Published in final edited form as:

ACS Sens. 2017 July 28; 2(7): 924–931. doi:10.1021/acssensors.7b00137.

Luminescent Probes for Sensitive Detection of pH Changes in Live Cells through Two Near-Infrared Luminescence Channels

Shuwei Zhang[†], Tzu-Ho Chen^{‡, ||}, Hsien-Ming Lee^{*, ‡}, Jianheng Bi[†], Avik Ghosh[§], Mingxi Fang[†], Zichen Qian[§], Fei Xie[†], Jon Ainsley[⊥], Christo Christov[†], Fen-Tair Luo^{*, ‡}, Feng Zhao^{*, §}, and Haiying Liu^{*, †}

[†]Department of Chemistry, Michigan Technological University, Houghton, Michigan 49931, United States

[§]Department of Biomedical Engineering, Michigan Technological University, Houghton, Michigan 49931, United States

[‡]Institute of Chemistry, and Chemical Biology and Molecular Biophysics, Taiwan International Graduate Program, Academia Sinica, No. 128 Academia Road, Section 2, Taipei 11529, Taiwan, Republic of China

^{||} Department of Chemistry, National Taiwan University, No. 1 Roosevelt Road Section 4, Taipei 10617, Taiwan, Republic of China

[⊥]Department of Applied Sciences, Northumbria University, Newcastle-upon-Tyne, NE1 8ST, United Kingdom

Abstract

Two water-soluble near-infrared luminescent probes, which possess both conventional intense Stokes fluorescence and unique single-photon frequency upconversion luminescence (FUC), were developed for sensitive and selective detection of pH changes in live cells. The water solubility and biocompatibility of these probes were achieved by introducing mannose residues through 2,2'-(ethylenedioxy)diethylamine tethered spacers to a near-infrared conventional fluorescence (CF) and FUC organic fluorophore. At a pH higher than 7.4, the probes have ring-closed spirocyclic lactam structures, thus are colorless and nonfluorescent. Nevertheless, they sensitively respond to acidic pH values, with a drastic structural change to ring-opened spirocyclic lactam forms, which cause significant absorbance increases at 714 nm. Correspondingly, their near-infrared CF and FUC intensities at 740 nm are also significantly enhanced when excited by 690 and 808 nm, respectively. The probes hold a variety of advantages such as high sensitivity, excellent reversibility and selectivity to pH over metal ions, low cellular autofluorescence background interference, good cell membrane permeability and photostability, as well as low

*Corresponding Authors: Dr. Hsien-Ming Lee, leehm1@gate.sinica.edu.tw., Dr. Fen-Tair Luo, luoft@gate.sinica.edu.tw., Dr. Feng Zhao, fengzhao@mtu.edu., Dr. Haiying Liu, hylu@mtu.edu.
Orcid: Feng Zhao: 0000-0003-4138-9027
Haiying Liu: 0000-0001-8351-2017

Associated Content: Supporting Information: The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acssensors.7b00137.

Notes: The authors declare no competing financial interest.

cytotoxicity. Our results have successfully proven that these probes can visualize intracellular lysosomal pH changes in live cells by monitoring both near-infrared CF and FUCL changes.

Graphical abstract

Keywords

luminescent probes; pH; near-infrared conventional fluorescence (CF); single-photon frequency upconversion luminescence (FUCL); lysosome; live cell imaging

Intracellular pH plays important roles in a variety of cellular events such as cellular metabolism, proliferation, phagocytosis, ion transport, homeostasis, endocytosis, signal transduction, chemotaxis, apoptosis, and enzymatic activity.^{1–4} Hence, it is crucial to maintain appropriate pH homeostasis in diverse subcellular compartments for all living organisms,⁵ while preserving different pH distributions in cells.⁶ Abnormal pH values can result in cellular dysfunction, inflammation, ischemic stroke, rheumatoid arthritis, cystic fibrosis, epileptic seizure, and cardiopulmonary and neurodegenerative diseases.^{7–9} The pH value of extracellular fluid is slightly basic (7.4) under normal mammalian physiological conditions, while it is significantly low (6.2–6.9) in tumor formation circumstances.⁹ Therefore, effective visualization of pH changes inside live cells can help explore cellular functions, and also provide an insightful understanding of cellular internalization pathways. Fluorescence microscopy using pH-sensitive fluorescent probes has obvious advantages compared to nuclear magnetic resonance, microelectrode,¹⁰ and absorbance spectroscopy methods because it enables the intact detection of intracellular and subcellular pH with operational simplicity, high sensitivity, real-time, and excellent three-dimensional and sequential resolution.^{11–28} Although many fluorescent probes have been developed for detection of pH over the years, only some of them are applied to monitor lysosomal pH in live cells.^{29–31} However, some of these probes potentially damage cells and experience cellular autofluorescence issues when their absorption and emission wavelengths are shorter than 600 nm.^{12,13,20,24,32,33} In order to address these issues, a few near-infrared fluorescent probes have been reported for detection of pH.^{26,27,34} However, all fluorescent probes, which are based on small organic fluorophores for pH detection in live cells, use conventional fluorescence (CF) instead of single-photon frequency upconversion luminescence (FUCL). Upconversion luminescent materials such as two-photon absorption organic dyes and lanthanide-doped upconversion nanoparticles are excited by longer wavelength light and emit in shorter regions.^{35,36} They have been commonly applied in cell

and tissue imaging because of their superior properties such as large anti-Stokes shift and low autofluorescence interference from biological samples. However, lanthanide-doped upconversion nanoparticles suffer from some toxicity in both in vivo and in vitro applications,³⁷ while two-photon absorption organic dyes often emit visible fluorescence and require expensive optical setup.³⁶ This category of upconversion luminescent organic dye with unique near-infrared FUC property has not attracted much attention.^{38,39} Because near-infrared FUC fluorophores require a low excitation power, the upconversion luminescence imaging technique excited by the near-infrared wavelength is anticipated to possess several advantages such as photodamage-free imaging of living organisms, less photobleaching of the dyes, rare cellular autofluorescence, high detection sensitivity, and optimized penetration depth in biological tissues because of the near-infrared luminescence (Scheme 1).

In this paper, we reported two water-soluble luminescent probes (A and B), which both possess near-infrared CF and FUC properties for the sensitive and selective detection of pH changes in live cells (Scheme 2). Our strategies for significantly increasing the probe solubility and biocompatibility are to introduce mannose residues to a two-channel near-infrared fluorophore with a spirocyclic structure through a 2,2'-(ethylenedioxy)diethylamine tethered spacer. In order to selectively accumulate the probes through the protonation of weak bases of amine groups in acidic lysosomes, the secondary and tertiary amine groups were introduced to 2,2'-(ethylenedioxy)diethylamine spacers bearing one and two mannose residues for probes A and B, respectively. These fluorescent probes are colorless and nonfluorescent under both neutral and basic conditions due to their retention of closed spirocyclic lactam forms. However, in acidic condition, they are highly fluorescent accompanying both significant near-infrared CF and FUC increases at 740 nm with excitation wavelengths at 690 and 808 nm (Scheme 1), respectively, due to significantly enhanced π -conjugation of the fluorophores with the opened spirocyclic lactam ring structures in acidic environment (Scheme 2). As a result, the fluorescent probes show extremely weak near-infrared CF and FUC intensities at the extracellular pH (7.4) and become highly bright with both significant near-infrared CF and FUC enhancements at 740 nm at the lysosomal pH (4.5). Fluorescent probes were applied to visualize lysosomal pH changes in live cells by detecting both near-infrared CF and FUC intensity changes. The results have demonstrated that the probes respond sensitively and selectively to pH changes with significant enhancement in both near-infrared CF and FUC intensities from basic to acidic pH, and display excellent photostability, low autofluorescence background interference from biological samples, as well as low cytotoxicity and good cell membrane permeability.

Experimental Section

Instrumentation

¹H NMR and ¹³C NMR spectra were collected by 400 MHz Varian Unity Inova NMR spectrophotometer in CDCl₃ and CD₃OD solutions. Chemical shifts (δ) were specified in ppm relative to solvent residual peaks as internal standards. High-resolution mass spectrometry data (HRMS) of the intermediates and the probes were measured by fast atom

bombardment (FAB) ionization mass spectrometer. Absorption and fluorescence spectra were measured by using a Perkin Elmer Lambda 35 UV/vis spectrometer and a Jobin Yvon Fluoromax-4 spectrofluorometer, respectively.

Cell culture and CF and Fucl imaging

Human dermal fibroblasts, HeLa, and KB cells were purchased from ATCC (Manassas, VA). The cells grown for a minimum of five passages were used in all experiments. The cells were seeded at a density of 10 000 cells/cm² on glass cover slides that were placed in 12-well culture plates and maintained in Dulbecco's modified eagle medium (DMEM, Thermo-Fisher, Waltham, MA) with 20% fetal bovine serum (FBS, Atlanta Biologicals, Flowery Branch, GA). After 16 h of incubation, the cell culture medium was replaced by freshly prepared serum-free medium with 2, 5, 10, 15, and 20 μ M of probe A or B. The cells were incubated further with 50 nM LysoTracker Green (Thermo-Fisher) for 30 min, and 1 mg/mL Hoechst for 5 min in order to confirm the specific target of our probes to lysosomes in cells. Live cell images were taken by a confocal fluorescence microscope. LysoTracker Green fluorescence, CF and FUCL images were taken in the same view field by appropriate excitation and emission to simultaneously visualize the extent of localization of our probes and LysoTracker Green in the same intracellular compartment. The exposure time for each filter was kept constant. The colocalization analysis based on Pearson's coefficient was obtained by the JACoP plugin from ImageJ.⁴⁰

Live Cell CF and FUCL Imaging at Different Intracellular pH Values

The HeLa and KB cell cultures were performed by using the same protocol described above. 10 μ M probe A or B at 37 °C was used to incubate with the cells for 30 min, further with 50 nM LysoTracker green for 30 min and 1 μ g/mL Hoeschst for 5 min, respectively. The cells were rinsed with neutral PBS buffer twice before they were treated with nigericin (5 μ g/mL) in a PBS buffer with pH values ranging from 4.5, 5.5, 6.5, to 7.5 for 30 min to equilibrate the intracellular and extracellular pH, which has been widely employed to calibrate the intracellular pH.^{41–45} CF and FUCL Images were taken by a confocal fluorescence microscope.

Materials

Unless specifically indicated, all reagents and solvents were purchased from commercial suppliers and used without further purification. Compounds 3 and 5³⁴ and 1-*O*-bromoethyl-2,3,4,6-tetra-*O*-acetyl- α -D-mannose (8)^{46,47} were synthesized according to reported procedures.

Results And Discussion

Synthetic Approach to Probes A and B

We used fluorescent dye (5) to prepare conventional and up-conversion near-infrared luminescent probes for sensitive and selective detection of lysosomal pH changes in live cells. This is because the fluorophore possesses a variety of advantages such as a large absorption extinction coefficient, high CF quantum yield (41% in methanol) at 740 nm, and FUCL peak at 740 nm, good photostability, and chemical stability (Scheme 3).^{34,39}

However, the fluorophore displays very poor water solubility and possesses severe cytotoxicity.³⁴ In order to overcome these drawbacks, we used mannose to enhance water solubility and biocompatibility of the fluorophore. We prepared two fluorescent probes bearing one and two mannose residues to study the effect of mannose residues on the probe sensitivity to pH changes. In order to introduce mannose residues to the near-infrared fluorophore (5), we chose 2,2'-(ethylenedioxy)-diethylamine (6) as a tethered spacer because we could easily attach one terminal of 2,2-(ethylenedioxy)diethylamine to the fluorophore through a condensation reaction of the carboxylic acid of the fluorophore with an amine group of compound 6 via an amide bond to form nonfluorescent compound 7 bearing a closed spirocyclic lactam ring at pH 7.4 (Scheme 3). Mannose residues were introduced to another amine terminal of compound 7 via a nucleophilic substitution reaction of an amine group of compound 7 with bromo group from 1-*O*-bromoethyl-2,3,4,6-tetra-*O*-acetyl- α -D-mannose (8) in the presence of potassium carbonate in *N,N*-dimethylformamide solution to yield compounds **9** and **10** bearing one and two protected mannose residues with the secondary and tertiary amines, respectively. Fluorescent probes **A** and **B** were obtained by the subsequent mild deacetylation of intermediates **9** and **10** in methanol containing potassium carbonate, respectively. The secondary and tertiary amines of fluorescent probes **A** and **B** may help selective accumulation of the probes in cellular lysosomal regions via lysosomotropism through protonation of these amine residues in an acidic cellular environment.¹⁸

Optical Responses of Probes A and B to pH Values

We first examined pH influence on absorption spectra of the probes. Probe A showed a very strong absorption peak centered at 378 nm, a weak absorption at 479 nm, and an extremely weak absorption peak at 713 nm in 40 mM citrate-phosphate buffer solution when the buffer pH is 7.4 (Figure 1a). However, absorbance of probe A at 713 nm was significantly enhanced, accompanying with a shoulder peak at 654 nm, and its absorbance at 378 nm considerably decreased after gradual decreases of pH from 8.0 to 4.4 (Figure 1a and 1b). These results indicate that the gradual pH decreases trigger the opening of the fluorophore spirocyclic lactam ring and significantly enhance π -conjugation of the fluorophore. As a result, the solution color of the fluorescent probe A changes from colorless to green when the solution pH decreases from 8.0 to 4.4. However, further decrease of pH below 4.4 results in gradual decrease in the absorbance at 713 nm (Figure 1b). The decrease of the absorbance at 713 nm at pH below 4.4 may arise from the lack of charge balance through its resonance structures of the fluorophore because the tertiary amine group of the fluorophore may be protonated at lower pH (Scheme 2). Probe **A** reversibly responds in the absorbance to pH ranging from 2.2 to 8.0 when it is treated with acid or base. Probe **B** shows similar absorbance responses to pH changes (Figure 1c,d, and Scheme 2).

The potential application of the probes in pH sensing was investigated by evaluating the pH effect on CF intensity of the probes. Figure 2a displays the CF spectra of the probe A at different pH values. When the buffer pH is higher than 7.4, probe A becomes nonfluorescent due to its retention of the closed form of the spirocyclic lactam ring. However, gradually changing pH from 7.4 to 4.4 causes the appearance of a new fluorescence peak at 740 nm, and significantly increases this peak fluorescence intensity when the probe was excited at

690 nm. The pH change from 7.4 to 4.4 triggers a more than 214-fold increase in the fluorescence intensity at 740 nm, which indicates that probe A is very sensitive to acidic pH changes because the acidic conditions result in spirocyclic lactam ring opening of the fluorophore (Figure 2a).

Moreover, probe A undergoes slight red shifts in the fluorescence peak when pH is decreased from 7.4 to 4.4 because of the enhanced π -conjugation of the probe. The Henderson–Hasselbach-type mass action equation was used to calculate the pK_{cycl} value of probe A related to the spirocyclic lactam ring opening of the fluorophore, and obtained a value of 5.8 (Scheme 2). Probe A displays excellent reversible fluorescence responses to pH from 4.0 to 7.4 with molar absorption coefficient of $3.0 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$ and fluorescence quantum yield of 8.4% at pH 4.4. Probe B exhibits slightly lower sensitivity to pH with 130-fold increases in the fluorescence intensity at 740 nm when the buffer pH was adjusted from 7.4 to 4.4 (Figure 2c,d). Probe B shows molar absorption coefficient of $3.0 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$ and fluorescence quantum yield of 8.1% at pH 4.4. The pK_{cycl} value of probe B related to the spirocyclic lactam ring opening was calculated to be 6.1. However, further enhancing acidic conditions from pH 4.4 to 2.0 causes significant fluorescence quenching of probe A (Figure 1b) since the potential protonation of amine group of the fluorophore could significantly reduce the electron donating ability of the group, and causes the lack of charge balance through the resonance structures of the fluorophore (Scheme 2). We used the Henderson–Hasselbach-type mass action equation to analyze the changes of fluorescence intensity of probe A on pH and obtained pK_a value of 2.8, which may be related to protonation of the nitrogen atom of the fluorophore (Scheme 2). The similar pH effect on fluorescence quenching of probe B was also observed with further enhanced acidic conditions from 4.4 to 2.0 (Figure 2d). The changes of fluorescence intensity of probe B yielded pK_a value of 3.3, which may be associated with the further protonation of the nitrogen atom of the fluorophore (Scheme 2).

We also investigated the pH effect on FUEL intensity of 5 μM probes A and B. Almost the same pH effect was observed on FUEL intensity of probes A and B (Figure 3) as that on the near-infrared CF of the probes (Figure 2). Probe A displayed almost no FUEL at pH higher than 7.4 because the probe retained its closed spirocyclic lactam ring in neutral and basic conditions. However, strong near-infrared FUEL peak at 740 nm was observed with an excitation wavelength at 808 nm when the buffer pH was changed from pH 7.4 to pH 4.4 (Figure 3a,b) because the acidic environment leads to spirocyclic lactam ring opening of the fluorophore. Probes **A** and **B** display FUEL quantum yields of 2.9% and 2.5% at pH 4.4, respectively (Table S1 in Supporting Information). Further decrease of pH from 4.4 to 2.6 caused a decrease of FUEL intensity at 740 nm (Figure 3b) because the strong acid condition may protonate the nitrogen atom of the fluorophore, significantly reduce the electron donating ability of the nitrogen atom, and lead to the lack of charge balance through the resonance structures of the fluorophores (Scheme 2). The same pH effect on FUEL intensity of probe B was also observed as that of probe A (Figure 3c,d). These results demonstrate that we can use both near-infrared CF and FUEL properties of the probes **A** and **B** to detect pH changes.

Selectivity of the Probes

We examined metal ion effect on selective fluorescent response of the probes to pH over metal ions by adding main group and transition metal ions (Figure 4). Both probes display slight responses to 200 μM alkali and alkaline earth metal ions as such Na^+ , K^+ , Ca^{2+} , and Mg^{2+} , as well as some transitional metal ions (200 μM) such as Cu^{2+} , Zn^{2+} , Fe^{2+} , Co^{2+} , Ag^+ , Ni^{2+} , and Mn^{2+} at pH 7.4 and 4.4 (Figure 4), which indicates that the probes exhibit good selectivity to pH over these alkali, alkaline earth ions, and transitional metal ions.

Photostability of the Probes

We investigated the photostability of the probes by exciting them continuously for 5 min intervals and measuring CF intensity every 10 min. Probe A showed good photostability with its fluorescence decrease by 5.5% under 1 h excitation and by 12.4% under 3 h excitation at 690 nm (Figure 5). Fluorescent probe B displayed similarly excellent photostability as probe A; its CF intensity decreased by 5.6% under 1 h excitation and by 11.3% under 3 h excitation (Figure 5). Use of lower excitation wavelength at 808 nm can avoid photobleaching of the probes and generate stable FUCL signals because FUCL intensities of the probes A and B remained almost unchanged during the same 3 h excitation at a lower energy wavelength of 808 nm (Figure 6).

Cytotoxicity of the Probes

The XTT assay was used to study the cytotoxicity of both probes to live cells during the staining process (Figure 7). Incubation of human dermal fibroblasts with the probes showed that the cell viability was higher than 70% even at a concentration of 50 μM . These results suggested that both probes A and B had low cytotoxicity, and could be used as live cell staining reagents.

Near-Infrared CF and FUCL Responses of the Probes to pH in Live Cells

In order to investigate the effect of the probe concentration on imaging, and also evaluate whether probes **A** and **B** could respond to intracellular pH and selectively target the inherently acidic compartments in live cells, we incubated HeLa and KB cells with 5, 10, 15, and 20 μM probe **A** or **B**, and compared CF and FUCL in both cell types (Figure 8 and Figures S24, S32). The probe A shows good CF and FUCL intensities in live cells, and the intensities increase with increasing probe concentrations (Figure 9 and Figures S25–S27 and S31–S36 in Supporting Information). Probe B displayed slightly lower CF and FUCL intensities in HeLa cells than probe A (Figures 9, 10, S24 and S28 in Supporting Information), which may be due to the lower intake of probe B in live cells than that of probe A. The merge CF and FUCL images of probe B showed that some cells have an extra high green ring (Figure 8), indicating that the cellular bright spots have pH around 5.0. The positively stained areas in the cells marked with probe **A** or **B** were well matched with those stained with LysoTracker Green. The colocalization analysis based on the Pearson's coefficient, which gave a value of 0.85 or higher for probes A and B with the commercial LysoTracker Green, verified that our probes and the commercial one were located in the same cellular compartment (Figures 9, 10 and Figures S25–S27, S29–S31, S33–36, and S38–S41 in Supporting Information). These results indicated that fluorescent probes A and

B were capable of targeting lysosomes in live cells as LysoTracker Green did. Both CF and FUCL intensities in live cells could be significantly enhanced by increasing probe concentrations (Figure 8, and Figures S24, S28, S32, and S37 in Supporting Information).

In order to investigate the sensitivity of our probes to pH changes in live cells, the fluorescence imaging in live cells with different intracellular pH values was conducted by incubating HeLa and KB cells with 10 μ M probe A or B. The cells were subsequently incubated with nigericin (5 μ g/mL) in buffer solution with different pH values ranging from 4.5, 5.5, 6.5, to 7.5 in order to equilibrate the intracellular and extracellular pH, respectively.^{41–45} Both fluorescent probes A and B showed very weak CF and FUCL in both HeLa and KB live cells around physiological pH (7.5) (Figures 11, 12, and Figures S52, S57, and S62 in Supporting Information). Decrease of pH from 7.5 to 4.5 resulted in gradual increases of CF and FUCL intensities (Figures 11 and 12). These turn-on responses of probes A and B in both CF and FUCL intensities to intracellular pH changes in live cells (Figures 11 and 12) were consistent with the trend of their corresponding responses to pH changes in buffer solution (Figures 2 and 3). In addition, probes A and B showed CF/FUCL ratiometric responses to pH changes from 4.5 to 7.5 because the merge images of CF and FUCL images in the third columns in Figures 11 and 12 underwent significant color changes from yellow to green with pH changes from 4.5 to 7.5. In contrast with probes A and B, the commercial probe displayed less sensitivity to intracellular pH changes, proving the superior pH detection capability of our synthesized probes (Figures 11, 12, and Figures S52, S57, and 63). Merge CF and FUCL image of 10 μ M probe B in a pH 7.5 buffer without nigericin in Figure 10 is less orange and more green than that of the probe in a pH 4.5 buffer containing 5 μ g/mL nigericin in Figure 13, indicating that decrease of intracellular pH led to higher CF and FUCL intensities.

Conclusion

In this work, we designed and synthesized two water-soluble near-infrared fluorescent probes bearing mannose residues through a 2,2'-(ethylenedioxy)diethylamine tethered spacers for sensitive and selective detection of pH changes in live cells. The fluorescent probes respond to pH changes based on the structural changes between the closed and opened forms of the spirocyclic lactam rings of the fluorophores. These probes are not fluorescent with a closed spirocyclic lactam ring structure at neutral or basic pH, but become highly fluorescent in both near-infrared CF and FUCL at 740 nm with opened spirocyclic lactam ring structures at acidic pH environment. Probe A displays slightly higher fluorescence quantum yield in pH 4.4 than probe B. The fluorescent probes possess good cell permeability, low cytotoxicity, and excellent photostability. Under near-infrared excitation at 690 and 808 nm, they provide effective monitoring of lysosomal pH changes through sensitive detection of near-infrared CF and FUCL changes in live cells, respectively.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The research reported in this publication was supported by the National Institute of General Medical Sciences of the National Institutes of Health under Award Number R15GM114751 (to H. Y. Liu).

References

1. Casey JR, Grinstein S, Orlowski J. Sensors and regulators of intracellular pH. *Nat Rev Mol Cell Biol.* 2010; 11(1):50–61. [PubMed: 19997129]
2. Kennedy RT, Huang L, Aspinwall CA. Extracellular pH is required for rapid release of insulin from Zn-insulin precipitates in beta-cell secretory vesicles during exocytosis. *J Am Chem Soc.* 1996; 118(7):1795–1796.
3. Hoyt KR, Reynolds IJ. Alkalinization prolongs recovery from glutamate-induced increases in intracellular Ca^{2+} concentration by enhancing Ca^{2+} efflux through the mitochondrial $\text{Na}^{+}/\text{Ca}^{2+}$ exchanger in cultured rat forebrain neurons. *J Neurochem.* 1998; 71(3):1051–1058. [PubMed: 9721729]
4. Anderson RGW, Orci L. A View of Acidic Intracellular Compartments. *J Cell Biol.* 1988; 106(3): 539–543. [PubMed: 3279044]
5. Matin A, Konings WN, Epstein W, Stock JB, Padan E, Schafer G, Booth IR, Rowbury RJ, Bennett GN, Poole RK, Park SF. PH homeostasis in acidophiles. *Novartis Found Symp.* 1999; 221:152–166. [PubMed: 10207918]
6. Roos A, Boron WF. Intracellular pH. *Physiol Rev.* 1981; 61(2):296–434. [PubMed: 7012859]
7. Izumi H, Torigoe T, Ishiguchi H, Uramoto H, Yoshida Y, Tanabe M, Ise T, Murakami T, Yoshida T, Nomoto M, Kohno K. Cellular pH regulators: potentially promising molecular targets for cancer chemotherapy. *Cancer Treat Rev.* 2003; 29(6):541–549. [PubMed: 14585264]
8. Davies TA, Fine RE, Johnson RJ, Levesque CA, Rathbun WH, Seetoo KF, Smith SJ, Strohmeier G, Volicer L, Delva L, Simons ER. Non-Age Related Differences in Thrombin Responses by Platelets from Male-Patients with Advanced Alzheimers-Disease. *Biochem Biophys Res Commun.* 1993; 194(1):537–543. [PubMed: 8333868]
9. Webb BA, Chimenti M, Jacobson MP, Barber DL. Dysregulated pH: a perfect storm for cancer progression. *Nat Rev Cancer.* 2011; 11(9):671–677. [PubMed: 21833026]
10. He S, Mason RP, Hunjan S, Mehta VD, Arora V, Katipally R, Kulkarni PV, Antich PP. Development of novel F-19 NMR pH indicators: Synthesis and evaluation of a series of fluorinated vitamin B-6 Analogues. *Bioorg Med Chem.* 1998; 6(9):1631–1639. [PubMed: 9801834]
11. Diwu ZJ, Chen CS, Zhang CL, Klaubert DH, Haugland RP. A novel acidotropic pH indicator and its potential application in labeling acidic organelles of live cells. *Chem Biol.* 1999; 6(7):411–418. [PubMed: 10381401]
12. Lv HS, Liu J, Zhao J, Zhao BX, Miao JY. Highly selective and sensitive pH-responsive fluorescent probe in living Hela and HUVEC cells. *Sens Actuators, B.* 2013; 177:956–963.
13. Zhu H, Fan JL, Xu QL, Li HL, Wang JY, Gao P, Peng XJ. Imaging of lysosomal pH changes with a fluorescent sensor containing a novel lysosome-locating group. *Chem Commun.* 2012; 48(96): 11766–11768.
14. Li Z, Song YL, Yang YH, Yang L, Huang XH, Han JH, Han SF. Rhodamine-deoxylactam functionalized poly styrene-alter-(maleic acid) s as lysosome activatable probes for intraoperative detection of tumors. *Chem Sci.* 2012; 3(10):2941–2948.
15. Ying LQ, Branchaud BP. Selective labeling and monitoring pH changes of lysosomes in living cells with fluorogenic pH sensors. *Bioorg Med Chem Lett.* 2011; 21(12):3546–3549. [PubMed: 21576021]
16. Smith DG, McMahon BK, Pal R, Parker D. Live cell imaging of lysosomal pH changes with pH responsive ratiometric lanthanide probes. *Chem Commun.* 2012; 48(68):8520–8522.
17. Ma LJ, Cao WG, Liu JL, Deng DY, Wu YQ, Yan YH, Yang LT. A highly selective and sensitive fluorescence dualresponsive pH probe in water. *Sens Actuators, B.* 2012; 169:243–247.

18. Galindo F, Burguete MI, Vigara L, Luis SV, Kabir N, Gavrilovic J, Russell DA. Synthetic macrocyclic peptidomimetics as tunable pH probes for the fluorescence imaging of acidic organelles in live cells. *Angew Chem, Int Ed.* 2005; 44(40):6504–6508.
19. DePedro HM, Urayama P. Using LysoSensor Yellow/Blue DND-160 to sense acidic pH under high hydrostatic pressures. *Anal Biochem.* 2009; 384(2):359–361. [PubMed: 18976626]
20. Hasegawa T, Kondo Y, Koizumi Y, Sugiyama T, Takeda A, Ito S, Hamada F. A highly sensitive probe detecting low pH area of HeLa cells based on rhodamine B modified beta-cyclodextrins. *Bioorg Med Chem.* 2009; 17(16):6015–6019. [PubMed: 19616959]
21. Lin HJ, Herman P, Kang JS, Lakowicz JR. Fluorescence lifetime characterization of novel low-pH probes. *Anal Biochem.* 2001; 294(2):118–125. [PubMed: 11444806]
22. Wang L, Xiao Y, Tian WM, Deng LZ. Activatable Rotor for Quantifying Lysosomal Viscosity in Living Cells. *J Am Chem Soc.* 2013; 135(8):2903–2906. [PubMed: 23409947]
23. Dong BL, Song XZ, Wang C, Kong XQ, Tang YH, Lin WY. Dual Site-Controlled and Lysosome-Targeted Intramolecular Charge Transfer-Photoinduced Electron Transfer-Fluorescence Resonance Energy Transfer Fluorescent Probe for Monitoring pH Changes in Living Cells. *Anal Chem.* 2016; 88(7):4085–4091. [PubMed: 26987045]
24. Wang QQ, Zhou LY, Qiu LP, Lu DQ, Wu YX, Zhang XB. An efficient ratiometric fluorescent probe for tracking dynamic changes in lysosomal pH. *Analyst.* 2015; 140(16):5563–5569. [PubMed: 26107774]
25. Wan QQ, Chen SM, Shi W, Li LH, Ma HM. Lysosomal pH Rise during Heat Shock Monitored by a Lysosome-Targeting Near-Infrared Ratiometric Fluorescent Probe. *Angew Chem, Int Ed.* 2014; 53(41):10916–10920.
26. Zhang JT, Yang M, Mazi W, Adhikari K, Fang MX, Xie F, Valenzano L, Tiwari A, Luo FT, Liu HY. Unusual Fluorescent Responses of Morpholine-Functionalized Fluorescent Probes to pH via Manipulation of BODIPY's HOMO and LUMO Energy Orbitals for Intracellular pH Detection. *ACS Sens.* 2016; 1(2):158–165. [PubMed: 27547822]
27. Zhang JT, Yang M, Li C, Dorh N, Xie F, Luo FT, Tiwari A, Liu HY. Near-infrared fluorescent probes based on piperazine-functionalized BODIPY dyes for sensitive detection of lysosomal pH. *J Mater Chem B.* 2015; 3(10):2173–2184.
28. Schaferling M. Nanoparticle-based luminescent probes for intracellular sensing and imaging of pH. *Wiley Interdiscip Rev Nanomed Nanobiotechnol.* 2016; 8(3):378–413. [PubMed: 26395962]
29. Han JY, Burgess K. Fluorescent Indicators for Intracellular pH. *Chem Rev.* 2010; 110(5):2709–2728. [PubMed: 19831417]
30. Yin J, Hu Y, Yoon J. Fluorescent probes and bioimaging: alkali metals, alkaline earth metals and pH. *Chem Soc Rev.* 2015; 44(14):4619–4644. [PubMed: 25317749]
31. Ni Y, Wu JS. Far-red and near infrared BODIPY dyes: synthesis and applications for fluorescent pH probes and bio-imaging. *Org Biomol Chem.* 2014; 12(23):3774–3791. [PubMed: 24781214]
32. Hu ZQ, Li M, Liu MD, Zhuang WM, Li GK. A highly sensitive fluorescent acidic pH probe based on rhodamine B diethyl-2-aminobutenedioate conjugate and its application in living cells. *Dyes Pigm.* 2013; 96(1):71–75.
33. Shen SL, Chen XP, Zhang XF, Miao JY, Zhao BX. A rhodamine B-based lysosomal pH probe. *J Mater Chem B.* 2015; 3(5):919–925.
34. Vegesna GK, Janjanam J, Bi JH, Luo FT, Zhang JT, Olds C, Tiwaria A, Liu HY. pH-Activatable Near-Infrared Fluorescent Probes for Detection of Lysosomal pH inside Living Cells. *J Mater Chem B.* 2014; 2:4500–4508.
35. Gorris HH, Wolfbeis OS. Photon-Upconverting Nanoparticles for Optical Encoding and Multiplexing of Cells, Biomolecules, and Microspheres. *Angew Chem, Int Ed.* 2013; 52(13):3584–3600.
36. Yao S, Belfield KD. Two-Photon Fluorescent Probes for Bioimaging. *Eur J Org Chem.* 2012; 2012:3199–3217.
37. Tian J, Zeng X, Xie XJ, Han SY, Liew OW, Chen YT, Wang LH, Liu XG. Intracellular Adenosine Triphosphate Deprivation through Lanthanide-Doped Nanoparticles. *J Am Chem Soc.* 2015; 137(20):6550–6558. [PubMed: 25923914]

38. Yang HR, Han CM, Zhu XJ, Liu Y, Zhang KY, Liu SJ, Zhao Q, Li FY, Huang W. Upconversion Luminescent Chemodosimeter Based on NIR Organic Dye for Monitoring Methylmercury In Vivo. *Adv Funct Mater.* 2016; 26(12):1945–1953.
39. Liu Y, Su QQ, Zou XM, Chen M, Feng W, Shi YB, Li FY. Near-infrared in vivo bioimaging using a molecular upconversion probe. *Chem Commun.* 2016; 52(47):7466–7469.
40. Bolte S, Cordelieres FP. A guided tour into subcellular colocalization analysis in light microscopy. *J Microsc.* 2006; 224:213–232. [PubMed: 17210054]
41. Thomas JA, Buchsbaum RN, Zimniak A, Racker E. Intracellular pH Measurements in Ehrlich Ascites Tumor-Cells Utilizing Spectroscopic Probes Generated In Situ. *Biochemistry.* 1979; 18(11):2210–2218. [PubMed: 36128]
42. Llopis J, McCaffery JM, Miyawaki A, Farquhar MG, Tsien RY. Measurement of cytosolic, mitochondrial, and Golgi pH in single living cells with green fluorescent proteins. *Proc Natl Acad Sci U S A.* 1998; 95(12):6803–6808. [PubMed: 9618493]
43. Fan L, Liu QL, Lu DT, Shi HP, Yang YF, Li YF, Dong C, Shuang SM. A novel far-visible and near-infrared pH probe for monitoring near-neutral physiological pH changes: imaging in live cells. *J Mater Chem B.* 2013; 1(34):4281–4288.
44. Song PS, Chen XT, Xiang Y, Huang L, Zhou ZJ, Wei RR, Tong AJ. A ratiometric fluorescent pH probe based on aggregation-induced emission enhancement and its application in livecell imaging. *J Mater Chem.* 2011; 21(35):13470–13475.
45. Han JY, Loudet A, Barhoumi R, Burghardt RC, Burgess K. A Ratiometric pH Reporter For Imaging Protein-dye Conjugates In Living Cells. *J Am Chem Soc.* 2009; 131(5):1642–1643. [PubMed: 19146412]
46. Xue C, Velayudham S, Johnson S, Saha R, Smith A, Brewer W, Murthy P, Bagley ST, Liu H. Highly Water-Soluble, Fluorescent, Conjugated Fluorene-Based Glycopolymers with Poly-(ethylene glycol)-Tethered Spacers for Sensitive Detection of Escherichia. coli *Chem - Eur J.* 2009; 15(10):2289–2295. [PubMed: 19142934]
47. Xue CH, Donuru VRR, Liu HY. Facile, versatile prepolymerization and postpolymerization functionalization approaches for well-defined fluorescent conjugated fluorene-based glycopolymers. *Macromolecules.* 2006; 39(17):5747–5752.

Figure 1.

Absorption spectra of 5 μ M probes A (a) and B (c) in citrate phosphate buffer (containing 1% ethanol) with different pH values, and plot of absorbance of the probes A (b) and B (d) at 713 nm versus pH with thrice-repeated measurements, respectively.

Figure 2. CF spectra of 5 μ M probes A (a) and B (c) at different pH values, and plot of fluorescence intensity of probes A (b) and B (d) at 740 nm versus pH with three repeated measurements and excitation wavelength at 690 nm, respectively.

Figure 3.

FUCI spectra of 5 μM probes A (a) and B (c) at different pH values in citrate–phosphate buffer solution, and plot of FUCI intensity of probes A (b) and B (d) at 740 nm versus pH with three repeated measurements and excitation wavelength at 808 nm, respectively.

Figure 4. Fluorescence responses of 5 μM probes A (a) and B (b) to different metal ions at pH 4.4 and 7.4 buffer solutions, respectively.

Figure 5. Relative CF intensities of 5 μM probes A (a) and B (b) in pH 4.4 buffer solutions as a function of time in 3 h under excitation at 690 nm.

Figure 6. Relative FUCI intensities of 5 μ M probes A (a) and B (b) in pH 4.4 buffer solutions as a function of time in 3 h under excitation at 808 nm.

Figure 7.

Cytotoxicity and cell proliferation of probes A and B tested by XTT assay. The human dermal fibroblasts were incubated with 2, 5, 10, 15, and 50 μM of probe A or B for 12 h, and cell viability was measured by adding XTT reagent and measuring at 475 nm. The cell viability was directly proportional to the absorbance measured at 475 nm and was normalized to control cells (no probe added). The error bars indicate $\pm$ SD from 6 replicates.

Figure 8. CF and FUCL images of KB cells incubated with 50 nM LysoTracker Green and 5, 10, 15, and 20 μM probes A and B, respectively. Images were taken by confocal fluorescence microscope at 40 $\times$ magnification, scale bars = 20 μm .

Figure 9.

CF and FUCL images of HeLa cells incubated with 10 μ M probe A. The cells were incubated with probe A for 2 h, post serum starvation (2 h), and imaged for colocalization with 50 nM LysoTracker Green and 1 μ g/mL Hoechst 33342 stains. Images were obtained by confocal fluorescence microscope at 40 $\times$ magnification, scale bars = 20 μ m.

Figure 10.

CF and FUCL images of HeLa cells incubated with 10 μM probe B. The cells were incubated with probe B for 2 h, post serum starvation (2 h), and imaged for colocalization with 50 nM LysoTracker Green and 1 $\mu\text{g}/\text{mL}$ Hoechst 33342 stains. Images were obtained by confocal fluorescence microscope at 40 $\times$ magnification, scale bars = 20 μm .

Figure 11.

CF and FUCL images of HeLa cells incubated with 10 μ M probe A in buffers with different pH values of 4.5, 5.5, 6.5, or 7.5 containing 5 μ g/mL nigericin. The cells were incubated with probe A for 2 h, post serum starvation (2 h), and imaged for colocalization with 50 nM LysoTracker Green. Images were conducted by confocal fluorescence microscope at 40 $\times$ magnification, scale bars = 20 μ m.

Figure 12.

CF and FUCL images of HeLa cells incubated with 10 μM probe B in buffers at different pH values of 4.5, 5.5, 6.5, or 7.5 containing 5 $\mu\text{g}/\text{mL}$ nigericin. The cells were incubated with probe B for 2 h, post serum starvation (2 h), and imaged for colocalization with 50 nM LysoTracker Green. Images were collected by confocal fluorescence microscope at 40 $\times$ magnification, scale bars = 20 μm .

Figure 13.

CF and FUCL images of HeLa cells incubated with 10 μM probe B in buffers at pH 4.5 containing 5 $\mu\text{g}/\text{mL}$ nigericin. The cells were incubated with probe B for 2 h, post serum starvation (2 h), and imaged for colocalization with 50 nM LysoTracker Green and 1 $\mu\text{g}/\text{mL}$ Hoechst 33342 stains. Images were obtained by a confocal fluorescence microscope at 40 $\times$ magnification, scale bars = 20 μm .

Scheme 1.

Conventional Fluorescence (CF) and Single-Photon Frequency Upconversion Luminescence (FUCL).^{38,39}

Scheme 2.

Structures of Probes A and B, and Their Closed and Opened Spirocyclic Lactam Hypothetical Forms upon pH Changes

Scheme 3.
Synthetic Route of Fluorescent Probes A and B