Fluorescent Indicators for Intracellular pH

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1. Introduction

Intracellular pH (pH_i)[1,2] plays many critical roles in cell, enzyme, and tissue activities, including proliferation and apoptosis,[3–8] multidrug resistance (MDR),[9] ion transport,[10–15] endocytosis,[5] and muscle contraction.[16,17] Monitoring pH changes inside living cells is also important for studying cellular internalization pathways, such as phagocytosis,[18] endocytosis,[19] and receptor ligand internalization.[20] Changes of pH_i effect the nervous system too, by influencing synaptic transmission, neuronal excitability, cell−cell coupling via gap junctions, and signal cascades.[21–26] Abnormal pH_i values are associated with inappropriate cell function, growth, and division and are observed in some common disease types such as cancer[27] and Alzheimer's.[28] Some organelles, e.g., endosomes[29] and plant vacuoles,[30] have intracompartmental pHs of 4−6. In cell biology, low intracompartmental pH values can serve to denature proteins or to activate enzyme and protein functions that would be too slow around pH 7.0. For instance, the acidic environments in lysosomes (pH 4.5−5.5)[31,32] can facilitate the degradation of proteins in cellular metabolism. Thus, cellular dysfunction is often associated with abnormal pH values in organelles.[29]

Intimate connections between the cell functions with intracellular pH means that precise measurement of intracellular pH can provide critical information for studying physiological and pathological processes down to a single organelle. Good resolution in the space and time dimensions, i.e., spatial and temporal, is highly desirable. Compared to other pH_i measurement methods such as microelectrodes, NMR, and absorbance spectroscopy, fluorescence spectroscopy has advantages with respect to spatial and temporal observation of pH_i changes. Moreover, fluorescence techniques have high sensitivities, they tend to be operationally simple, and they are in most cases nondestructive to cells.

Qualitative measurements of pH_i can be achieved using fluorescent indicators that switch on or off at sharply defined pH values. However, such measurements may be influenced by many factors, including optical path length, changes of temperature, altered excitation intensities, and varied emission collection efficiencies. The alternative is to use "ratiometric detection".

Ratiometric spectroscopic methods require fluorescent sensors that are differentially sensitive to the analyte (i.e., protons for pH probes) for at least two excitation or emission wavelengths (Figure 1).[33,34] For instance, for a suitable fluorescent dye, emission at one carefully chosen wavelength may be enhanced or diminished relative to the emission at another. Ratios between these signals then can be calibrated

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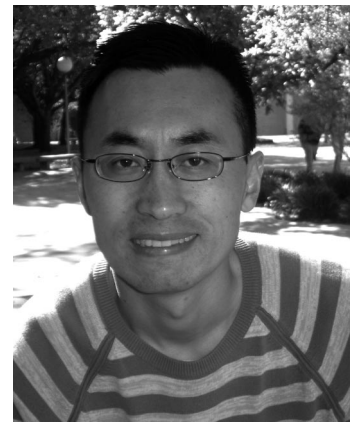
Kevin Burgess has been co-author of the *Highlights* section in *Chemistry and Industry* for over 20 years and has recently published a workbook for graduate students studying organic chemistry: *Organic Chemistry By Inquisition* (www.byinquisition.org). He is the Rachel Professor of Chemistry at Texas A&M University, where he has been since 1992. His research interests focus on peptidomimetics for mimicking or disrupting protein−protein interactions, asymmetric organometallics catalysis, and fluorescent dyes for applications in biotechnology. All these projects are related to high-throughput and combinatorial chemistry. Motivation to write this review came from a realization of the importance of being able to measure intracellular pH values.



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to indicate pH_i values. Advantages when using ratiometric methods are accrued because parameters such as optical path length, local probe concentration, photobleaching, and leakage from the cells are irrelevant. This must be so since both signals come from the probe in exactly the same environment.

This review is about intracellular pH sensors, including small fluorescent organic molecules, nanoparticles, and fluorescent proteins, e.g., GFP. It focuses on their preparations, photophysical properties, and advantages/disadvantages for intracellular pH measurements. The discussion is limited to fluorescent indicators that have been applied to measure intracellular pH values since the1980s; relatively few indicators were used to measure intracellular pH values before that date, and those are now largely redundant.[2]

2. Methods for Delivering Fluorescent Dyes Into Cells

A variety of methods can be used for importing highly charged fluorescent compounds into cells. These include microinjection,[35] scrape loading,[36] hypertonic lysis,[37] and carrier-mediated endocytosis.[38] All of these approaches perturb the cell resting state physiology.

Another strategy for import of fluorescent compounds into cells uses concepts similar to the "prodrug approach".[39] This strategy involves chemical modification of charged, non-cell-permeable dyes outside cells to neutral cell-permeable ones after import. Thus, transport of masked forms into the cell allows endogenous cellular esterases to liberate the charged fluorescent form of these compounds. The archetypical example of this is the use of nonfluorescent acetoxylmethyl (AM) or acetate esters of fluoresceins as pH_i indicators. These compounds diffuse into cells and are then hydrolyzed by nonselective intracellular esterases to afford the free, charged, fluorescent dyes (Figure 2). In fact, conversion of nonfluorescent AM esters into fluorescent free dyes has been used for cell viability assays.[3] Application of this approach is probably less disruptive to the cells than the methods mentioned above, but it is not totally innocuous. Hydrolysis of AM esters yields acetic acid and methanol; both byproducts may induce abnormal cellular events. Moreover, the fluorescent dyes can localize in any cellular compartment, and in this approach the fluorescent compounds may particularly tend to accumulate in organelles having high concentrations of esterases. The AM ester and liberated dye also may be cytotoxic to some extent. Nevertheless, these undesirable effects may be tolerable for many applications.

Acetoxymethyl (AM) esters all tend to be synthesized via the same strategy. This features reaction of hydroxyl and/or carboxylic acid groups on the free dye with freshly prepared bromomethyl acetate[40] in the presence of diisopropylethylamine in anhydrous chloroform (Figure 2).[41,42]

Fluorescence intensities of the free pH indicators inside cells are reduced if the fluorescent molecules are somehow expelled from the cells. Rates of dye leakage from cells are related to the net charge on the dyes; more highly charged ones are expelled slower. For instance, fluorescein has a higher leakage rate relative to 5-(and 6)-carboxyfluorescein because the former has one less negative charge.[43] Dye−dextran (a complex, branched polyglucose with varying lengths from 10 to 150 kDa), −biomolecule, or −nanoparticle conjugates can circumvent the leakage problem because passage of the dyes from the inside to the outside of the cells is unfavorable, and the concentration decreases only due to cell division.[44] Cells labeled with the pH indicator BCECF (see next section) on dextran have been shown to produce much more stable fluorescent signals, reduced probe compartmentalization, and 10-fold greater resistance to light-induced damage when compared with dye AM-labeled cells.[44] Overall, pH indicators that are coupled to carrier molecules that do not cross the cell membrane may be particularly useful for long-term experiments where retention of the probe in cells is an issue.

Interactions of probes with biomolecules or organelles in cells can significantly change their spectral properties relative to aqueous saline solutions.[45,46] Consequently, ex-vivo calibration is required for more accurate pH_i measurements. Thomas and co-workers in 1979 introduced the method that is most widely used for pH_i calibration.[47] In this approach, intracellular pH is assumed to be equal to extracellular pH when the cells are treated with the K^+/H^+ ionophore, nigericin (5 μL/mL). Nigericin makes cells permeable to H^+ {and K^+}, thus equilibrating the intra- and extracellular pH.

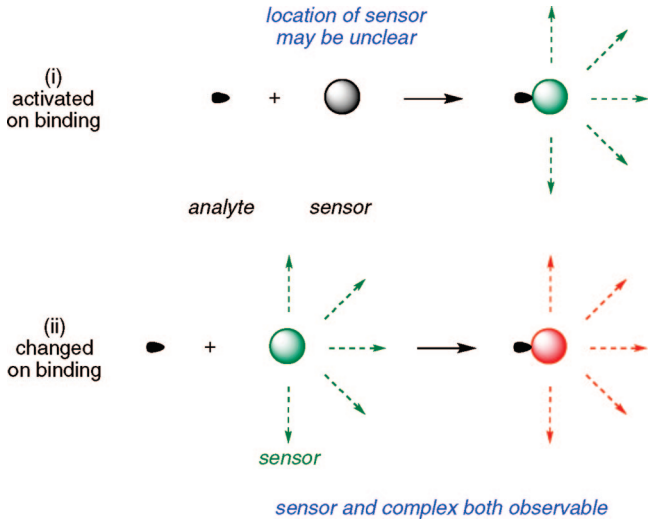


Figure 1. Fluorescent sensors may be activated (i) by analytes. Ratiometric ones (ii) change the wavelength of fluorescence emission on binding.

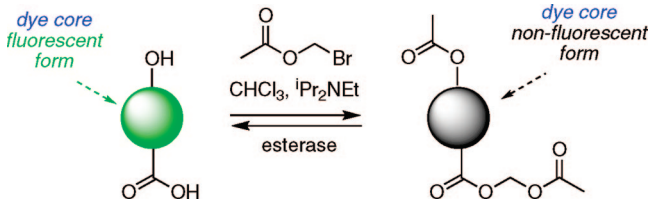


Figure 2. Synthesis and hydrolysis of AM and acetate esters.

3. Fluorescein-Based pH_i Indicators

3.1. Most Widely Used pH_i Indicator: BCECF

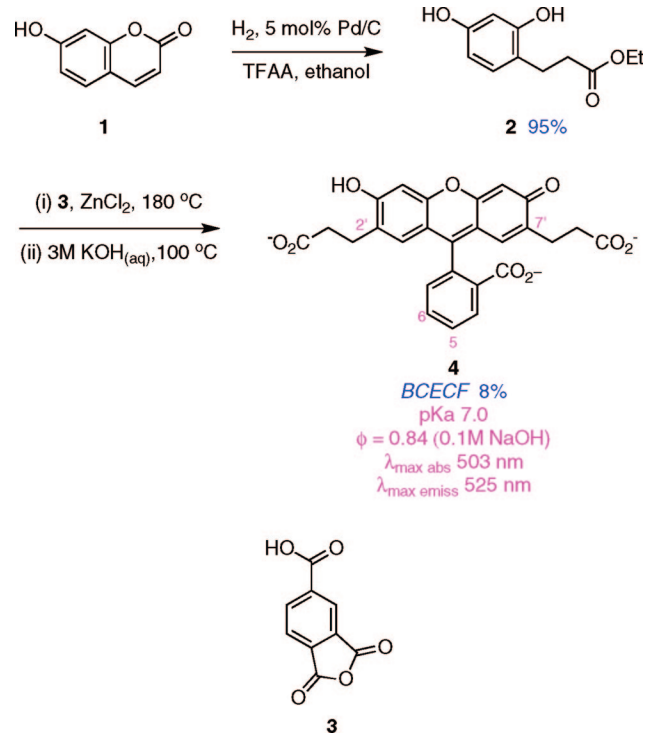
2′,7′-Bis-(2-carboxyethyl)-5-(and-6-)carboxyfluorescein **4** (BCECF; Scheme 1) was introduced for measuring cytoplasmic pH by Roger Tsien and co-workers in 1982.[43] Since then it has been widely used for mammalian or plant cells,[3,10,11,48,49] living tissues,[50–52] and individual organelles,[53] such as the endoplasmic reticulum.[10,54]

BCECF **4** is synthesized via condensation of ethyl 3-(2,4-dihydroxyphenyl)-propionate **2** (from hydrogenation of commercially available 7-hydroxycoumarin, **1**, in ethanol containing a catalytic amount of trifluoroacetic anhydride) with trimellitic acid anhydride **3** in the presence of anhydrous $ZnCl_2$ at 180 °C (Scheme 1).[43] The commercially available acetoxymethyl ester of BCECF is a mixture of three compounds **5**−**7** (BCECF AM I−III, Figure 3). These three regioisomeric BCECF AM esters can be transformed into the free BCECF by nonselective esterase inside living cells.

Like fluorescein, carboxyfluorescein, and fluorescein sulfonic acid, the absorbance of BCECF is sensitive to the pH. Absorption of BCECF **4** red shifts from pH 3.6 to 9.2, and its molar absorptivity is much larger in the phenolate anion form than in the phenolic form.[55] However, indicators based on fluorescence are far more sensitive than those that use absorption, so the remainder of our discussion focuses on this.

BCECF **4** is often used as a ratiometric excitation (or dual excitation) pH indicator because the absorption profile for the dye changes significantly with pH.[34,52,55,56] In this approach fluorescence intensity ratios corresponding to excitation at two different wavelengths are measured, and these data are correlated to pH via ex-vivo calibration using Thomas' method.[47]

Scheme 1. Synthesis of BCECF[a]



[a] TFAA: trifluoroacetic anhydride.

An alternative to ratiometric excitation for pH measurements is the ratiometric emission method. This strategy involves measuring fluorescence intensities at two different wavelengths when the indicator is excited at one wavelength. BCECF is unsuitable for this approach since its relative emissions at any two different wavelengths are not significantly dependent on pH. An example of a dye that can be used in this mode is carboxy.SNARF, i.e., C.SNARF-1 (see below).

BCECF **4** is widely applied in cell biology because of several attributes. First, the free dye is retained well inside cells because it has 4−5 negative charges at physiological pH values (~7.4). Second, the pK_a of BCECF **4** (7.0) is ideal for sensing cytosolic pHs, which are normally in the range of 6.8−7.4. Third, BCECF AM esters **5**−**7** are cell membrane permeable, and this facilitates noninvasive loading of the dye into cells. Conversion of nonfluorescent BCECF AM esters **5**−**7** into fluorescent BCECF **4** acid form is efficient, so much so that this transformation has been used for cell viability assays.[3] Fourth, ionic strengths of solutions surrounding BCECF **4** do not have much influence on the spectral properties of the dye.[43] Free BCECF **4** inside cells does not usually accumulate in any particular cellular compartment.

There are also some problems associated with BCECF in measurements of pH_i values. For instance, even though the rate of leakage of this dye from cells is relatively slow, it can still be ca. 10% over 10−20 min at 25 °C and more at 37 °C.[43] To circumvent this issue, the BCECF−dextran conjugate might be used; this exhibits excellent intracellular retention and much lower cytotoxicity effects, but it is not cell membrane permeable and has to be delivered into cells via relatively destructive techniques, e.g., microinjection. Another disadvantage of BCECF **4** is that it, like most fluorescein-based dyes, photobleaches relatively quickly;

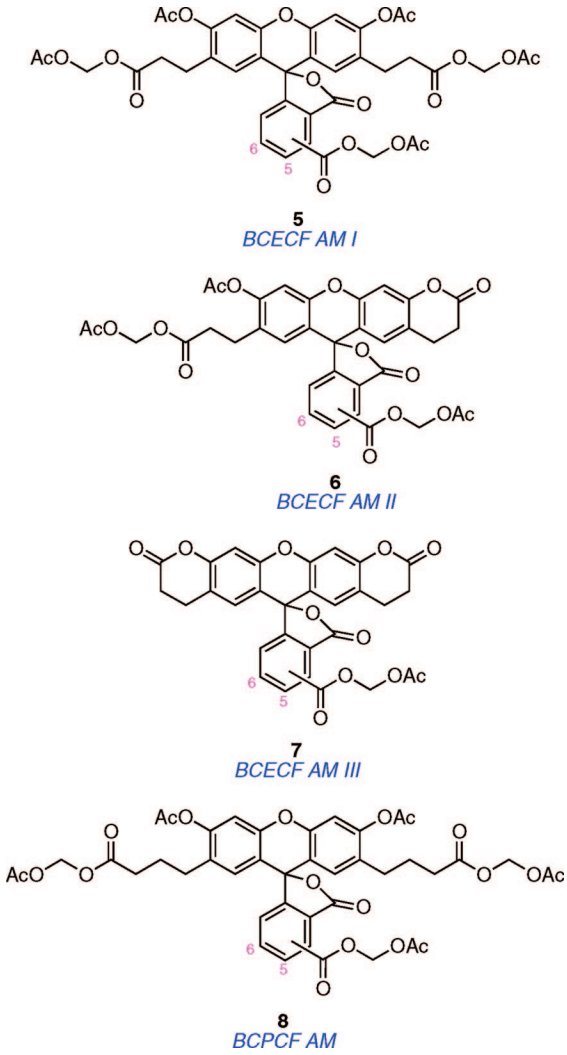


Figure 3. Structures of BCECF/BCPCF AM esters.

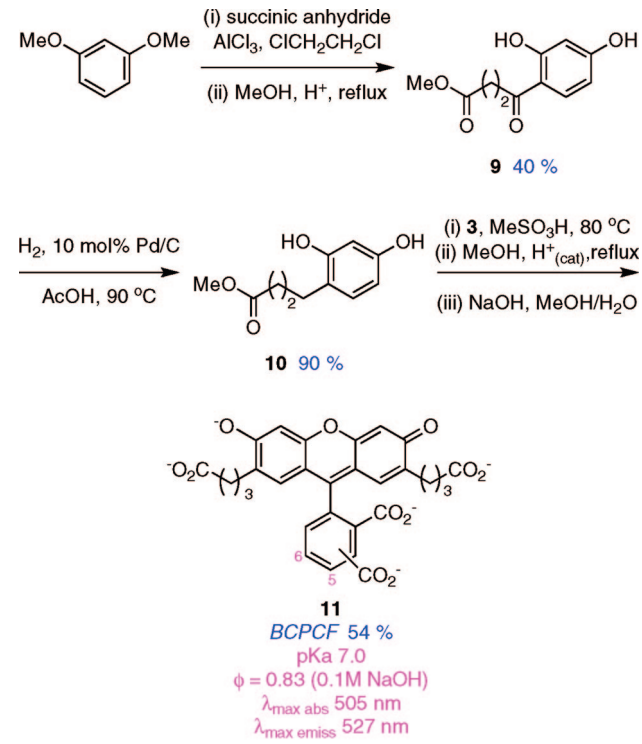
hence, erroneous pH_i measurements can result.[50] Moreover, such photobleaching reactions of this kind can damage cells.

3.2. BCPCF

BCPCF **11**, 2′,7′-bis-(2-carboxypropyl)-5-(and-6-)-carboxyfluorescein (Scheme 2), is a homolog of BCECF **4**. BCPCF **11** has 2-carboxypropyl substituents at the 2′- and 7′-xanthene positions, whereas BCECF **4** has 2-carboxyethyl groups there. The original synthesis of BCPCF **11** is shown in Scheme 2.[57] In this, 1,3-dimethoxybenzene was subjected to Friedel−Crafts acylation with succinic anhydride followed by in-situ demethylation and Fisher esterification to yield the γ-ketone ester **9** in 40% yield. The ketone group of the compound **9** was reduced to **10**, which has a chain of three methylene groups. Condensation of this resorcinol derivative **10** with trimellitic acid anhydride **3** (see Scheme 1 for structure) in methanesulfonic acid eventually gives BCPCF **11** as a mixture of two regioisomers. In fact, the intermediate acid is esterified solely to facilitate chromatographic separation; then this ester is converted back to the carboxylic acid form.

Commercially available BCPCF AM esters **8** predominantly exist in a form shown in Figure 3. BCECF **4** and BCPCF **11** have very similar pK_a values, absorption and emission maximum wavelengths, and quantum yields, just as expected for such structurally similar compounds. The

Scheme 2. Synthesis of BCPCF



previous section notes that ratiometric excitation pH_i measurements featuring BCECF are usually achieved by determination of the fluorescence intensity ratios at 535 nm corresponding to excitation at 503 and 439 nm. The absorbance of BCECF **4** at 439 nm corresponds to its isosbestic point; this is generally ideal for ratiometric methods except that in this case the absorptivity of **4** at 439 nm is quite weak. Application of BCPCF **11** overcomes this disadvantage of BCECF **4**. The isosbestic point of BCPCF **11** is red shifted to 454 nm compared with BCECF **4**; this corresponds to a stronger absorbance; hence, BCPCF **11** tends to be a better ratiometric dual-excitation probe.[55,57]

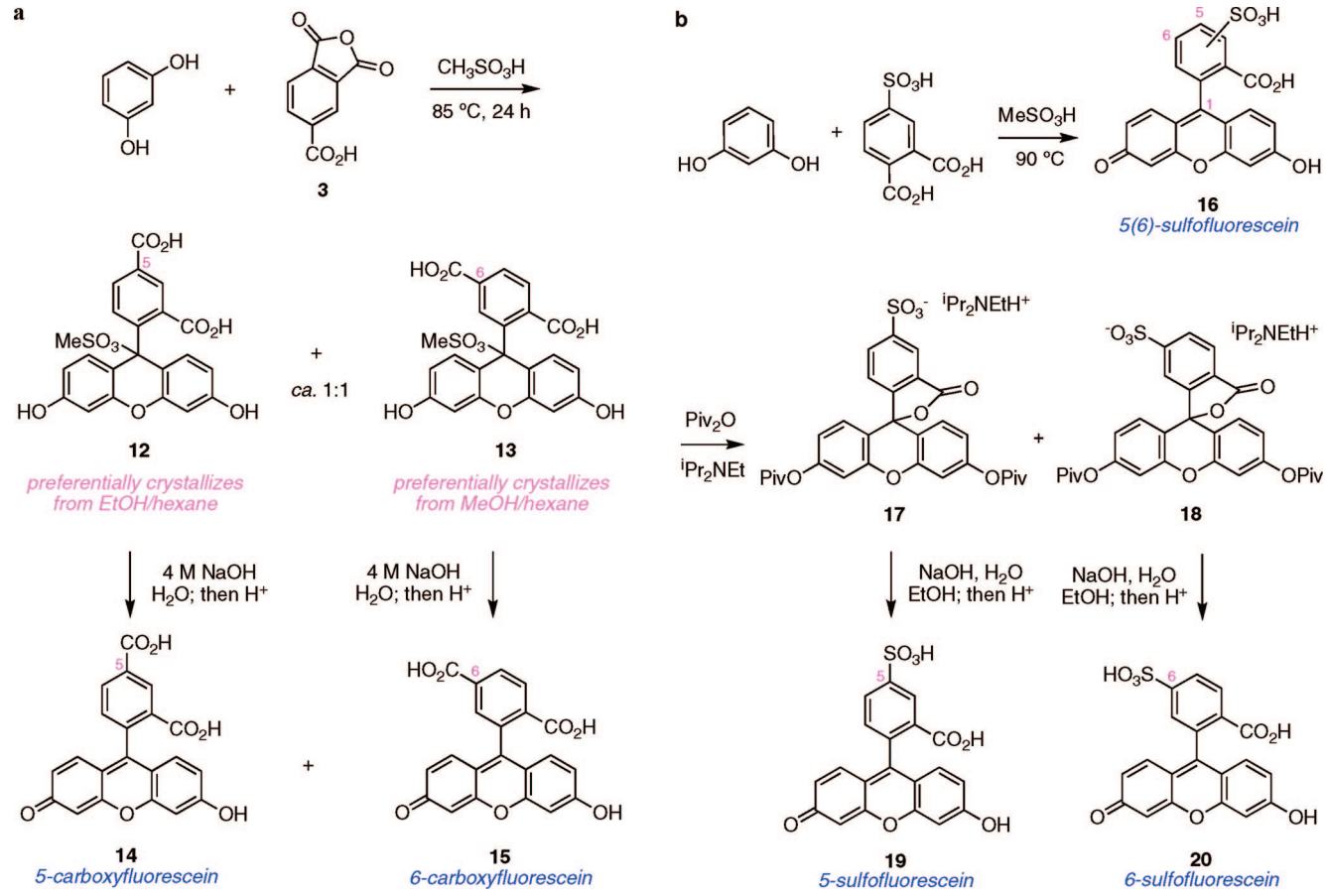
BCECF **4** and BCPCF **11** share a common disadvantage for pH_i measurements. Their fluorescence emission intensities are dependent on the concentration of the probes. Thus, if the dyes accumulate in certain regions of the cell then they can indicate different pH_i values indicative of dye, not proton, concentration differences.[55,58]

3.3. Fluorescein, Carboxyfluorescein, and Fluorescein Sulfonic Acid

BCECF and BCPCF are preferred for intracellular pH measurements, but fluorescein, fluorescein sulfonic acid, and, especially, carboxyfluorescein are still widely used for pH_i determination presumably because they are easy and cheap to prepare via standard condensation methods.[59–61]

Condensation of resorcinol with trimellitic acid anhydride **3** and 4-sulfophthalic acid produced a mixture of 5(6)-carboxyfluorescein (**14** and **15**) and 5(6)-sulfofluorescein (**19** and **20**), respectively (Scheme 3).[62,63] The two isomers exhibit essentially identical pH-dependent spectral properties with a pK_a of ~6.5; therefore, the mixture is sufficiently good for pH_i determination. Fluorescein sulfonic acid moves through the paracellular space inside live cells since it is water soluble and is cell membrane impermeant; it can be used for determination of barrier permeability.[64]

Scheme 3. Synthesis of (a) Carboxyfluorescein and (b) Sulfofluorescein Regioisomers



On rare occasions it may be desirable to use regioisomerically pure substituted fluorescein probes. Fortunately, methods for the preparation of single isomers are available.[59,65,66] For instance, preparation of 5- and 6-carboxyfluorescein begins by condensation of trimellitic anhydride **3** with resorcinol in the presence of methanesulfonic acid at 85 °C. The reaction affords a 1:1 mixture of isomeric compounds **12** and **13**. The 6-isomer (**13**) is selectively precipitated with an isomeric purity over 98% when this mixture is recrystallized twice in methanol/hexane. The compounds left in the filtrate are recrystallized from ethanol/hexane two times to give the 5-isomer (**12**) also in greater than 98% purity. Hydrolysis of the isomerically pure methanesulfonic acid adducts **12** and **13** under basic conditions affords 5-carboxyfluorescein **14** and 6-carboxyfluorescein **15**, respectively (Scheme 3a). Preparation of isomerically pure 5-sulfofluorescein **19** and 6-sulfofluorescein **20** can be achieved via a similar approach. Fluorescein-5(6)-sulfonic acid was converted into dipivaloyl esters **17** and **18** (Scheme 3b). Then, the di-isopropylethylamonium salt of the 6-isomer **18** was separated via crystallization from dichloromethane and diethyl ether solution. The isomerically pure salt **17** was isolated via subsequent recrystallization from the filtrate of **18**. Basic hydrolysis of these pivaloyl esters **17** and **18** yields the isomerically pure 5-sulfofluorescein **19** and 6-sulfofluorescein **20**, respectively.

Fluorescein diacetate is occasionally used for measuring pH_i values;[67] the main disadvantage of this is that once fluorescein is liberated via intracellular hydrolysis it can rapidly leak out of cells; hence, it is not easy to discern if fluorescence intensity decreases were induced by leakage or pH changes. The more charged derivatives, 5- and 6-carboxyfluorescein applied as cell-permeable carboxyfluorescein AM esters, are more often used for pH_i measurements because they are better retained in living cells. Even so, at 37 °C, intracellular concentrations of 5- and 6-carboxyfluorescein have been observed to diminish by 30−40% in the first 10 min after washing.[43] 5- and 6-Sulfofluoresceins are more water-soluble and even better retained inside cells or organelles compared with carboxyfluorescein. However, these sulfonic acid derivatives are not commonly used as pH_i indicators because their diacetate forms cannot easily diffuse into cells. Some other fluorescein derivatives such as dimethylcarboxyfluorescein[58] can be used as pH_i indicators, but many of these are not particularly photostable or well retained in living cells.

One approach to the problem of leakage of fluorescein derivatives from cells is to import them in activated form that will nonspecifically conjugate to intracellular biomolecules. For instance, fluorescein isothiocyanate[54,68] and 5-(6)-carboxyfluorescein diacetate succinimidyl ester[69] have both been used in this way.

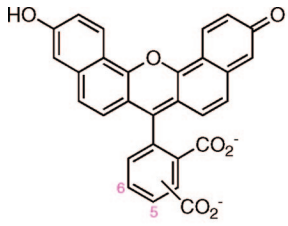
The xanthene parts of the pH_i indicators discussed above have very similar pK_a values to fluorescein, ~6.4.[70] The detailed spectral properties of fluorescein, carboxyfluorescein, and fluorescein sulfonic acid can be obtained on the Web site of Life Technologies.[71]

3.4. Miscellaneous Fluorescein Derivatives

3.4.1. 5- (and 6)-Carboxynaphthofluorescein

Some fluorescein derivatives can be used in pH_i measurements.[72] For instance, 5- (and 6)-carboxynaphthofluorescein

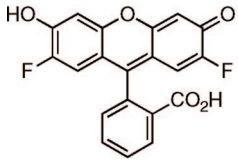
21 is a dual-emission ratiometric pH probe that is functional at near-neutral conditions (pK_a of ~7.6).[73,74] In its acidic form, this compound absorbs and fluoresces at 509 and 572 nm, respectively. Its basic form has a red-shifted (bathochromic) absorption and emission peaks with maxima peak at 598 and 668 nm, respectively. These pH-sensitive, long-wavelength, dual-emission spectra have been applied for determination of physiological pH_i.[73] However, this and other fluorescein derivatives are not widely used, presumably because they photobleach easily, and naphthofluorecein derivatives tend to have poor quantum yields.[72,75]



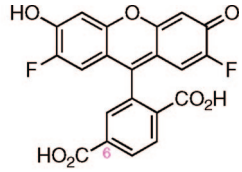
21
5(6)-carboxynaphthofluorescein
pKa 7.6
ϕ = NA
acidic/basic form
$\lambda_{max\ abs}$ 509/598 nm
$\lambda_{max\ emiss}$ 572/668 nm

3.4.2. Halogenated Fluoresceins

Fluorescein derivatives with different pK_a values can be used to monitor changes of proton concentrations that are centered around other pH values.[61] Electron-withdrawing groups on xanthenes lower their pK_a values. For instance, the halogenated fluoresceins in the Oregon Green series **22**−**24**[76] and 5(6)-carboxydichlorofluorescein (CDCF)[77] **25** all have pK_a's of ~4.8. Otherwise, the pH-dependent absorbance and fluorescence spectral characteristics of these dyes are similar to fluorescein; hence, dual-excitation ratiometric measurements of pH_i are possible. CDCF **25** has been used for determination of light-dependent pH changes in various acidic cellular compartments of plants.[78,79] Detailed information about applications of these dyes can be obtained through the Life Technologies Web site.[71]



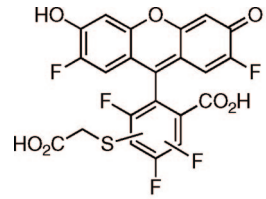
22
Oregon Green 488
pKa 4.8
ϕ = 0.97 (pH 9 buffer)
$\lambda_{max\ abs}$ 490 nm
$\lambda_{max\ emiss}$ 514 nm



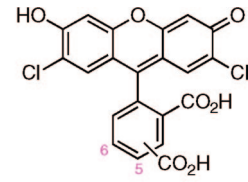
23
6-carboxyl Oregon Green 488
pKa 4.8
ϕ = 0.92 (pH 9 buffer)
$\lambda_{max\ abs}$ 492 nm
$\lambda_{max\ emiss}$ 514 nm

3.4.3. Rhodols

"Rhodols" are hybrids of a rhodamine and a fluorescein; they have the same backbone as rhodamine dyes, but one of the NR_2 groups is replaced by oxygen.[76,80−82] These fluorophores have high molar absorptivities in the visible region and, with appropriate *N*-substituents, high quantum yields in the range of 520−580 nm. They are more photostable than fluorescein derivatives. Absorbance spectra of these dyes

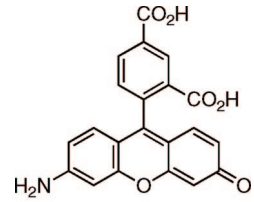


24
Oregon Green 514
pKa 4.8
acidic/basic form
ϕ = 0.22/0.65
$\lambda_{max\ abs}$ NA/506 nm
$\lambda_{max\ emiss}$ NA/529 nm



25
CDCF
pKa 4.8
acidic/basic form
ϕ =NA
$\lambda_{max\ abs}$ 495/504 nm
$\lambda_{max\ emiss}$ 504/525 nm

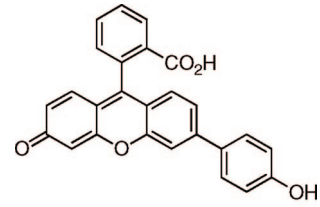
exhibit significant shifts with variation of pH values; thus, they are suitable for dual-excitation ratiometric pH measurements. Some of these compounds have pK_a values between 4.5 and 6.5, ideal for pH measurements in acidic environments.[76,82] For instance, the conjugate of rhodol **26** with ethylenediamino-ouabain (ouabain is a glycosylated steroid) has been used for probing the pH values in the acidic microenvironment at the cardiac glycoside-binding site of Na^+/K^+-ATPase.[83]



26
pKa 5.59
ϕ = 0.89 (pH 8-9)
$\lambda_{max\ abs}$ = 494 nm
$\lambda_{max\ emiss}$ = 520 nm

3.4.4. Anthofluorescein

Anthofluorescein **27** is highly sensitive to pH changes between 7 and 10.[84] Both the absorption and the emission maxima for this compound are pH dependent, in the ranges of 460−530 and 530−580 nm. The quantum yield of this dye in pH 8 aqueous buffer is only 0.02, which is not useful for applications where sensitivity is an issue. However, its quantum yield is highly dependent on the viscosity of the surrounding media; it improves to 0.3 when 20% glycerol was added, probably because the internal conversion rate between the resonance structures is decreased. A nonfluorescent, diacetate form of compound **27** has been synthesized. Incubation of this cell-permeable diacetate with Hela cells resulted in high fluorescence within the cells, probably due, in part, to the relatively high viscosity in that environment.[84]



27
pKa NA
ϕ = 0.02 (pH 8)
= 0.3 (20% glycerol, pH 8)
$\lambda_{max\ abs}$ = 460 -530 nm
$\lambda_{max\ emiss}$ = 530 -580 nm

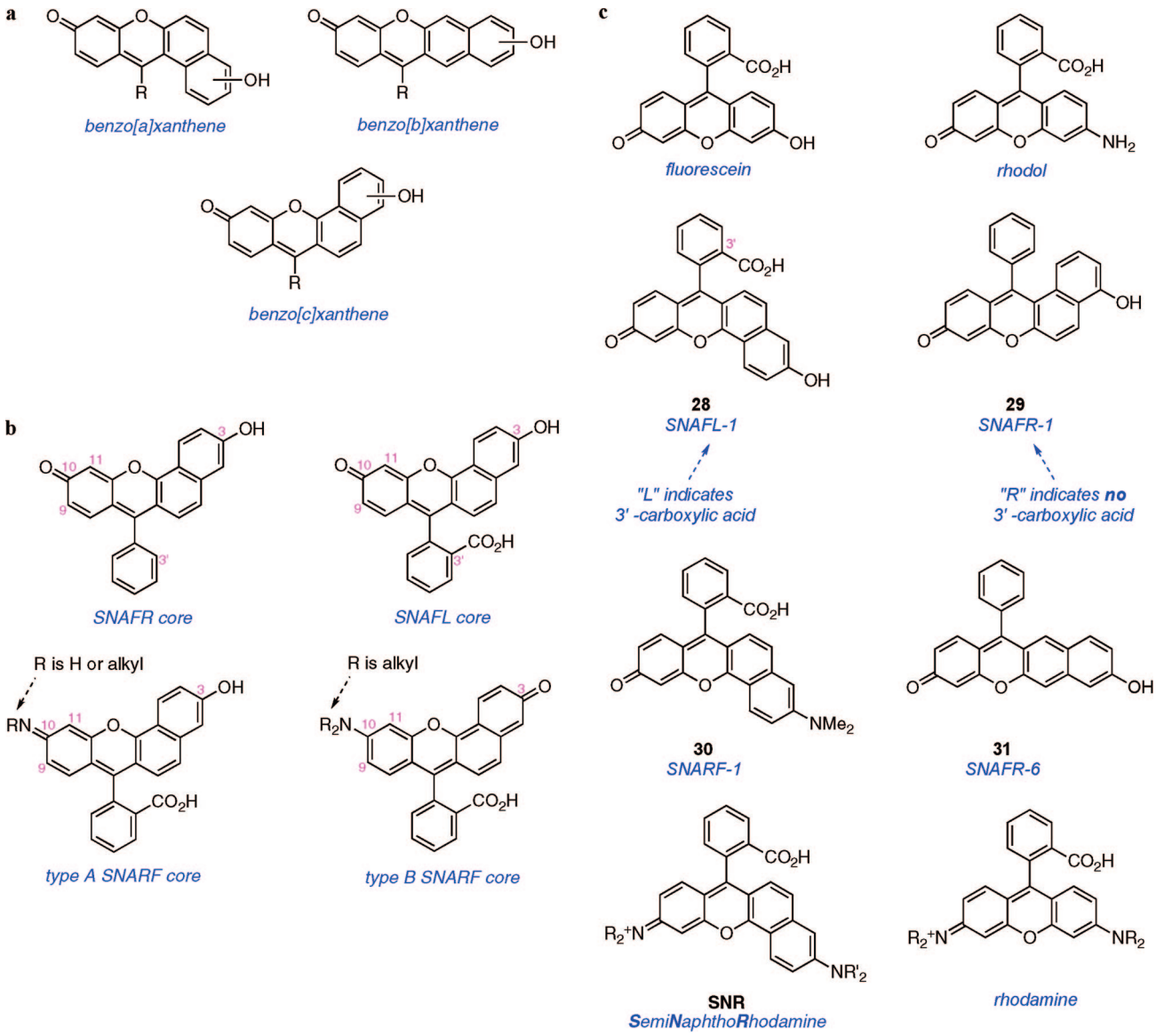


Figure 4. (a) Three types of benzoxanthenes, (b) three types of benzo[*c*]xanthenes that have different heterocyclic substituents, and (c) evolution of benzoxanthenes from fluorescein and rhodamine.

4. Benzoxanthene Dyes

4.1. Nomenclature Origin and Structures of SNAFLs, SNAFRs, and SNARFs

There are three possible isomers of benzoxanthene dyes that differ via their orientation of annulation (Figure 4a). Representatives of all three compound types have been prepared, and their spectral and photophysical properties have been studied. Benzo[*c*]xanthenes were introduced by Molecular Probes (formerly Invitrogen, and now Life Technologies) in the early 1990s.[42] These dyes include the seminaphthofluorones (SNAFRs), seminaphthofluoresceins (SNAFLs), and seminaphthorhodafluors (SNARFs); all these dyes have one benzene and a naphthalene component in the fluorophore (Figure 4b). SNAFLs, SNAFRs, and SNARFs are long-wavelength fluorescent pH_i indicators with oxygen and nitrogen 10-substituents, respectively.[42,85]

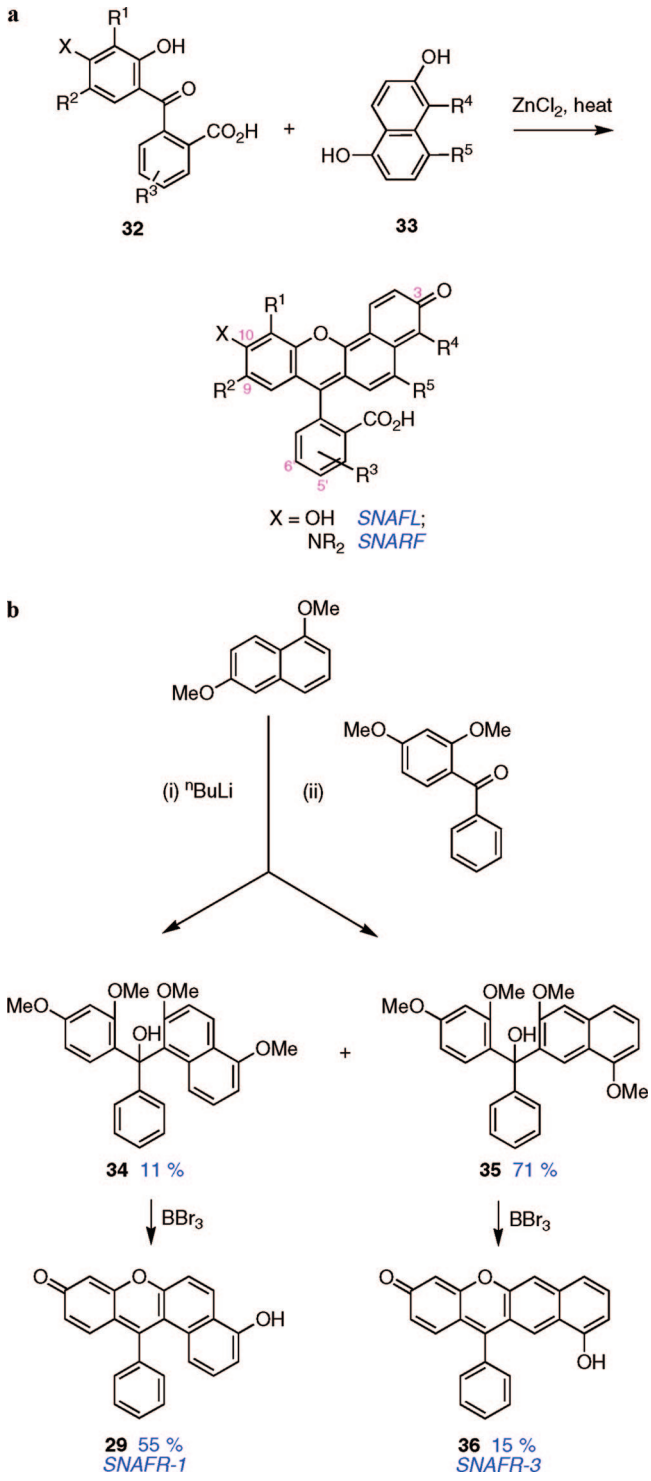
The mnemonics for these compounds are so similar that it is bewildering to use them, but understanding the following generalities may make these abbreviations more useful. Throughout, "**SNA**" stands for "**S**emi**NA**phtho-"SNA**FR**s and SNA**FL**s share similar molecular structures except that SNA**FR**s do not possess a carboxyl substituent at the 3′ position (they are **F**luo**R**ones), whereas SNA**FL**s are **FL**uorescein derivatives, and SNA**RF**s are derived from **R**hoda**F**luors. Figure 4c delineates how these dyes are related to fluorescein and rhodamines. Consideration of this graphic also makes it evident that there are other permutations of the annulation structure and *O*/*N*-substitution patterns that correspond to compounds that are not used as pH indicators and may even not have been prepared.

Semiempirical computer calculations (AM1) have been used to predict whether bathochromic shifts should be observed in the absorption and fluorescence spectra of the type [*a*] and [*b*] benzoxanthene isomers.[86] Some compounds in that series have recently been prepared, and they do indeed have red-shifted absorbance and emission maxima, but they have not yet been used for pH_i measurements.[85,87]

4.2. Synthesis and General Optical Properties of SNAFLs, SNAFRs, and SNARFs

Scheme 4a shows syntheses of benzo[*c*]xanthene dyes, i.e., SNARFs and SNAFLs, via condensation of 1,6-dihydrox-

Scheme 4. (a) Original Syntheses of SNARFs and SNAFLs, and (b) Illustrative Syntheses of a Benzo[*a*]xanthene and a Benzo[*b*]xanthene



ynaphthalenes **33** with the appropriately substituted benzophenone derivatives **32**; these in turn were made via coupling of resorcinol or 3-aminophenol with phthalic anhydride derivatives in toluene. For instance, carboxySNARF-4F **38** was synthesized via acid-catalyzed condensation of 5-fluoro-1,6-dihydroxynaphthalene with 2,4- (and 2,5)-dicarboxy-3′-dimethylamino-2′-hydroxybenzophenone.[88] Syntheses of benzo[*a*] and [*b*]xanthene dyes were only recently achieved.[85,87] In that preparation, lithiated 1,6-dimethoxynaphthalene was coupled with 2,4-dimethoxybenzophenone to produce compounds **34** and **35**. SNAFR-1 **29**

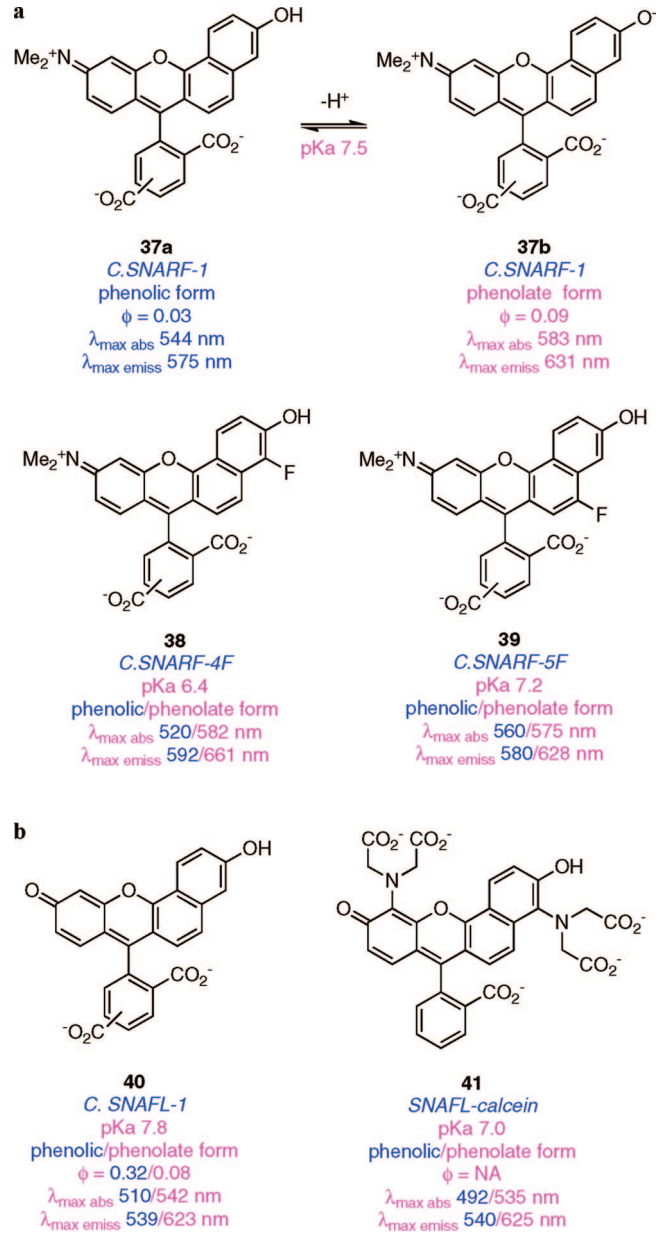


Figure 5. Spectral and photophysical properties of some commercially available benzo[*c*]xanthenes that have been used as pH indicators: (a) type B SNARFs and (b) SNAFL-1 derivatives.

and SNAFR-6 **36** were isolated in 55% and 15% yield after treatment with BBr_3.[87]

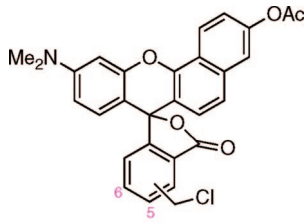
Molar absorptivities of SNARFs and SNAFLs are highest under basic conditions, and their absorbance maxima shift to the red; this is true of most fluorescein derivatives. However, unlike most fluorescein-based pH indicators, their emission spectra also show significant pH-dependent shifts. The protonated form emits in the yellow-orange region (540−580 nm), whereas deep red emissions (620−640 nm) are observed for the basic form. Both the absorbance and fluorescence spectra of SNARFs and SNAFLs show sharp, pH-independent, isosbestic points at ∼530 and ∼585 nm, respectively; these are desirable properties for dual-absorbance and dual-emission ratiometric measurements. SNARFs and SNAFLs have been used as dual-emission pH indicators[89] for determination of intracellular pH values via flow cytometry[90] and confocal spectroscopy.[89] *N,N*-Dialkyl SNARFs (i.e., type B SNARFs in Figure 5a) are more fluorescent in basic solutions where they exist predominantly as their

anionic forms (quantum yields of 0.05−0.20) than in their neutral forms (quantum yields of 0.02−0.07). For instance, the anionic and neutal forms of C.SNARF-1 **37a** and **37b** have quantum yields of 0.03 and 0.09, respectively. Conversely, SNAFLs and type A SNARFs (Figure 4b) have higher quantum yields (up to 0.5) in the neutral form (i.e., under acidic conditions; Figure 5b).

4.3. Long-Wavelength Dual-Emission pH_i Indicators: C.SNARF-1, C.SNARF-4F, and C.SNARF-5F

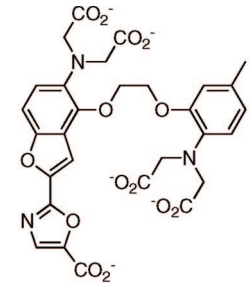
Carboxy-SNARF-1 **37** (or "C.SNARF-1")[91] is probably the *second* most widely used pH_i indicator behind BCECF (see above). It has been applied to determine absolute cytosolic,[15,92,93] mitochontrial,[89] and nuclear[15] pH values in living cells using flow cytometry,[90] microplate readers,[38] confocal imaging,[89] or microspectrofluorometry.[45]

Dyes like C.SNARF-1 **37** have several attributes that may explain why they are so widely used. First, C.SNARF-1 **37** can be temporarily shielded as an AM ester, facilitating import into living cells. Furthermore, the cell-permeable chloromethyl SNARF-1 acetate **42** slowly reacts with intracellular thiols, forming conjugates that are retained inside cells and facilitating long-term pH studies. Second, the ratiometric properties of C.SNARF-1 **37** are not significantly dependent on its concentration or on the ionic strength of the surrounding aqueous media; these are desirable properties for general-use pH_i indicators.[55] When C.SNARF-1 was irradiated to photobleach the compound, the ratio of the fluorescence intensities at 580 and 640 nm was shown to be essentially invariant; this makes the dye more suitable for extended experiments than it would otherwise be.[89] Furthermore, the fluorescence spectrum of C.SNARF-1 has been shown to be sufficiently different than the Ca^{2+} sensor fura-2 **43**[94] and the Na^+ sensor SBFI **44**,[95] facilitating simultaneous measurement of H^+, Ca^{2+}, and Na^+ concentrations in cells. Finally, C.SNARF-1 can be excited at longer wavelengths (514 or 536 nm) than some other probes, reducing cell damage due to radiation and circumventing some disadvantageous effects of intracellular autofluorescence when observing these compounds.

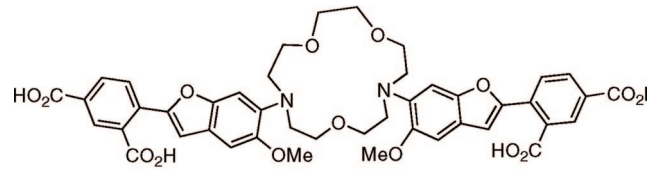


42
chloromethyl SNARF-1 acetate

There are also some drawbacks to using C.SNARF-1 as a pH indicator. It has a low quantum yield, especially under acidic conditions (neutral form; $\phi = 0.03$). Intracellular pH values under 7.0 cannot be measured accurately using this dye because its pK_a is too high (7.5). Moreover, the spectral properties of C.SNARF-1 are significantly influenced by temperatures and environments in living cells.[45] The quantum yield of the probe decreases by 25% when the temperature increases from 25 to 37 °C. Further, the brightness of the dye inside living cells is diminished probably because of its interaction with intracellular proteins. C.SNARF-4F **38**[88] and



43
Fura-2



44
SBFI

C.SNARF-5F **39** have lower pK_a values, 6.4 and 7.2, respectively, and are recommended by Life Technologies as replacements for C.SNARF-1 to measure acidic or cytosolic pH_i.[96,97] Some other C.SNAFLs, like C.SNAFL-1 **40** and SNAFL-calcein **41**, for example, have been used for measuring pH_i too, but their pK_a's are usually bigger than 7.6. Finally, the fast photobleaching rate of these dyes, especially at 37 °C, restricts the application of SNAFLs for the measurement of pH_i in living systems.[98]

5. Cyanine-Based pH_i Indicators

5.1. Design of pH-Sensitive Cyanine Dyes

Cyanine-based stains tend to absorb and emit in the near IR region; this is an advantage because those wavelengths cause minimal cell damage and are clear of cell autofluorescence and tissue absorption.[20,99–101] These factors also increase detection sensitivities and depth of light penetration in tissues. Sulfonated pentamethine and septamethine cyanine dyes have been widely used as labeling reagents because of their good water solubilities, quantum yields (tend to be >0.1), and high molar extinction coefficients (>200 000 L mol^{-1} cm^{-1}). One disadvantage of cyanine-based dyes is that they tend to photobleach faster than ones based on anthracenes or BODIPY.

There are two types of pH-sensitive cyanine dyes for biological applications. The first one features non-*N*-alkylated indolium structures (Figure 6a). These are almost totally nonfluorescent when the nitrogen atom is not protonated, but they are highly fluorescent as cations; thus, they are used as probes that reveal pH via their fluorescence brightness rather than shifts in emission wavelengths. Further, a new blue-shifted absorption peak emerges when the pH is increased. Subtle changes to the structure of these cyanine-based probes can change their pK_a values; hence, ones for use at near-neutral and acidic pHs have been obtained.

The second type of pH-sensitive cyanine probe is based on photoinduced electron transfer (PeT) (Figure 6b); these consist of a fluorophore and a nitrogen-containing modulator. Turning on and off the fluorescence is achieved by suppressing or allowing PeT processes by protonation/deprotonation of the modulator. Again, the fluorescence emission wavelength is largely unaffected by pH changes. In contrast

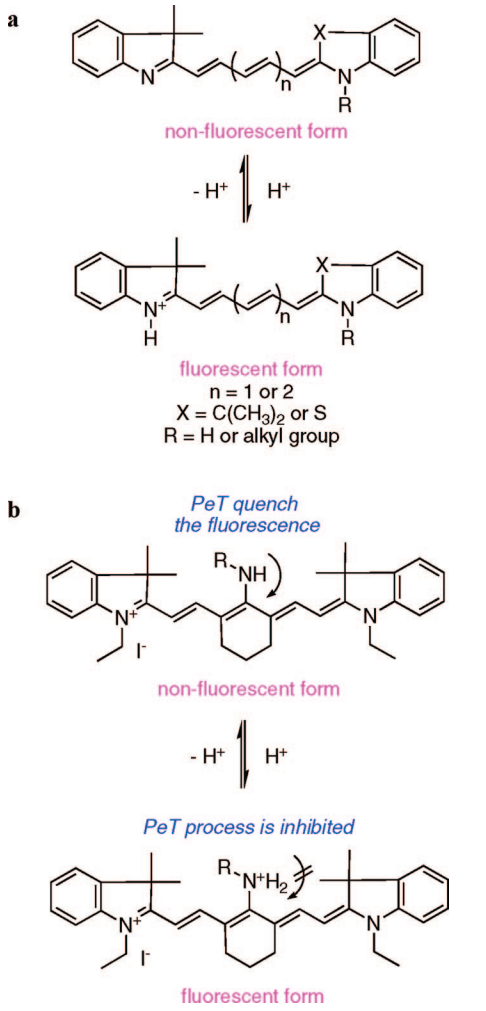


Figure 6. Two types of design of pH probes based on polymethine cyanines: (a) non-*N*-alkylated indolium Cy-5 and Cy-7 dyes and (b) modulator-regulated tricarbocyanines.

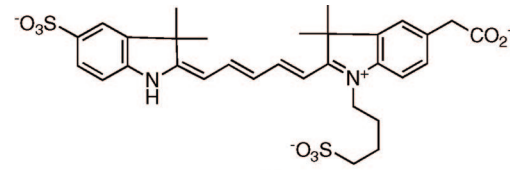
to the first type of pH indicators, however, the maximum absorption peak has a large red shift when the pH is increased.

5.2. Near-Neutral Cyanine-Based pH Indicators

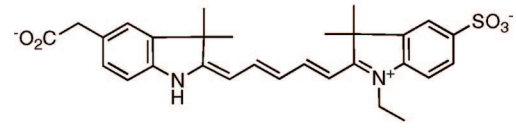
pH-neutral pentamethine cyanine dyes **45**−**48** with pK_a's 7.1−7.5 fluoresce at ~665 nm and have maximum absorption peaks at ~645 nm.[20,102,103] These pH-sensitive Cy-5 dyes have desirable properties for visualization of small pH changes inside live cells. Sulfonic acid functional group is included to improve their solubilities in aqueous media and to prevent aggregation. Linkage of these pH probes to biomolecules has been obtained via activation of the carboxylic acid functional group as a *N*-hydroxysuccinimidyl ester.

A derivative of indocyanine green in which one *N*-butylsulfonic acid is replaced by a hydrogen atom gives the pH-sensitive probe **49**, H-ICG.[104] This norcarbocyanine fluoresces around 800 nm, and this emission shows small but noticeable red shifts at increased pH values; a pK_a of 7.2 has been calculated for this process. However, this compound is not very soluble in aqueous media (it has only one sulfonic acid), and it does not have a group for conjugation to biomolecules.

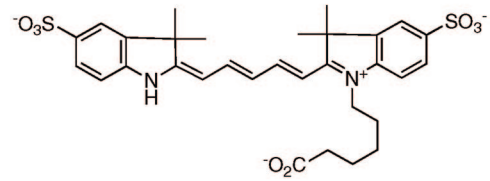
The neutral pH fluorescent probe **50**[100] has a tricarbocyanine (Cy) fluorophore coupled with a 4′-(aminomethylphenyl)-2,2′:6′,2″-terpyridine (Tpy) receptor. At pH 10, the brightness of **50** was observed to be low (ϕ = 0.008), presumably due to quenching via PeT, involving electrons of the Tpy group. Protonation of the N atoms circumvents these PeT processes, and the dye fluoresces brightly (ϕ = 0.13) at 750 nm with a pK_a in aqueous buffer of ca. 7.1. Compound **50** imported into HepG2 cells (it is cell permeable) is more fluorescent at pH 7.0 than at 7.8 ex vivo; this property has been used to follow minor pH changes in the



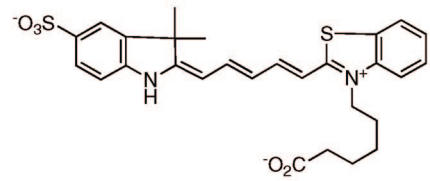
45
pKa ~ 7.5
ϕ = NA
$\lambda_{max\ abs}$ = 645 nm (pH 4-5)
$\lambda_{max\ emiss}$ = 665 nm (pH 4-5)



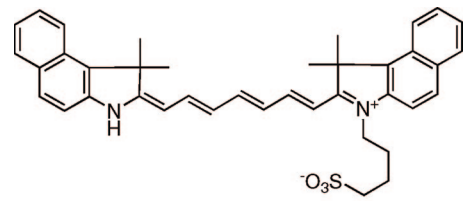
46
pKa ~ 7.5
ϕ = NA
$\lambda_{max\ abs}$ = 645 nm (pH < 6)
$\lambda_{max\ emiss}$ = 665 nm (pH < 6)



47
pKa ~ 7.1
ϕ = NA
$\lambda_{max\ abs}$ = 653 nm (pH 4-5)
$\lambda_{max\ emiss}$ = 660 nm (pH 4-5)

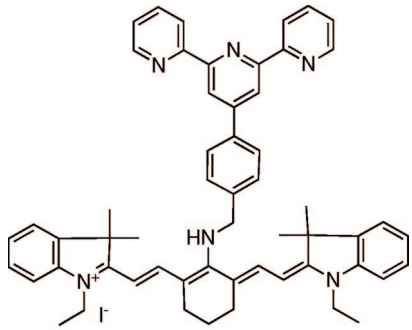


48
pKa ~ 7.1
ϕ =NA
$\lambda_{max\ abs}$ = 640 nm (pH 4-5)
$\lambda_{max\ emiss}$ = 670 nm(pH 4-5)



49
H-ICG
pKa ~ 7.2
ϕ = NA
$\lambda_{max\ abs}$ = 780 nm (pH < 4)
$\lambda_{max\ emiss}$ = ~ 800 nm (pH < 4)

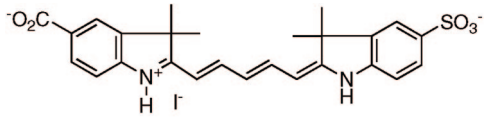
6.7−7.9 range.[100] Moreover, this probe was shown to have low cytotoxicity and good photostability.



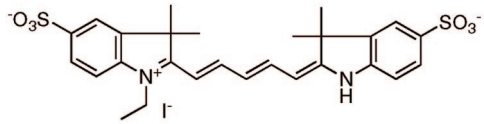
50
pKa ~ 7.1
ϕ = 0.13 (in acidic solution)
$\lambda_{max\ abs}$ = 648 nm
$\lambda_{max\ emiss}$ = 750 nm

5.3. Acidic Cyanine-Based pH Indicators

Acidic cyanine dyes tend to absorb and fluoresce above 630 nm; this is an advantage relative to most other acidic pH probes, such as Lysosensors (see the next section). The non-*N*-alkylated indolium septacarbocyanines **51** (CypHer 5) and **52** have wavelength emission maxima around 665 nm that do not shift with pH, but their intensities change.[102] This is because the protonated probe absorbs at ~650 nm but at 450−520 nm in the basic form with a pK_a of ~6.1. That pK_a response has proved useful for monitoring cellular internalization of G protein-coupled receptors (GPCRs)[20] and viral particles.[19] Thus, CypHer 5 **51** on *c-myc* or *anti*-VSV glycoprotein complex with *N*-terminally tagged epitopes on the *c-myc*-*δ*-opioid receptor or the thyrotropin-releasing hormone receptor, respectively. Agonists for this class of receptor (e.g., DADLE or TRH) stimulate their internalization into endosomes, resulting in fluorescence increases compared to cells that are not treated with such agonists.[20]

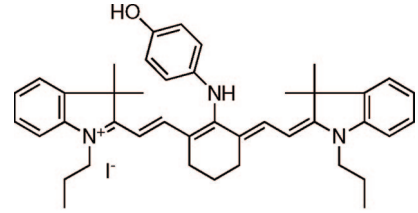


51
CypHer 5
pKa 6.1
ϕ = NA
$\lambda_{max\ abs}$ = 655 nm (pH 4-5)
$\lambda_{max\ emiss}$ = 665 nm (pH 4-5)



52
pKa 6.4
ϕ = NA
$\lambda_{max\ abs}$ = 650 nm (pH 4-5)
$\lambda_{max\ emiss}$ = 665 nm (pH 4-5)

Cyanine *Ap-Cy* **53** is cell permeable. It has an aminophenol-based modulator; hence, it has an optimal pH response around ~5.1.[105] When protonated, the dye has a maximum absorbance at 558 nm and fluoresces at 615 nm. Its fluorescence intensity increases about 10-fold when the pH is decreased from 6.5 to 4.0. These characteristics have been exploited when *Ap-Cy* **53** was used for monitoring pH_i within HepG2 cells.[105]



53
Ap-Cy
pKa ~ 5.1
ϕ = NA
$\lambda_{max\ abs}$ = 558 nm (pH 4-6.5)
$\lambda_{max\ emiss}$ = 615 nm (pH 4-6.5)

6. Miscellaneous Small Molecule pH_i Indicators

This section covers small molecule pH_i indicators that cannot be grouped into the categories discussed above. The first four considered in this subsection (europium complexes **54**, a fluorene derivative **55**, 1,4-DHPN **57**, and HPST **58**) are indicators for near-neutral environments. The rest of the dyes in this section are useful under more acidic conditions; they are based on anthracene, BODIPY, or rhodamine structures to give emission maxima that occur at longer wavelengths.

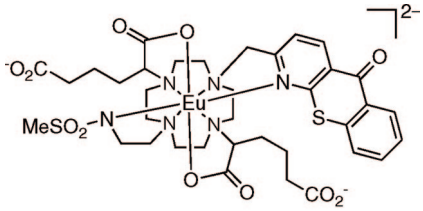
6.1. Various Indicators for Near-Neutral pH Values

6.1.1. Europium Complex

Emissive europium(3+)[106] complexes such as **54** may be applied for measurement of pH_i.[107] In molecule **54**, the sensitizing group azathiaxanthone allows excitation in the range from 360 to 405 nm. Fluorescence of this complex between 680 and 710 nm is hypersensitive to *N*-ligation of the sulfonamide which, unlike the sensitizing group, dissociates from the metal as the pH is lowered (see structure **54b**). Thus, the fluorescence intensity at 680 nm is quite strong in basic aqueous solutions (pH ≈ 8) and diminished in acidic media (pH 4−5). This characteristic makes the complex suitable for ratiometric pH measurement based on fluorescence intensity ratios at 587 and 680 nm as a function of pH. The complex possesses a large Stokes shift of ~200 nm and fluoresces in the near-IR region where cell autofluorescence is less problematic. Moreover, complex **54** is cell permeable and nontoxic. When the dyes are used to stain cells, confocal fluorescence microscopy indicates that both the europium emission (ca. 570 nm) and the azathiaxanthone fluorescence (450 nm) eminate mainly from the nucleus, implying that the intact complex is localized there. A disadvantage of **54** is that, like most lanthanide complexes, it has a relatively low quantum yield (0.06) spread over multiple fluorescence emissions.

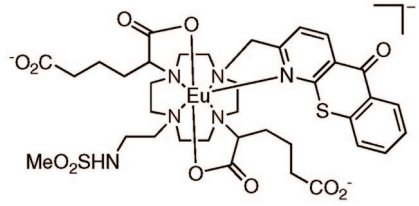
6.1.2. Fluorene Derivative

Fluorene derivatives usually fluoresce in high efficiency and exhibit excellent photostability. The donor-π-acceptor fluorene derivative **55**[108] is a near-neutral pH_i indicator with a pK_a of ~7. It is water soluble, cell permeable, and diffuses into the cytosol. Also, it has low cytotoxicity (in the 0.1−100 μM concentration range)[108] as indicated by the Alamar Blue reduction analysis (a method to test cell viability).[109] Sharp



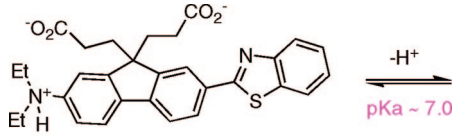
54a

pKa ~ 6.2

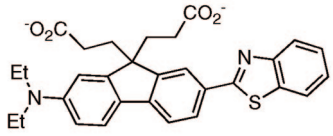


54b

isosbestic points are observed in the absorbance and emission spectra of this dye (at 355 and 492 nm, respectively); as we have already commented, isosbestic points are highly desirable for ratiometric measurements because they are indicative of well-proportioned spectral transformations between two {pH} states. Furthermore, this probe has been applied for imaging of two-photon excitation with a relatively large two-photon absorption cross section (100 GM at ~800 nm) in its neutral form **55b**.



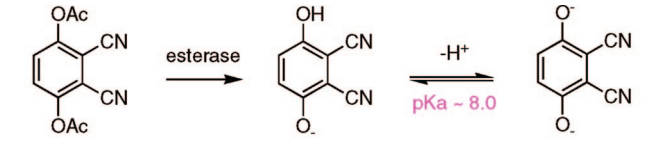
55a *N-protonated form*
$\phi = 0.21$
$\lambda_{max\ abs} = 341$ nm
$\lambda_{max\ emiss} = 391$ nm



55b
neutral form
$\phi = 0.56$
$\lambda_{max\ abs} = 382$ nm
$\lambda_{max\ emiss} = \sim 580$ nm

6.1.3. 1,4-Dihydroxyphthalonitrile (1,4-DHPN)

1,4-DHPN **57**[110,111] was a commonly used pH_i indicator in the early 1980s before it was largely superseded by BCECF **4** and C.SNARF-1 **37**. The spectral properties of this compound are more desirable for intracellular pH_i measurements than fluorescein derivatives. This is because the fluorescence emission maximum for 1,4-DHPN shifts with pH, whereas fluoresceins tend not to have this characteristic; hence, they are used to give changes of fluorescence intensities at one single wavelength.[47] The maximum fluorescence wavelength in the emission spectra of 1,4-DHPN shifts from 450 to 476 nm as pH is increased from 3 to 10, and this permits the dual-emission ratiometric measurements. The ratio of the fluorescence intensities at 512 and 455 nm does not significantly change with dye concentration and the ionic strength of the medium. Further, the dye is not toxic to cells (at least as assessed by monitoring oxygen consumption, an older method to test cell viability).[111] The parent dye is not especially cell permeable, but the corresponding diacetate, 1,4-diacetoxyphthalonitrile (1,4-DAPN) **56**,[112] is, and it can be hydrolyzed into 1,4-DHPN **57** by the enzyme esterase. 1,4-DHPN **57** has been used to sense the pH_i regulatory responses when A6 cells are incubated with acid and base.[111]



56
1,4-DAPN
cell permeable

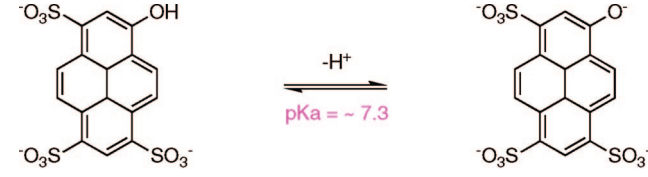
57a
1,4-DHPN
phenolic form
$\lambda_{max\ abs} = 342$ nm
$\lambda_{max\ emiss} = 453$ nm

57b
1,4-DHPN
phenolate form
$\lambda_{max\ abs} = 402$nm
$\lambda_{max\ emiss} = 483$ nm

There are, however, several drawbacks associated with applications of 1,4-DHPN **57** in pH_i measurements. First, the dye is rapidly cleared from living cells because it only has 1−2 negative charges at physiological pH values.[113] Second, the low UV excitation wavelengths typically used for this dye (350−365 nm) might perturb the cells. Third, the emission spectrum does not have a well-behaved isosbestic point; hence, this dye is not ideal for ratiometric methods based on differences in emission wavelengths. Overall, dyes like the BCECF and SNARFs are more favorable with respect to these parameters; hence, they tend to be preferred over 1,4-DHPN for pH_i measurements.

6.1.4. 8-Hydroxypyrene-1,3,6-trisulfonic acid (HPTS)

HPTS is a highly water-soluble dye compound[114] with low toxicity,[115] and it is also very cheap compared with most other indicators. It has been used for measurement of cytoplasmic pH[116] or acidic organelle pH[117] in many cell types. Excitation ratio imaging is possible using HPTS **58** since it has absorbance maxima at 405 and 465 nm that increases and decreases, respectively, when the solution pH is varied from 5 to 8. Furthermore, this tri- or tetra-anionic dye is retained well inside living cells at physiological pH values. The main limitation to the use of HPTS **58** as an intracellular indicator is its lack of cell permeability, and there is no convenient pro-drug-like form to facilitate transport of this dye into cells. This accentuates the general need for sulfonic acid protecting groups that are cleaved by esterases. At present, HPTS **58** is only useful for pH_i measurements when loaded inside living cells via microinjection, electroporation,[118] and scrape loading,[116] which might damage the cells.



58a
HPTS
phenolic form
$\phi = 1.0$
$\lambda_{max\ abs} = 405$ nm
$\lambda_{max\ emiss} = 514$ nm

58b
HPTS
phenolate form
$\phi = 1.0$
$\lambda_{max\ abs} = 465$ nm
$\lambda_{max\ emiss} = 514$ nm

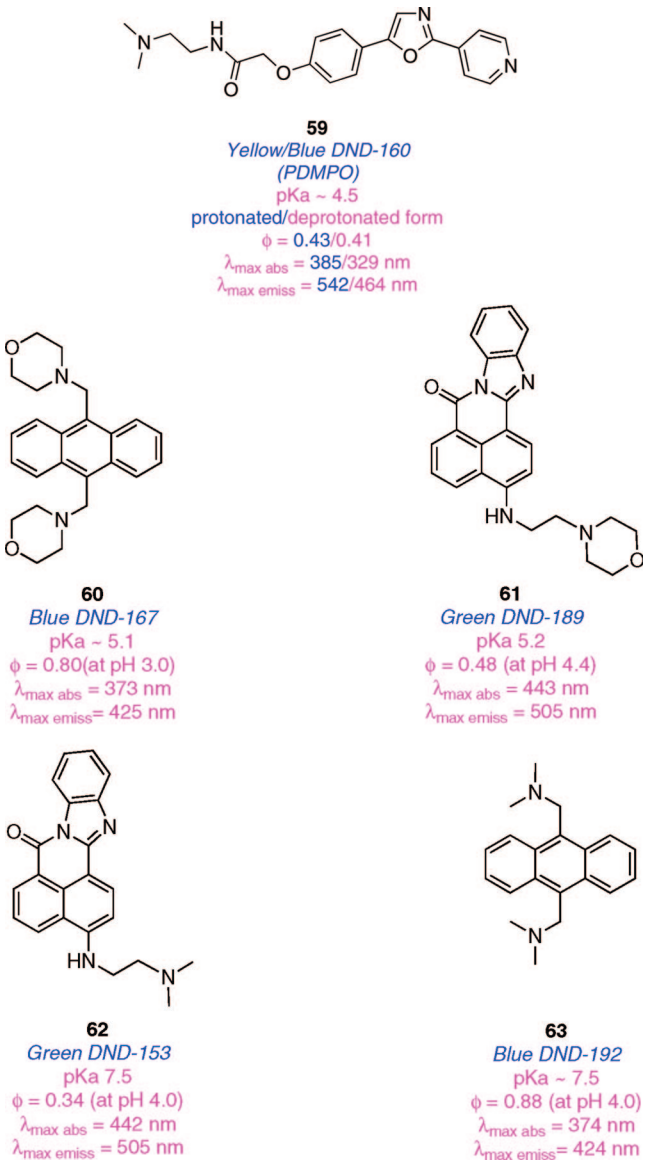


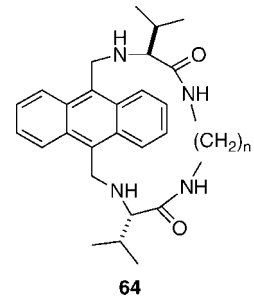
Figure 7. Commercialized lysosensors for acidic environments.

6.2. Various pH Indicators for Acidic Environments

6.2.1. Commercialized Lysosensors, and Anthracene Dyes

Lysosome interiors tend to be acidic; hence, indicators that detect in this region are sometimes referred to as "lysosensors". Many probes are available from Life Technologies for measurement of acidic pH_i values. A pyridyl oxazol probe Yellow/Blue DND-160 PDMPO **59**,[119] the anthrathene-based sensor DND-167 **60**,[120] DND-189 **61**, DND-153 **62**, and DND 192 **63**,[120–122] (Figure 7) are dyes that work on the principle of that electronic excited states can be quenched before they fluoresce by electron transfer from amines; this process is known as PeT.[123,124] Dyes of this type become more emissive in acidic environments when proximal amines are protonated. Of all the lysosensors shown in Figure 7, DND-160 **59** is unique because it is brightly fluorescent in protonated and deprotonated forms ($\phi \approx 0.4$ for both forms), and its absorbance and fluorescence spectra are significantly blue shifted with isosbestic points at 365 and 470 nm as the pH values are increased. The acidic form DND-160 **59** fluoresces brightly in yellow light with a peak at 542 nm. The basic form emits strongly blue light with a maximum at 464 nm. Furthermore, it showed pH-dependent lifetime responses, indicating a good probe for lifetime imaging to determine lysosomal pH.[124] The pK_a of DND-160 **59** is about 4.5. DND-160 **59** has been applied for dual-emission imaging for lysosomal pH. Advantages of the DND dyes and of anthracene derivatives in particular are that they tend to be relatively photostable and cell permeable. Conversely, a disadvantage associated with that particular dye type is that anthracene absorbs and emits at relatively short wavelengths (377 and 430 nm),[120] leading to cell damage and undesirable artifacts from autofluorescence. LysoSensor DND-189 **61** is exceptional insofar as its fluorescence sharply decreases in acidic environments, i.e., at pH values from 4.0 to 2.0.[125] DND-153 **62** and DND-192 **63** with a pK_a of 7.5 are sensitive to neutral pH but still have strong emission in green light at pH 8.[124] Presumably PeT does not quench the fluorescence of these dyes completely because the oxidation/reduction potentials of the fluorophore and the amine are not well matched for this.

A group of macrocyclic peptidomimetics FG-H503 **64a**, FG-H504 **64b**, FG-H506 **64c**, and FG-H508 **64d** derived from the 9,10-dimethylanthracene moiety were reported in 2005.[125] All of these probes **64** have very similar absorbance (at 377 nm) and fluorescence maxima (ca. 430 nm) in aqueous solution but have tunable pH properties for the fluorescence imaging of acidic organelles in live cells. The peptidic parts differ only in the size of the cyclic systems they form around the anthracene (*n* changes from 3 to 8): this structural change modifies the pK_a values of the amine parts from 5.06 to 5.43. Thus, these peptidomimetics are useful in a pH region that is *not* covered effectively by lysosensors DND-167 **60** and DND-189 **61** (pK_a of 5.1 and 5.2). It was concluded that FG-H503 **64a** localizes in acidic organelles after being taken up by macrophage Raw 264.3 cells, because it colocalized with lysosomal probes DND-189 **61** and DND-26 **67**.



64

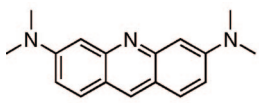
	n	pKa
a	3	5.06
b	4	5.24
c	6	5.41
d	8	5.43

6.2.2. Acridine Dyes

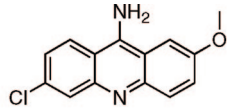
Lipophilic, weak bases, such as monoamine, diamine acridine orange **65** (AO), and 9-amino-6-chloro-2-methoxyacridine **66** (ACMA), have been used to stain various organelles. These dyes are cell permeable in their neutral forms but much less so when protonated. The absorbance and fluorescence spectra of AO **65** are dependent upon its concentration.[126] As a nonaggregated monomer, AO **65** absorbs at ~492 nm and emits green light around 530 nm. A red emission at 655 nm has been attributed to dimers or oligomers of AO **65**; these aggregates have blue-shifted absorbance maxima, ca. 465 nm.[127,128] Fluorescence ratios in the green/red (530/655 nm) were shown to be dependent on dye concentrations in the acidic compartments of living cells. Concentrations of AO **65** in membrane vesicles are

dependent on the pH difference between the cytosol and the interior of the vesicles. In fact, relative acidities of vesicles in sensitive or in multidrug-resistant cancer cells have been appraised by determination of the red/green ratio of AO **65**.[127]

A major limitation of AO **65** as a pH probe is that its spectral properties are significantly affected by temperature and the presence of anions;[128,129] for instance, NO_3^- anion can induce aggregation of AO **65**. For this reason, AO **65** is not used for quantitative determination of pH_i.



65
Acridine orange
ϕ = 0.46 EtOH, 0.01M HCl
monomer/dimer
$\lambda_{max\ abs}$ = 495/465nm
$\lambda_{max\ emiss}$ = 530/655 nm

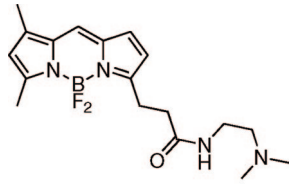


66
ACMA
pKa 8.6 (monocation)
ϕ = 0.66 (pH 7.2)
$\lambda_{max\ abs}$ = ~ 419nm
$\lambda_{max\ emiss}$ = 484 nm

ACMA **66**[130] is a nucleic acid stain. It exists mainly in its monocation form (pK_a 8.6) in physiological environments. This dye, unlike AO **65**, does not dimerize in aqueous solution, even at concentrations as high as 200 μM.[131] Its fluorescence is quenched by pH or potential gradients across cell membranes.[131,132]

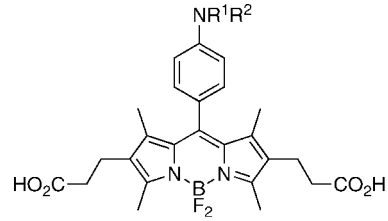
6.2.3. BODIPY-Based Dyes

Cell-permeable LysoTrackers, i.e., a BODIPY derivative Green DND-26 **67**, could also be used for imaging acidic compartments in live cells. This dye tends to absorb and emit at longer wavelengths than the anthracene derivatives, and it is a brighter probe because its molar absorptivity is higher. Comparing to lysosensors, which exhibit a pH-dependent increase in fluorescence intensity upon acidification, lysotrackers, i.e., DND-26 **67**, do not have enhanced fluorescence intensity at acidic pH. LysoTraker probes with varied fluorescent colors are available in Life Technologies.



67
Green DND-26
pKa NA
ϕ = NA
$\lambda_{max\ abs}$ = 504 nm
$\lambda_{max\ emiss}$ = 511 nm

A series of pH probes based on BODIPYs **68** (i.e., NH_2BDP **68a**, DiMeNBDP **68b**, EtMeNBDP **68c**, and DiEtNBDP **68d**) were recently reported for imaging acidic endosomes in cancer cells.[133] These compounds **68** are almost nonfluorescent in basic media (ϕ < 0.002) due to PeT quenching by the *meso*-aminophenol substituent. However, they are highly fluorescent in acidic environments (ϕ = 0.55−0.60) when the aniline amine is protonated. The pK_a values of these BODIPY dyes **68** range from 3.8 to 6.0; this range is possible by changing the alkyl group on the nitrogen. Monoclonal antibody trastuzumab labeled with these acidic pH-sensitive dyes selectively target the human epidermal growth factor type 2 receptor and are then internalized. Confocal spectroscopy revealed that the antibody−probe conjugates are not fluorescent outside cells at neutral pH values. However, 2 h after they are combined with appropriate cells, the pH probe−antibody conjugates fluoresce in endosomes. Only viable cells are visualized under these conditions because the acidic pH in lysosomes is maintained by an energy-consuming proton pump; this factor can be an advantage for some analyses.



	R^1	R^2	pKa
a	H	H	3.8
b	Me	Me	4.3
c	Me	Et	5.2
d	Et	Et	6.0

68

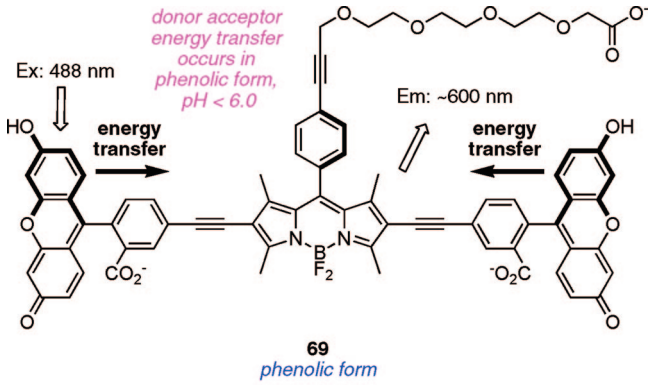
6.2.4. pHrodo Indicators

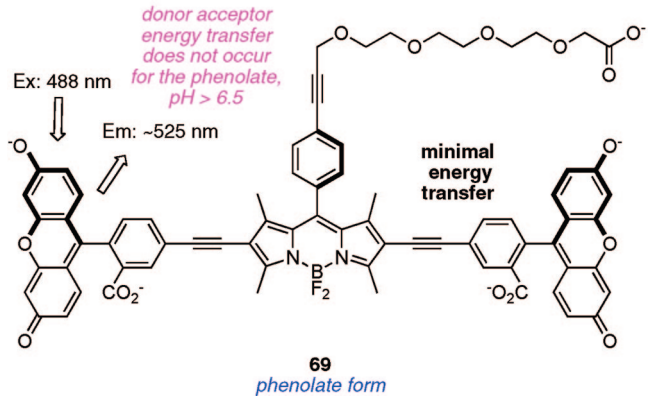
pHrodo is a new rhodamine-based probe introduced by Life Technologies, but its exact structure is not given by them. Its fluorescence is dramatically increased in acidic environments. pHrodo−biomolecule conjugates have pK_a values of ~6.5 and absorption/fluorescence maxima at 560 and 585 nm, respectively. Background subtraction is not usually required because this acidotropic probe is nonfluorescent at near-neutral environments but gives intense red signals in acidic vesicular compartments. It has been used for determining the engulfment of apoptotic cells by macrophages.[18]

7. Energy-Transfer Cassette

Compound **69** based on through-bond energy-transfer cassettes[134–137] has been used for probing pH_i in COS-7 cells.[38] Probe **69** consists of two xanthene donors, one BODIPY acceptor, and a triethylene glycol carboxylic acid linker. The linker part is designed to increase the water solubility of the compound in aqueous solution and allow attachment to biomolecules. Energy-transfer efficiency from the donors to the acceptor is modulated by the oxidative potentials of the xanthene part, which in turn depend on its protonation state. Thus, when the system is excited at wavelengths that correspond to the donor the fluorescence of the whole system is sensitive to the pH of the medium. At pH 5.5 or less, the xanthene donors exist in the phenolic state, the oxidation potential is ideal for energy transfer, and the probe fluoresces via the acceptor, i.e., red, around 600 nm.[138] Conversely, the xanthene donors exist predominantly in the phenolate form under basic conditions pH > 7. In that state the donor and acceptor oxidation potentials are not well matched for energy transfer, and the sensor fluoresces almost exclusively from the donors parts (green, i.e., around 520 nm). If the pH is between 5.5 and 6.5, the cassette emits from donors as well as the acceptor. Overall, the cassette remains fluorescent as the pH is changed.

A recent discovery from our laboratories shows that Pep-1-mediated import into COS-7 cells tends to deposit the dye-labeled protein cargoes into the cytosol and endosome when the experiment is performed at 4 and 37 °C, respectively.[139] Thus, **BSA-69** conjugate under these conditions would be expected to fluoresce with different red-to-green ratios when **BSA-69** is distributed within the cytosol with pH at ~7.2 and the endosome with pH around 5−6. An ex-vivo





calibration curve was generated for the cassette shown above (Figure 8); this facilitated its use to determine pH values for the endosomes and the cytosol. The pH values of endosome and cytosol, obtained from the red/green ratio (5.03 and 2.03), were 5.4 and 7.2, respectively; these data are consistent with those expected for such intracellular regions.

Imaging of protein-**69** inside cells was possible using this probe. We favor reserving the word probe for labels that can be conjugated with biomolecules to track them within cells. This distinction is important when differentiating these from stains. We reserve the word stains for dyes like C.SNARF-1 **37** that are usually used in solutions to bathe the cells and stain their interiors. Dyes like C.SNARF-1 **37** are usually not attached to biomolecules then imported into cells for several reasons. These reasons relate to their low quantum yields, making them hard to visualize at low concentrations, and photobleaching effects.

8. pH Indicators Based on Nanoparticles, Lipobeads, and Microspheres

Nanoparticles can have unique properties resulting from their large surface-to-volume ratios and small sizes; consequently, they have some potential as sensors in medicine and biotechnology. Probes for pH based on nanoscaffolds can possess several advantages over small molecule pH sensors. First, multiple indicators can be attached to single particles; hence, the localized brightness of the system is increased. Second, particles can simultaneously support pH-sensitive and -insensitive dyes to facilitate ratiometric measurements. Third, nanoparticles may be less vulnerable to leakage through cell membranes and to cellular compartmentalization. Fourth, some nanoparticles are more photostable than small organic dyes. Finally, the physical properties of the nanoparticles can be modulated and manipulated

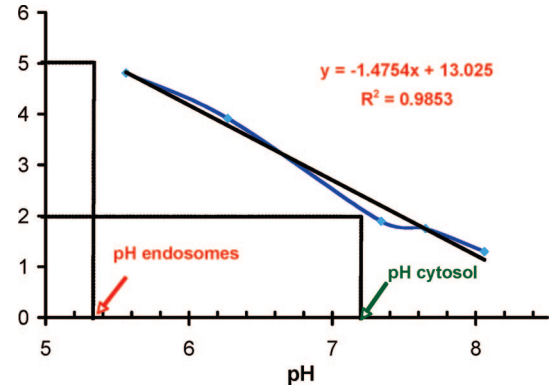


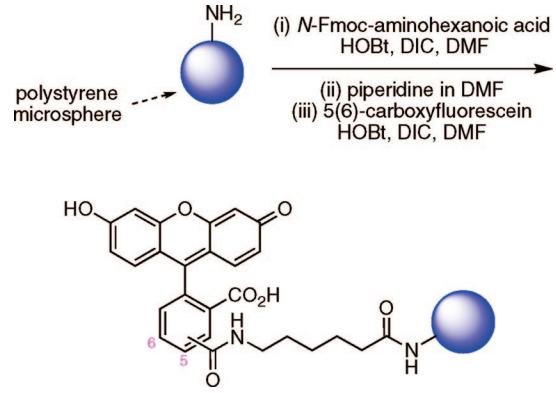
Figure 8. Ex-vivo calibration curve with pH values corresponding to those observed within endosomes (red/green = 5.03; import at 37 °C) and the cytosol (red/green = 2.03; import at 4 °C).

by adjusting their core structures, for instance, by choosing between bacteriophage, silica, and coated polystyrenes.

8.1. Polystyrene Microspheres

Fluorescein-loaded onto amino-functionalized polystyrene microspheres, ca. 2 μM diameter, have been used for real-time detection of H^+ concentrations inside living cells.[140] These microspheres were shown to be cell permeable and noncytotoxic to cells at any concentration tested. These beads have an aminohexanoic acid linker between the bead and the fluorescent label {formed from 5(6)-carboxyfluorescein, Scheme 5}.

Scheme 5. Synthesis of Fluorescein-Capped Polystyrene Microspheres



8.2. Bacteriophage Particles

M13 bacteriophage particles (see Figure 9) functionalized with cyanine dyes have been used for determination of intracellular pH.[141] These particles provide a flexible heterofunctional platform that is approximately 880 × 6.6 nm in size. They contain ca. 2700 copies of the p8 coat protein; hence, the surface of the particle displays amine groups that may be used for conjugation to other molecules. In this particular case those amines were coupled with the cyanine dyes, HCyC-646 (pH-sensitive) **70** and Cy-7 dyes **71** (pH insensitive, Figure 9). When protonated, HCyc-646 **70b** absorbs at 646 nm and emits at 670 nm with a quantum yield of 0.08 in aqueous solution. In neutral or basic environments, the dye is deprotonated **70a**, there is an hypsochromic (blue) shift of the absorbance to 506 nm, and the near-IR fluorescence is lost. The pK_a of HCyc-646 **70** is 6.2, which is suitable for sensing acidic environments in live cells. The

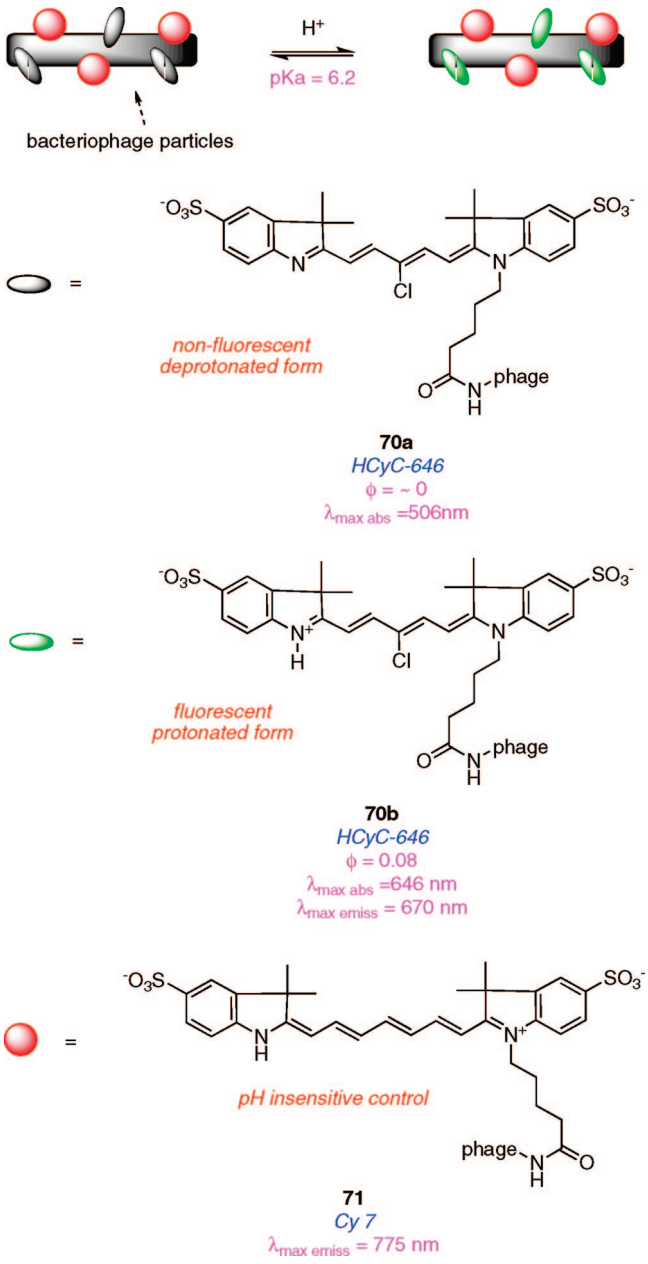


Figure 9. HCyC-646, **70**, and Cy-7, **71**, loaded onto bacteriophage particles.

pH-insensitive dye Cy-7 **71** emits at 775 nm, and this fluorescence provides an in-built control on the nanoparticle that can be used to calibrate the fluorescence changes of the other dye. Typically 400−500 copies of HCyC-646 **70** and Cy-7 **71** combined were attached to the bacteriophage. In one experiment, incubation of the labeled bacteriophage with RAW cells for 1 h gave internalization of the particles into acidic organelles where they had a pH of 5.0−6.5; such values are to be expected for intracellular vesicles such as lysosomes, endosomes, and phagosomes.

Imaging through tissue was also achieved using dye-labeled bacteriophage particles.[141] Good correlations were observed between ratiometric pH readings from these particles and the values measured via an electrode. However, a limitation of this system is that fluorescence emissions from HCyC-646 **70** and Cy-7 **71** at (670 and 775 nm, respectively) penetrate tissue with different efficiencies; hence, a correction factor must be applied for accurate measurements of pH.

8.3. CdSe/ZnSe/ZnS Quantum Dots

Colloidal luminescent mercaptoacetic acid capped CdSe/ZnSe/ZnS quantum dots are pH sensitive and have been applied to sensing intracellular pH in human ovarian cancer cells.[142] The CdSe core emits visible light, and the two ZnSe/ZnS shells stabilize the photoluminescence properties of the quantum dots by preventing oxidation of the core. Capping the dots with mercaptoacetic acid also serves to increase their water solubilities. Fluorescence intensities of these quantum dots in cells increase monotonically with increasing pH, i.e., it is quenched in acidic solutions. In living cells these particles are around 10-fold less fluorescent at pH 4 than at pH 10. Further, their high resistance to photobleaching facilitates long-term cell tracking and monitoring of the intracellular pH.

8.4. Silica Nanoparticles

Fluorescent dyes encapsulated in silica nanoparticles, "fluorescent core-shell silica nanoparticles", have been produced for quantitative chemical sensing in live cells. The fluors encapsulated in these particles tend to be brighter and more photostable than the corresponding free dyes in solution.[143,144] Dual-emission sensor nanoparticles can combine a pH-sensitive fluorescein dye and a pH-insensitive dye like tetramethylrhodamine. Such particles have been shown to be endocytosed by RBL mast cells upon the addition of the macropinocytosis stimulator, phorbol 12,13-dibutyrate. Following uptake, the particles were trapped in endosomes that later matured into lysosomes. The pH values of various intracellular locations indicated by confocal fluorescence images varied from 6.5 (endosome) to 5.0−5.5 (lysosome). The rhodamine internal standard for the pH_i measurements also acts as an indicator of the particle location even in acidic pH conditions where the fluorescein component is less emissive.

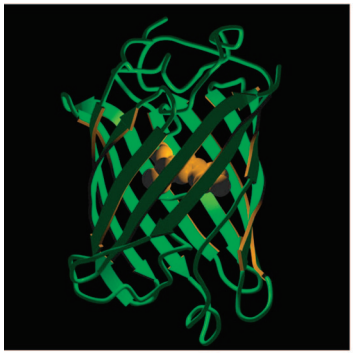
8.5. Lipobeads

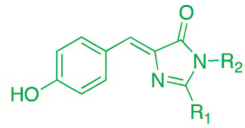
Micrometric phospholipid-coated polystyrene particles, also called "lipobeads", have been used for determination of pH_i in murine macrophage cells.[145] Again, just as in the work described above, pH-sensitive fluorescein and pH-insensitive tetramethylrhodamine were used for these ratiometric pH measurements; the liposome-encapsulated dyes display sensing properties similar to those observed in aqueous solution. In this case those fluors were covalently attached to the phospholipids coats on the polystyrene particles, thus preventing leakage of dye molecules into the microenvironment. The lipobeads were shown to be noninvasively ingested by macrophage cells and delivered into lysosomes. However, use of macrophage cells is not a stringent test of the ability of particles to permeate cell walls or of their cytotoxic effects; this is because macrophage cells easily ingest foreign material, and they are relatively robust. Bright field images of the particles in these cells indicated they were not significantly aggregated. Lysosome pH values deduced using these lipobeads were 5.7 ± 0.1; this is a reasonable value.

9. Fluorescent Proteins

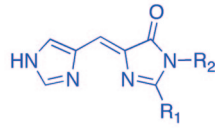
Green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* is widely used as a reporter for gene expression[146] and as a marker for biomolecules.[147] GFP has a

cylindrical 11-strand β-barrel structure encapsulating the chromophore *p*-hydroxybenzylideneimidazolidinone **72**. This fluorescent part is formed by autocatalytic condensation, cyclization, and oxidation of three consecutive amino acids Ser-Tyr-Gly from the 65−67 parent protein. The β-barrel forms a relatively rigid, hydrophobic environment which enhances the quantum yield of the chromophore.[148]





72
GFP chromophore



73
BFP chromophore

Photophysical properties of GFP and similar fluorescent proteins can be modified by mutagenesis.[149–151] For instance, replacement of the S66 tyrosine residue in GFP with histidine gives the blue fluorescent protein BFP that contains the chromophore **73**. The spectral properties of both native GFP and its mutants are strongly pH dependent in aqueous solutions,[152] suggesting pH-sensing roles and applications in cell compartments. The S65T-GFP chromophore has a pK_a of 6.0 and absorbance maxima at ~382 and 490 nm. The intensities of these peaks change with solution pH; in acidic environments, absorbance at 382 nm predominates, but in basic media, the 490 nm peak predominates.

Two GFP mutants S65T and F64L/S65T, also termed GFPmul1, have been used for measurement of pH of cell compartments in living cells.[153,154] Similar pH_i values were deduced using GFPmul1 and pH_i indicator BCECF. Another pH-sensitive GFP mutant, called enhanced yellow fluorescent protein (EYFP), has a pK_a of 7.1, suggesting that this protein is suitable for pH_i measurements in pH range of 6.5−7.5.

One advantage of fluorescent proteins is that they can be targeted to specific organelles such as cytosol, nucleus, mitochondria, trans-Golgi, and endoplasmic reticulum by expressing them in conjugation with appropriate targeting peptides or proteins.[155–157] The fact that they are expressed in cells, rather than imported into them, can also be an advantage in some situations. Disadvantages of using fluorescent proteins as indicators are that it takes appreciable amounts of work to engineer cells to express these proteins and the range of fluorescence wavelengths available is limited.

10. Conclusions

Probes for pH_i measurements can be used to study pH-dependent biological and pathological processes, such as cell death, cancers, and cell proliferation. BCECF **4** and carboxy-SNARF-1 **37** are the two most widely used pH_i indicators since they have desirable photophysical properties for the determination of near-neutral intracellular H^+ concentrations. Fluorescein and fluorescein derivatives, e.g., carboxyfluorescein, are common pH_i indicators; however, they rapidly leak from the cytosol through cell membranes, and this can lead to erroneous pH measurements. HPTS **58**, another widely used intracellular pH probe, tends to be retained inside living cells because it has three sulfonate groups, and it can be applied for measurements of acidic and near-neutral pH values. However, HPTS **58** is not cell permeable and must be injected into cells if it is to be observed there. Other organic fluors that have been used as stains in pH measurements have suboptimal properties in terms of photostabilities or quantum yields. Table 1 shows the useful photophysical properties of most of the pH_i indicators mentioned above. Table 2 lists the photophysical properties of acidic pH indicators. Figure 10 gives a "pH spectrum" for the most widely used cellular pH-sensitive stains.

Table 1. Photophysical Properties of Near-Neutral pH Indicators

indicator	$\lambda_{max,abs}$ (nm)	$\lambda_{max,em}$ (nm)	pK_a	dual excitation or emission[a]	ϕ	refs
BCECF, **4**	503	525	7.0	excitation	0.84 (0.1 M NaOH)	43, 49, 55
BCPCF, **11**	505	527	7.0	excitation	0.83 (0.1 M NaOH)	55, 57
C.fluorescein	492	516	6.5	excitation	0.93 (0.1 M NaOH)	58, 158
C.SNARF-1, **37**[b]	544	575	7.5	both	0.03 (pH 5−6)	5, 42, 94, 159
C.SNARF-1, **37**[c]	583	631			0.09 (pH 10−12)	
C.SNARF-4F, **38**[b]	520	592	6.4	both	na	88, 96
C.SNARF-4F, **38**[c]	582	661				
C.SNARF-5F, **39**[b]	560	580	7.2	both	na	88, 97
C.SNARF-5F, **39**[c]	575	628				
C.SNAFL-1, **40**[b]	510	539	7.8	both	0.32 (pH 5−6)	42
C.SNAFL-1, **40**[c]	542	623			0.08 (pH 10−12)	
SNAFL-calcein, **41**[b]	492	540	7.0	both	na	98
SNAFL-calcein, **41**[c]	535	625				
45 and **46**	645	665	7.5	na	na	102, 103
47	653	660	7.1	na	na	102
48	640	670	7.1	na	na	102
50	648	750	7.1	na	0.13 (*N*-protonated form)	100
55[b]	341	391	7.0	both	0.21 (pH 4 buffer)	108
55[c]	382	~580			0.56 (pH 10 buffer)	
1,4-DHPN, **57**[b]	342	402	8.0	both	na	110, 111
1,4-DHPN, **57**[c]	453	483				
HPTS, **58**[b]	405	514	~7.3	excitation	1.0 (pH 5.5)	114, 115, 160
HPTS, **58**[c]	465	514			1.0 (pH 9.0)	
69[c]	489/576	607	6.5	emission	0.18 (pH 4.1 buffer)	38
69[c]	499/576	525			0.15 (pH 8.8 buffer)	

[a] Favored method. [b] Acidic or phenolic form. [c] Basic or phenolate form. [d] Monomer. [e] Dimer or oligomer. [f] Protonated form. [g] Deprotonated form.

Table 2. Photophysical Properties of Acidic pH Indicators

indicator	$\lambda_{max,abs}$ (nm)	$\lambda_{max,em}$ (nm)	pK_a	dual excitation or emission[a]	ϕ	refs
Oregon Green 488, **22**	490	514	4.8	excitation	0.97 (pH 9 buffer)	61
6-carboxyl Oregon Green 488, **23**	492	514	4.8	excitation	0.92 (pH 9 buffer)	61
Oregon Green 514, **24**	506	529	4.8	excitation	0.22[f] 0.65[g]	76
CDCF, **25**	503	525	4.7	excitation	na	77, 161
26	494	520	5.6	emission	0.89 (pH 8−9)	82, 83
C.SNARF-4F, **38**[b]	520	582	6.4	both	na	88, 96
C.SNARF-4F, **38**[c]	592	661				
51	655	665	6.1	na	na	102
52	650	665	6.4	na	na	102
53 (Ap-Cy)	558	615	5.1	na	na	105
HPTS, **58**[b]	405	514	7.3	excitation	1.0 (pH 5.5)	114, 115, 160
HPTS, **58**[c]	465	514			1.0 (pH 9.0)	
DND-160 (PDMPO), **59**[b]	385	542	4.5	both	0.31 (pH 3.0)	124
DND-160 (PDMPO), **59**[c]	329	464			0.41 (pH 7.7)	
Blue DND-167, **60**	373	425	5.1	na	0.80 (pH 3.0)	120, 123, 124
Green DND-189, **61**	443	505	5.2	na	0.48 (pH 4.4)	124
Green DND-153, **62**	442	505	7.5	na	0.34 (pH 4.0)	124
Blue DND-192, **63**	374	424	7.5	na	0.88 (pH 4.0)	124
Acridine Orange, **65**[d]	495	530	na	emission	0.46 EtOH (0.01 M HCl)	127–129
Acridine Orange, **65**[e]	465	655				
ACMA, **66**	419	484	8.6	na	0.66 (pH 7.2)	130–132, 162
Green DND-26, **67**	504	511	na	na	na	125
68	~506	~516	3.8−6.0	na	0.55−0.60 (protonated)	163
69[b]	489/576	607	6.5	emission	0.18 (pH 4.1)	38
69[c]	499/576	525			0.15 (pH 8.8)	
pHrodot	560	585	6.5	na	na	18

[a] Favored method. [b] Acidic or phenolic form. [c] Basic or phenolate form. [d] Monomer. [e] Dimer or oligomer. [f] Protonated form. [g] deprotonated form.

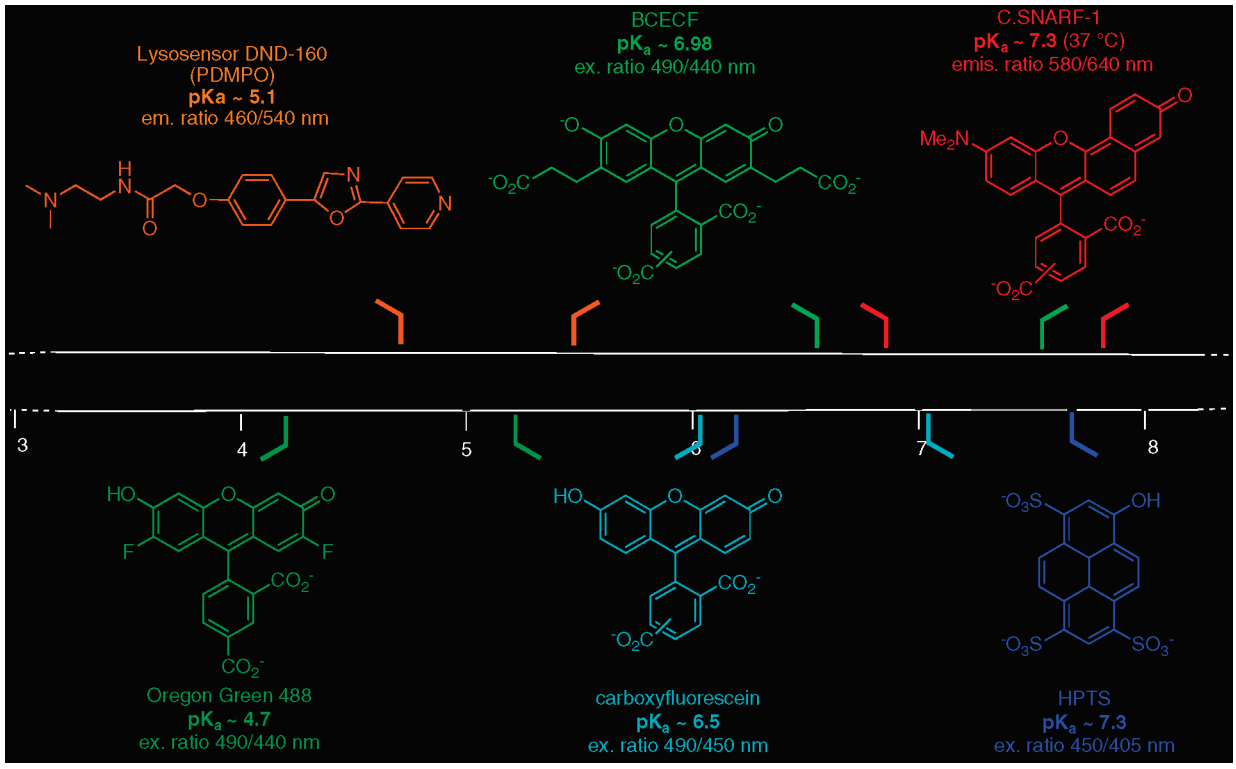


Figure 10. pH-sensitive ranges of the most widely used cellular pH-sensitive stains.

Most pH_i measurements are ratiometric. They can be dual excitation (changes at one fluorescent wavelength are observed) or dual emission. Methods based on a single-excitation wavelength (dual emission) have a significant advantage insofar as they are most easily used on different equipment (e.g., confocal microscopes, plate readers, and flow cytometers) where only one or limited excitation wavelengths are available.

Fluorescent proteins can be used to measure the pH_i of specific cell organelles (e.g., the mitochondria, ER, and Golgi) after fusing them to targeting entities. This is a big advantage when probing the pH of specific organelles, but it is a significant amount of work to construct suitably genetically encoded cells.

Other methods for pH_i determination are more futuristic. The near IR cyanine-based dye **49** might be a good potential candidate for in vivo pH measurement since it exhibits long-wavelength absorbance and emission spectra at neutral environments. However, cyanine dyes are well known to be photobleached quickly, and this might limit its future

application. Newly synthesized series of SNAFR (**29**, **31**, and **36**) are potential near IR ratiometric pH_i indicators. There are limited data about their photophysical properties, e.g., pK_a's and quantum yields, in literature. Desired photophysical properties for pH_i measurements might be significantly obtained via modification, e.g., halogenation, of the core structure of SNAFRs. Nanoparticles, e.g., CdSe quantum dots, dye-doped silica nanoparticles, and dye-labeled bacteriophage, can be more photostable and brilliant than small fluorescent organic dyes. However, they tend to be endocytosed into cells, and thus, they can be trapped in acidic vesicles or endosomes. Moreover, there are more convenient ways to *stain* cells, and the disadvantage of using these indicators as *probes* bioconjugated to proteins is that they tend to be as big or bigger than the protein itself.

Most of the molecules used for measurements of intracellular pH_i values are stains, i.e., entities that color the whole cell. The xanthene-BODIPY cassette **69** has the potential to be used as a probe, i.e., it can be attached to proteins and then imported into cells to track that protein. This is possible because **69** has a higher quantum yield than C.SNARF-1 **37** both in acidic and basic environments and because it has a functional group to allow bioconjugation. There is clearly an opportunity to devise other pH_i probes for tracking spatial and temporal protein function inside live cells and the way pH changes around them.

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