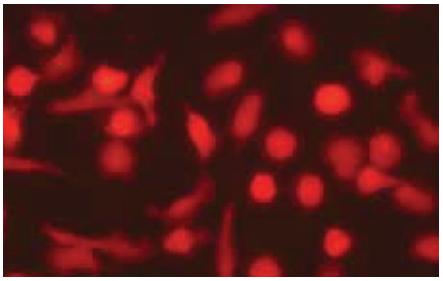

Figure 20.2.24 Human neutrophils loaded with 5-(and-6)-chloromethyl SNARF®-1 acetate (C6826).

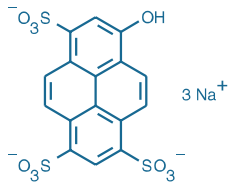

Figure 20.2.25 8-hydroxypyrene-1,3,6-trisulfonic acid, trisodium salt (HPTS; pyranine, H348).

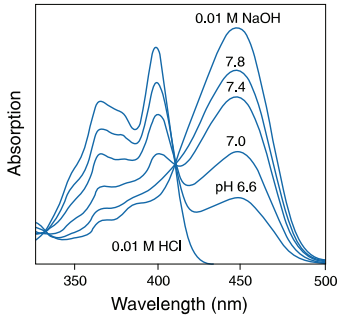

Figure 20.2.26 The pH-dependent absorption spectra of 8-hydroxypyrene-1,3,6-trisulfonic acid (HPTS, H348).

Amine- and Thiol-Reactive SNARF® Dyes

Our 5-(and 6-)chloromethyl SNARF®-1 acetate (C6826, Figure 20.2.23) contains a chloromethyl group that is mildly reactive with intracellular thiols, forming adducts that improve cellular retention of the SNARF® fluorophore (Figure 20.2.24). As with CellTracker™ Green CMFDA, improved retention of this conjugate in cells may permit monitoring of intracellular pH over longer time periods than is possible with other intracellular pH indicators. Similarly, amine-reactive SNARF®-1 succinimidyl ester (S22801, Section 20.4) is useful as an intracellular pH indicator [95] in addition to its more common application as a cell tracer.[96]

8-Hydroxypyrene-1,3,6-Trisulfonic Acid (HPTS)

8-Hydroxypyrene-1,3,6-trisulfonic acid (HPTS, also known as pyranine; H348; Figure 20.2.25) is an inexpensive, highly water-soluble, membrane-impermeant pH indicator with a pK_a of ~7.3 in aqueous buffers.[97] The pK_a of HPTS is reported to rise to 7.5–7.8 in the cytosol of some cells.[98] Unlike indicators based on the SNARF® and fluorescein dyes, there is no membrane-permeant form of HPTS available. Consequently, HPTS must be introduced into cells by microinjection, electroporation [99] or liposome-mediated delivery,[100–102] through ATP-gated ion channels [103] or by other relatively invasive means (Table 14.1, Section 14.1). HPTS exhibits a pH-dependent absorption shift (Figure 20.2.26), allowing ratiometric measurements using an excitation ratio of 450/405 nm.[49,104] Because the excited state of HPTS is much more acidic than the ground state,[105] it is frequently used as a photoactivated source of H^+ in mechanistic studies of bacteriorhodopsin and other proton pumps.[106–108]

Nigericin

Intracellular calibration of the fluorescence response of cytosolic pH indicators is typically performed using the K^+/H^+ ionophore nigericin (N1495), which causes equilibration of intracellular and extracellular pH in the presence of a depolarizing concentration of extracellular K^+ [22,23] (Loading and Calibration of Intracellular Ion Indicators—Note 19.1). Nett and Deitmer have compared this technique with calibrations performed by direct insertion of pH-sensitive microelectrodes in leech giant glial cells.[26]

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DATA TABLE 20.2 PROBES USEFUL AT NEAR-NEUTRAL pH

				Acidic Solution				Basic Solution						
Cat. No.	**MW**	**Storage**	**Soluble**	**Abs**	**EC**	**Em**	**Solvent**	**Abs**	**EC**	**Em**	**Solvent**	**pK_a**	**Product***	**Notes**
B1150	~615	F,D	DMSO	<300		none							B1151	1
B1151	520.45	L	pH >6	482	35,000	520	pH 5	503	90,000	528	pH 9	7.0		2, 3
B1170	~615	F,D	DMSO	<300		none							B1151	1
B3051	~615	F,D	DMSO	<300		none							B1151	1, 4
C194	376.32	L	pH >6, DMF	475	28,000	517	pH 5	492	75,000	517	pH 9	6.4		2, 3
C195	460.40	F,D	DMSO	<300		none							C194	
C652	476.44	L	pH >6, DMF	512	11,000	563	pH 6	598	49,000	668	pH 10	7.6		2, 3, 5
C1157	557.47	F,D	DMF, DMSO	<300		none							C1311	
C1270	453.45	L	pH >6	548	27,000	587	pH 6	576	48,000	635	pH 10	7.5		2, 3, 6
C1271	567.55	F,D	DMSO	<350		none							C1270	
C1272	567.55	F,D	DMSO	<350		none							C1270	
C1354	532.46	F,D	DMSO	<300		none							C1359	
C1904	376.32	L	pH >6, DMF	475	29,000	517	pH 5	492	78,000	517	pH 9	6.4		2, 3, 7
C2925	464.86	F,D	DMSO	<300		none							see Notes	8
C6826	499.95	F,D	DMSO	<350		none							see Notes	9
C7025	464.86	F,D	DMSO	<300		none							see Notes	8
C13196	560.52	F,D	DMSO	<300		none							C652	
F1130	478.32	D,L	H_2O, DMF	476	31,000	519	pH 5	495	76,000	519	pH 9	6.4		2, 3
F1300	332.31	L	pH >6, DMF	473	34,000	514	pH 5	490	93,000	514	pH 9	6.4		2, 3
F1303	416.39	F,D	DMSO	<300		none							F1300	
H348	524.37	D,L	H_2O	403	20,000	511	pH 4	454	24,000	511	pH 9	7.3		2, 3, 10
N1495	724.97	F,D	MeOH	<300		none								
S1129	518.43	F,D	DMSO	<300		none							F1130	
S23920	471.44	L	pH >6	552	27,000	589	pH 5	581	48,000	652	pH 9	6.4		2, 3
S23921	585.54	F,D	DMSO	<350		none							S23920	
S23922	471.44	L	pH >6	555	27,000	590	pH 5	579	49,000	630	pH 9	7.2		2, 3
S23923	585.54	F,D	DMSO	<350		none							S23922	

For definitions of the contents of this data table, see "Using *The Molecular Probes® Handbook*" in the introductory pages.

* Cat. No. of product generated *in situ* in typical intracellular applications.

Notes

1. MW value is approximate. BCECF, AM is a mixture of molecular species.
2. pK_a values may vary considerably depending on the temperature, ionic strength, viscosity, protein binding and other factors. Unless otherwise noted, values listed have been determined from pH-dependent fluorescence measurements at 22°C.
3. Spectra are in aqueous buffers adjusted to >1 pH unit above and >1 pH unit below the pK_a.
4. This product is supplied as a ready-made solution in the solvent indicated under "Soluble."
5. Data on pH dependence of C652 spectra obtained in our laboratories. Additional relevant data are reported elsewhere. (Mikrochim Acta (1992) 108:133)
6. Values of pK_a for these SNARF® indicators are as reported in published references. (Anal Biochem (1991) 194:330)
7. This product is specified to equal or exceed 98% analytical purity by HPLC.
8. Acetate hydrolysis of this compound yields a fluorescent product with pH-dependent spectral characteristics similar to C1904.
9. C6826 and S22801 are converted to fluorescent products with spectra similar to C1270 after acetate hydrolysis.
10. The pK_a for H348 was determined in 0.066 M phosphate buffers at 22°C. (Fresenius Z Anal Chem (1983) 314:119)