Near-infrared, dual-ratiometric fluorescent label for measurement of pH

Yevgen A. Povrozin [a], Larysa I. Markova [a], Anatoliy L. Tatarets [a], Vadim I. Sidorov [a], Ewald A. Terpetschnig [b,c,*], Leonid D. Patsenker [a,b]

[a] SSI "Institute for Single Crystals" of the National Academy of Sciences of Ukraine, 61001 Kharkov, Ukraine
[b] SETA BioMedicals, Urbana, IL 61802, USA
[c] ISS, Champaign, IL 61822, USA

ABSTRACT

We describe the spectral properties of an amine-reactive, pH-sensitive, long-wavelength ratiometric fluorescent label having a pK_a in the physiological pH range. The label exhibits its main absorption and emission in the near-infrared (NIR) region. On deprotonation, a blue shift of the excitation maximum is observed. Importantly, both the protonated and deprotonated forms of the label are fluorescent, with the deprotonated form having an extremely large Stokes shift of more than 100 nm. The spectral and photophysical properties of this pH label are compared with the properties of the protein-conjugated forms. Due to the observed pK_a shift to the acidic pH range upon conjugation to proteins, such labels are ideal for studying phagocytic events and their regulation by drugs and/or environmental factors.

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Most of the commercially available pH labels for biomedical applications are known to emit between 350 and 600 nm. The most well-known dye for intracellular pH measurements that fits this category is BCECF, a ratiometric, cell-membrane-permeable label that has its pK_a in the physiological pH range [1]. Unfortunately, there are no long-wavelength analogs available from this compound. The majority of pH-sensitive fluorescent probes employed in intracellular studies provide a reduced fluorescent signal in acidic media or, alternatively, the pK_a of the probe is outside the critical intracellular pH window between pH 5.0 and pH 8.0 [2].

A series of pH labels, the so-called CypHer dyes [3–7], were developed by Amersham and are commercially available from GE Healthcare. These dyes combine long-wavelength absorption and emission. Importantly, the deprotonated forms of these dyes are nonfluorescent. The protonated forms of the free dyes absorb between 645 and 655 nm with a high extinction coefficient (ε) of more than 200,000 $M^{-1} cm^{-1}$ and emission maxima between 660 and 670 nm [7]. Deprotonation of the dye leads to a bathochromic red shift of the absorption maximum and a loss in fluorescence, rendering the deprotonated form of the molecule nonfluorescent.

Due to the presence of ionic charges in the molecule, CypHer dyes will not passively penetrate through the cell membrane. Dyes like CypHer dyes, therefore, are almost exclusively used for labeling of the outer cell membranes or cell surface receptors, for example, G-protein-coupled receptors. Agonist activation of this class of receptor results in the internalization of the receptor from the plasma membrane (pH 7.4) to the endosomal pathway, and this is accompanied by a change in pH [7]. This pH-sensitive label has only one fluorescent form and, therefore, is useful for studies where a change in fluorescent emission can be observed easily and rapidly. The long emission wavelength of this pH-sensitive pentamethine cyanine dye is advantageous for biological studies.

We have investigated and spectrally, as well as photophysically, characterized a pH-sensitive fluorescent label, Square-650-pH, which is commercially available as a mono-NHS[1] (N-hydroxysuccinimide) ester. This cyanine-type pH label has spectral properties similar to those of the CypHer dyes but is fluorescent in both the protonated and deprotonated forms, displays an extremely large Stokes shift for the deprotonated form, and enables excitation and emission ratiometric measurement of pH.

Materials and methods

Chemicals, proteins, and media

Square-650-pH (K8-1407) is commercially available from SETA BioMedicals (Urbana, IL, USA) and was used as is. Immunoglobulin G (IgG) from bovine serum (reagent grade ⩾ 95%) and Sephadex G50 were purchased from Sigma. All other chemicals and solvents were obtained from Merck. All chemicals were used without further purification.

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* Corresponding author. Address: SETA BioMedicals, Urbana, IL 61802, USA. Fax: +1 217 344 7615.
E-mail address: ewaldte@juno.com (E.A. Terpetschnig).

[1] *Abbreviations used:* NHS, N-hydroxysuccinimide; IgG, immunoglobulin G; PB, phosphate buffer; DMF, dimethyl formamide; *D/P*, dye-to-protein ratio; RT, room temperature; QY, quantum yield; FLIM, fluorescence lifetime imaging; NIR, near-infrared; *c,* concentration.

Phosphate buffer (PB, pH 7.4, 67 mM) was prepared by mixing 808 ml of $Na_2HPO_4·2H_2O$ (11.876 g L^{-1}) and 192 ml of KH_2PO_4 (9.078 g L^{-1}). Bicarbonate buffer (pH 9.0, 50 mM) was obtained by dissolving 2.1 g of $NaHCO_3$ in 500 ml of distilled water.

General protein labeling procedures, purification of dye–IgG conjugates, and estimation of dye-to-protein ratios

A stock solution of 1 mg of the NHS-activated dye in 100 μl of anhydrous dimethyl formamide (DMF) was prepared. After 5 mg of IgG was dissolved in 1 ml of a 50-mM bicarbonate buffer (pH 9.0), 5, 10, 25, and 50 μl of dye stock solution were added to this solution. The mixture was allowed to stir for 3 h at 25 °C.

Separation of the dye–IgG conjugate from nonconjugated dye was achieved using gel permeation chromatography on a 1.5×25-cm column (stationary phase: Sephadex G-25; eluent: 67 mM PB, pH 7.4). The fraction with the shortest retention time containing the dye–IgG conjugate of bluish color was collected.

Each dye–protein conjugate was diluted with PB (pH 7.4) to obtain an absorbance ($A_{conj(\lambda max)}$) of 0.15 to 0.20 in a 5-cm quartz cell. Subsequently, the absorbances at the absorption maxima of the dye conjugate ($A_{conj(\lambda max)}$) and at 278 nm ($A_{conj(278)}$) were measured. The dye-to-protein (D/P) ratios were calculated using the following formula (with the assumption that the extinction coefficients for the free dye and the dye–IgG conjugate are the same) [8,9]:

$$D/P = \frac{A_{conj(\lambda max)}\varepsilon_{IgG}}{(A_{conj(278)} - x A_{conj(\lambda max)})\varepsilon_{dye}}, \tag{1}$$

where ε_{IgG} = 201,700 M^{-1} cm^{-1} is the extinction coefficient of IgG at 278 nm, $x = A_{dye(278)}/A_{dye(\lambda max)}$, and ε_{dye} is the extinction coefficient of the dye at the long-wavelength maximum.

Absorption spectra and extinction coefficients

The absorption spectra were measured at room temperature (RT) on a PerkinElmer Lambda 35 UV/Vis spectrophotometer, and the absorption maxima were recorded with an accuracy of 0.5 nm.

For the determination of the extinction coefficients, the dye (7–10 mg) was dissolved in 50 ml of PB (pH 7.4). The stock solution was diluted (1:2000), and the absorbance (A) was measured in a 5-cm standard quartz cell. The extinction coefficients were calculated according to Lambert–Beer's law. The reproducibility for determining the extinction coefficients is 1000 M^{-1} cm^{-1}.

Emission spectra and quantum yields

The emission spectra and quantum yields (QYs) of the dye and dye conjugates were measured in a universal buffer at RT on a Varian Cary Eclipse spectrofluorometer in standard 1-cm quartz cells. All acid forms of the dye were excited at 610 nm (λ_{exc}) and the base forms were excited at 540 nm with an excitation slit width of 2.5 nm and an emission slit width of 5 nm. The spectra are corrected. All concentrations were chosen to be between 0.5 and 1.0×10^{-6} M.

For the determination of the QYs, the integrated relative intensities of the dye or dye–protein conjugates were measured against Cy5 as the reference. The absorbancies at the excitation wavelength (610 and 540 nm) were chosen to be in the range between 0.12 and 0.18 (when measured in a 5-cm cell). The fluorescence spectra of the solutions were measured, and the QYs (φ) were determined relative to Cy5 (φ = 27%) [9] using the following formula [10]:

$$\varphi = \varphi_{Cy5} \cdot (F/F_{Cy5}) \cdot (A_{Cy5}/A), \tag{2}$$

where φ_{Cy5} is the QY of the Cy5, F, and F_{Cy5} are the integrated areas of the fluorescence spectra ($F = \int I(\lambda)d\lambda$), and A_{Cy5} and A are the absorbancies at the excitation wavelength of Cy5 and the fluorophore under examination, respectively.

The QY of each sample was measured independently three or four times, and the average value was calculated.

Fluorescence lifetime measurements

Fluorescence lifetime measurements were acquired with ChronosFD, a laser-diode-based frequency domain instrument (ISS, Champaign, IL, USA). A 635-nm laser diode was used as the excitation source. Two synthesizers (IFR2023) providing modulation frequencies in the range from 9 KHz to 1.2 GHz were used for the measurements. Data analysis was done with Vinci—Multidimensional Fluorescence Spectroscopy, a comprehensive software package for the analysis of time-resolved data (ISS). The analysis of the time-resolved fluorescence data was carried out using the traditional nonlinear least-squares method. This method evaluates how close a model selected by the user matches the data acquired with the instrument. A Marquardt–Levenberg algorithm was used as the minimization routine of the χ^2 function that compares the selected model with the experimental data. In all lifetime measurements, the dye concentrations were between 0.5 and 1.0×10^{-6} M.

pH titration experiments and pK$_a$ calculation

For the pH titration experiments, a series of universal buffer solutions with different pH values was prepared. Buffer solutions with the required pH were prepared by adding 0.2 N NaOH to equal volumes of phosphoric acetic and boric acid (each acid content was 0.04 M).

The pH value of each buffer was measured and controlled with a Hanna pH 213 Microprocessor pH meter with an accuracy of ±0.01. Then a stock solution of the dye in water was prepared. The concentrations of the stock solutions were chosen to obtain a final concentration between 0.5 and 1.0 μM. In the titration, 3 ml of universal buffer solution was placed in the cuvette and 10 μl of dye stock solution was added, followed by measurement of the absorption and emission spectra.

The calculation of the pK_a value for the free dye was performed using a nonlinear fitting method according to the following equation [11]:

$$I = \frac{I_{max} + I_{min}10^{(pH-pK_a)}}{10^{(pH-pK_a)} + 1}, \tag{3}$$

where I is a measured fluorescence intensity of the system, I_{max} is the fluorescence intensity of the fully protonated system, I_{min} is the fluorescence intensity of the deprotonated system, and the pK_a is the variable fitting parameter, which is the pH where 50% of the dye population in solution is protonated.

The pK_a value calculation for the IgG-conjugated dye was performed using a sigmoidal, nonlinear fitting method [12].

Results and discussion

Fig. 1 shows the absorption and emission spectrum of Square-650-pH under acidic and basic conditions normalized to the absorption spectra. The protonated form of the dye absorbs at 653 nm with an extinction coefficient of 135,000 M^{-1} cm^{-1}, whereas the base form absorbs at 535 with an extinction coefficient of 48,000 M^{-1} cm^{-1}. The Stokes shift of 18 nm in the emission spectrum of the protonated form of the dye is comparable to Stokes shifts found in other polymethine-type dyes (7–20 nm) [13–16].
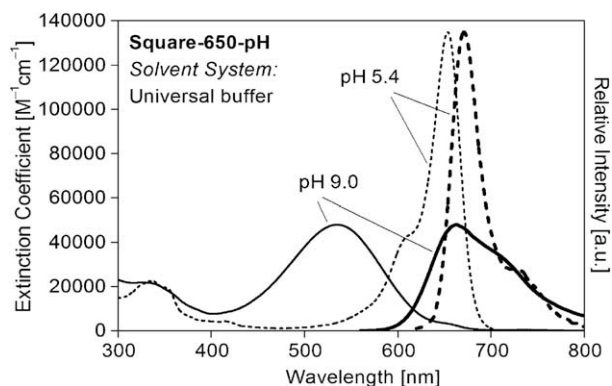
Fig. 1. Absorption and emission spectrum of Square-650-pH ($c = 0.5 \times 10^{-6}$ M) at pH 5.4 and pH 9.0.

Fig. 3. Normalized, pH-dependent fluorescence intensity of Square-650-pH ($c = 0.7 \times 10^{-6}$ M).

Nevertheless, the basic form of Square-650-pH exhibits a remarkably large Stokes shift (128 nm), but a shift that has been observed before in cyanine dyes [17]. The pH profile of Square-650-pH (Fig. 2) reveals an excitation isosbestic point at 585 nm, whereas the pK_a was identified with both excitation and absorption measurements to be in the physiological pH range ($pK_a = 7.11$) (Fig. 3). Obviously, the ground state pK_a coincides with the excited state pK_a. At pH 9.0, the emission spectrum of Square-650-pH is fairly broad, spanning from 600 to 800 nm with its maximum at 663 nm. Upon titration with acid, the intensity of the emission spectrum increases more than threefold as the pH decreases from 9.0 to 5.4 and its shape resembles more that of a typical cyanine dye. One can also observe a small shift of 8 nm between the emissions maxima of the two forms (Table 1).

As can be seen from its excitation (Fig. 2) and emission spectrum (Fig. 4), Square-650-pH displays ratiometric features in both spectral modes. The advantages of ratiometric labels for use in biological imaging applications are well known [18,19] in that they allow one to correct unwanted effects based on variations of indicator concentrations, illumination intensity, and detector sensitivity [18,20].

We also obtained time-resolved data with the pH label in acidic and basic environments. The frequency responses (phase–modulation curves) for a distinct frequency range were collected for the pH label at pH 2.0 (where the highest fluorescence intensity is observed, i.e., fully protonated form) and pH 9.0, and the fluorescent lifetimes were calculated. Importantly, the fluorescence lifetimes of the protonated and deprotonated forms of Square-650-pH are distinctly different (Table 2 and Fig. 5). Both frequency responses fit well to a monoexponential lifetime. The lifetime of the acid form
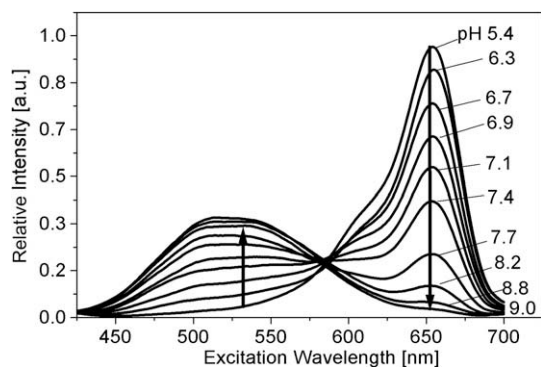
Table 1
Spectral properties of the protonated and deprotonated forms of Square-650-pH and a representative Square-650-pH–IgG conjugate

Compound	pH	Absorption maximum (nm)	Extinction coefficient ($M^{-1} cm^{-1}$)	Emission maximum (nm)	Quantum yield (%)
Square-650-pH	5.6	653	135,000	671	16
Square-650-pH	9.0	535	48,000	663	9
Square-650-pH–IgG ($D/P = 0.81$)	2.0	662	135,000	677	7
Square-650-pH–IgG ($D/P = 0.81$)	9.0	544	48,000	665 715	9

Fig. 4. Relative pH-dependent emission spectra of Square-650-pH ($\lambda_{exc} = 589$ nm, $c = 0.7 \times 10^{-6}$ M).

is 2.2 times longer than that of the base form. These two distinct lifetimes of the label suggest that Square-650-pH would also be a useful label for fluorescence-lifetime-based applications and would allow assessment of pH based on the measurement of the phase angle and/or the demodulation.

The photophysical properties of Square-650-pH were also measured after conjugation to protein (IgG). Although the excitation and emission spectra of the pH label at pH 2.0 are only slightly affected by the presence of protein, shifting them approximately

Fig. 2. pH-dependent excitation spectra of Square-650-pH ($c = 1.0 \times 10^{-6}$ M). The emission was collected at 710 nm.

Table 2
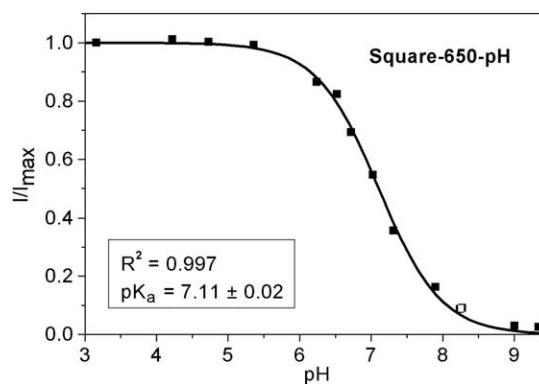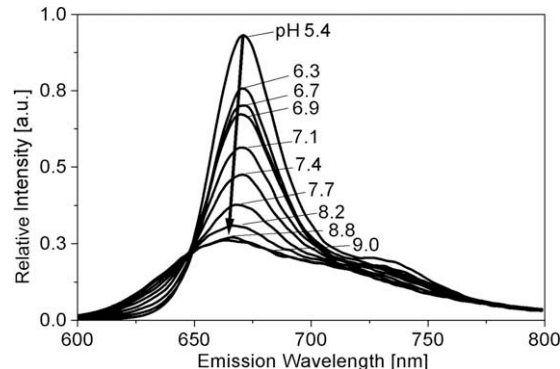Fluorescence lifetimes of Square-650-pH at pH 2.0 and pH 9.0.

Compound	pH	τ_1 (ns)	f_1	χ^2
Square-650-pH	2.0	1.17	1.00	1.58
Square-650-pH	9.0	0.53	1.00	0.93

Fig. 5. Frequency–response curves (phase angle and demodulation) of Square-650-pH at pH 2.0 and pH 9.0.

Fig. 7. Relative pH-dependent emission spectra of Square-650-pH–IgG conjugates ($D/P = 0.8$, $c = 0.7 \times 10^{-6}$ M).

10 nm to the red to 662 and 677 nm after covalent conjugation to IgG, the base form of the IgG conjugate shows a slightly smaller Stokes shift (121 nm) as compared with the free dye (128 nm). In addition, conjugation of the pH label to IgG causes a pK_a shift of 0.8 pH units from 7.11 to 6.28.

Fig. 6 shows the absorption and emission spectra for the protonated and deprotonated forms of the IgG conjugates for a D/P ratio of 0.8, whereas Fig. 7 shows the relative intensities of the relevant protein conjugate for various pH levels.

As we were trying to fit the pH titration curves of the free and protein-bound labels, we noticed that these two display very different behavior. The pH titration data for the free dye (Fig. 3) can be described well with the Bjerrum equation (Eq. 3) [12], whereas the pH titration curve for the Square-650-pH–IgG conjugate (Fig. 8) could not be fitted well with the Bjerrum equation but gave a better sigmoidal fit. For further evaluation of the differences between the pH titration data of the free dye and the IgG conjugate, we used Scatchard plots [21] with $v/[H^+]$ and v as coordinates, where v is the fraction of protonated Square-650-pH (which is equal to I/I_{max}) and $[H^+]$ is the proton concentration.

Unlike in solution where the fluorescent probe is in a homogeneous environment (Fig. 9, free dye), the environment of a covalently attached probe varies depending on the location of the probe molecules on the protein surface. Attached to a protein, the protonation (pK_a) of the dye is strongly influenced by the local probe environment that is visualized in the Scatchard plot (Fig. 9, IgG conjugate).

The lifetimes of both the protonated and deprotonated forms for Square-650-pH increase on labeling to an antibody (Table 3),
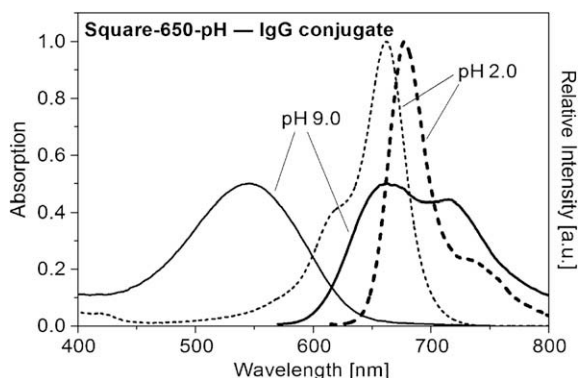

Fig. 8. Normalized, pH-dependent fluorescence intensity of Square-650-pH–IgG conjugate ($D/P = 0.8$, $c = 0.7 \times 10^{-6}$ M).

Fig. 9. Scatchard plots for the free Square-650-pH probe in solution (free dye) and for the IgG conjugate.

Table 3
Fluorescence lifetimes of square-650-pH–IgG conjugates at pH 2.0 and pH 9.0.

D/P ratio	pH	τ_1 (ns)	f_1	τ_2 (ns)	χ^2	τ_{mean} (ns)
0.8	2.0	0.79	0.25	1.78	1.18	1.52
0.8	9.0	0.61	0.77	1.89	1.27	0.89

Fig. 6. Absorption and emission spectra of Square-650-pH–IgG conjugate ($D/P = 0.8$, $c = 0.7 \times 10^{-6}$ M) at pH 2.0 and pH 9.0.

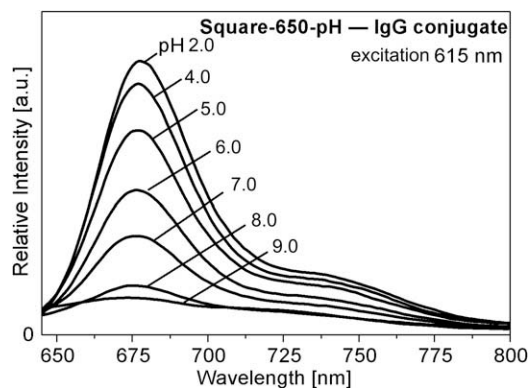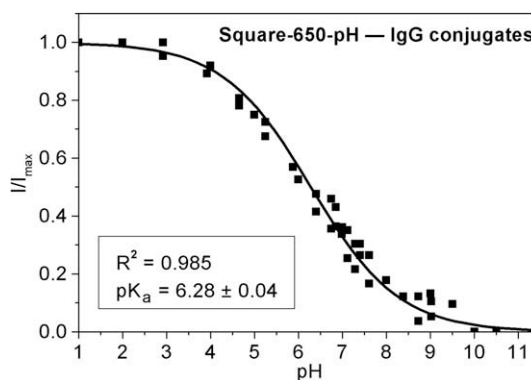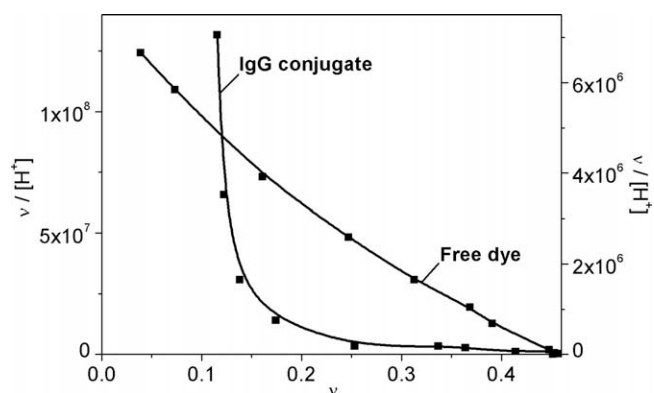and the initially monoexponential decays for the free dye become biexponential on conjugation to the carrier molecule. The conjugated label shows an 18° phase angle change between the protonated and deprotonated species at a modulation frequency of
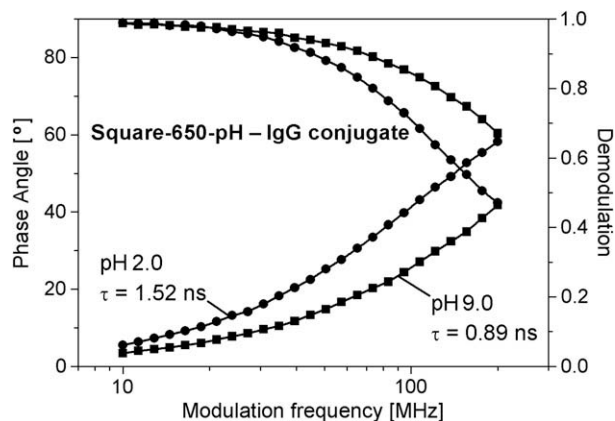
Fig. 10. Frequency–response curves (phase angle and demodulation) of the Square-650-pH–IgG conjugate ($D/P = 0.8$) at pH 2.0 and pH 9.0.

100 MHz (Fig. 10). This implies that the labeled form of Square-650-pH would also be suitable for fluorescence-lifetime-based pH sensing and fluorescence lifetime imaging (FLIM) applications, which became widely used during past decades [22–24]. Currently, the expected accuracy of phase–modulation measurements is 0.1° in phase angle and 0.005 units in modulation [25].

Conclusion

We have characterized a near-infrared (NIR) pH label that shows ratiometric behavior in both the excitation and emission modes of measurement. The free label has a pK_a of 7.11, and the antibody-conjugated label has a pK_a of 6.28. Conjugated to microorganisms, such labels are used as a tool to identify phagocytic events from nonspecific binding to cells. Because the fluorescence of Square-650-pH dramatically increases as the pH of its environment decreases from neutral to acidic, it is an ideal tool for studying such phagocytic events and their regulation by drugs and/or environmental factors. Another potential application is acidity regulation in mammalian cells. Monitoring pH changes is an important way to study signal transduction pathways and ligand interactions with G-protein-coupled receptors.

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