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FRET-Based Small-Molecule Fluorescent Probes: Rational Design and Bioimaging Applications

LIN YUAN, WEIYING LIN,* KAIBO ZHENG, AND SASA ZHU

State Key Laboratory of Chemo/Biosensing and Chemometrics, College of Chemistry and Chemical Engineering, Hunan University, Changsha, Hunan 410082, People's Republic of China

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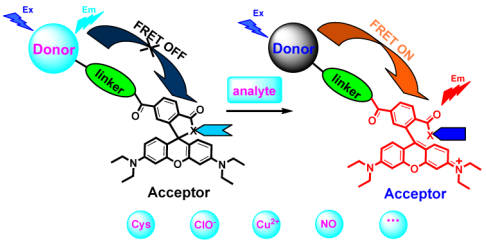
CONSPECTUS

Fluorescence imaging has emerged as a powerful tool for monitoring biomolecules within the context of living systems with high spatial and temporal resolution. Researchers have constructed a large number of synthetic intensity-based fluorescent probes for bio-imaging. However, intensity-based fluorescent probes have some limitations: variations in probe concentration, probe environment, and excitation intensity may influence the fluorescence intensity measurements. In principle, the use of ratiometric fluorescent probes can alleviate this shortcoming. Förster resonance energy transfer (FRET) is one of the most widely used sensing mechanisms for ratiometric fluorescent probes. However, the development of synthetic FRET probes with favorable photophysical properties that are also suitable for biological imaging applications remains challenging.



In this Account, we review the rational design and biological applications of synthetic FRET probes, focusing primarily on studies from our laboratory. To construct useful FRET probes, it is a pre-requisite to develop a FRET platform with favorable photophysical properties. The design criteria of a FRET platform include (1) well-resolved absorption spectra of the donor and acceptor, (2) well-separated emission spectra of the donor and acceptor, (3) donors and acceptors with comparable brightness, (4) rigid linkers, and (5) near-perfect efficiency in energy transfer.

With an efficient FRET platform in hand, it is then necessary to modulate the donor–acceptor distance or spectral overlap integral in an analyte-dependent fashion for development of FRET probes. Herein, we emphasize our most recent progress on the development of FRET probes by spectral overlap integral, in particular by changing the molar absorption coefficient of the donor dyes such as rhodamine dyes, which undergo unique changes in the absorption profiles during the ring-opening and -closing processes. Although partial success has been obtained in design of first-generation rhodamine-based FRET probes via modulation of acceptor molar absorption coefficient, further improvements in terms of versatility, sensitivity, and synthetic accessibility are required. To address these issues with the first-generation rhodamine-based FRET probes, we have proposed a strategy for the design of second-generation probes. As a demonstration, we have developed FRET imaging probes for diverse targets including Cu^{2+}, NO, HOCl, cysteine, and H_2O_2. This discussion of the methods for successfully designing synthetic FRET probes underscores the rational basis for further development of new FRET probes as a molecular toolbox for probing and manipulating a wide variety of biomolecules in living systems.

1. Introduction

Fluorescence imaging is one of the most powerful techniques for real-time, noninvasive monitoring of biomolecules of interest in their native environments with high spatial and temporal resolution, and it is instrumental for revealing the fundamental insights into the production, localization, trafficking, and biological roles of biomolecules in complex living systems.[1−3] Fluorescent probes are essential molecular tools for bioimaging. In this Account, fluorescent probes refer to synthetic organic dyes that interact specifically with a target of interest to induce an observable change in fluorescence properties. The development of fluorescent probes has facilitated the recent significant advances in cell biology and medical diagnostic imaging.[1−5] To date, a large number of small-molecule fluorescent probes have been constructed, and they can be classified into two types: intensity-based and ratiometric probes. A main limitation of intensity-based fluorescent probes is that variations in

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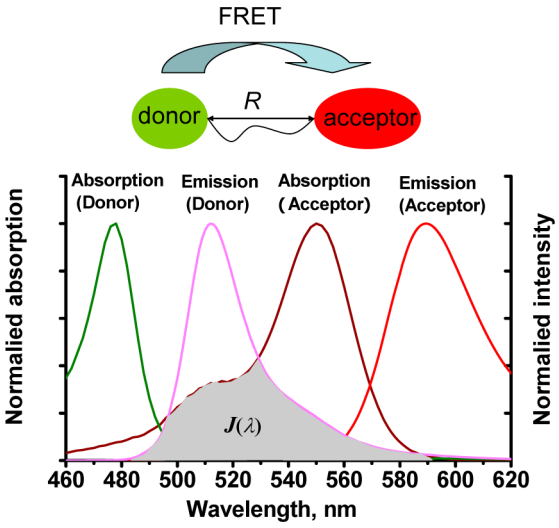


FIGURE 1. Schematic of the FRET process.

probe concentration, probe environment, and excitation intensity may influence the fluorescence intensity measurements. In principle, this problem can be alleviated by using ratiometric fluorescent probes, which allow the measurement of fluorescence emission/excitation intensities at two wavelengths, such that the ratios of signals will be independent of the environmental effects.[5−7]

Intramolecular charge transfer (ICT)[5,6] and FRET[5,7] are the two most commonly exploited sensing mechanisms for design of ratiometric fluorescent probes. An elegant example of ICT-type ratiometric fluorescent probes is PL1, a hydrogen peroxide ratiometric fluorescent probe, in which a H_2O_2-mediated reaction is used to modulate ICT efficiency.[6] However, some ICT-type ratiometric fluorescent probes have very broad emission spectra, which often lead to serious overlap in the emission peaks before and after interaction with target analytes. This shortcoming may elicit errors in the measurement of fluorescence intensity ratios. On the other hand, FRET has been extensively employed to construct protein- or nucleic acid-based bioprobes and small-molecule probes.

FRET is a nonradiative process in which an excited dye donor transfers energy to a dye acceptor in the ground state through long-range dipole−dipole interactions (Figure 1).[8−10] In this Account, we will restrict the discussion to FRET systems based on organic fluorophores due to the space limitation. Generally, the donor emission should have efficient spectral overlap with the acceptor absorption in FRET systems. FRET efficiency (E) is given by eq 1,[10]

$$E = R_0^6/(R_0^6 + R^6) \quad (1)$$

where R_0 is the Förster distance at which the transfer efficiency $E = 50\%$; R is the distance between the energy donor and acceptor. The Förster distance, R_0, can be calculated by simplified eq 2,

$$R_0 = 0.211[k^2 n^{-4} \Phi_D J_{DA}]^{1/6} (\text{in Å}) \quad (2)$$

where n is the refractive index, Φ_D is the quantum yield of the donor, k denotes the average squared orientational part of a dipole−dipole interaction (it is typically assumed $k^2 = 2/3$ for common organic fluorophores provided that both partners are freely moveable and thus randomly oriented),[10] and J_{DA} expresses the degree of spectral overlap between the donor emission and the acceptor absorption, which is given by eq 3,

$$J_{DA} = \int_0^\infty I_D(\lambda)\varepsilon_A(\lambda)\lambda^4 \, d\lambda \quad (3)$$

where $I_D(\lambda)$ is the donor normalized fluorescence emission spectrum, $\varepsilon_A(\lambda)$ is the acceptor molar absorption coefficient, and λ is the wavelength.

To design small-molecule FRET ratiometric fluorescent probes, it is a prerequisite to formulate a FRET energy transfer platform, which consists of an energy donor, a linker, and an energy acceptor. The donor emission spectrum should have reasonable overlap with the acceptor absorption spectrum, and the HOMO and LUMO energy levels of the acceptor should be located within those of the donor to avoid quenching of the acceptor fluorescence by photoinduced electron transfer (PET).[11] Furthermore, for practical applications, some design criteria involving energy donors, acceptors, and linkers should be considered: (1) The absorption spectrum of the donor should be well separated from that of the acceptor to ensure independent excitation at the absorption wavelengths of the donor and acceptor, respectively. (2) The emission spectrum of the donor should be resolved from that of the acceptor for high accuracy in the measurement of fluorescence intensity ratios. (3) The donor fluorophore and the acceptor dye should have comparable brightness ($\varepsilon \times \Phi$), which may impart two well-separated emission bands with comparable intensities before and after interaction with an analyte. (4) Appropriate linkers should be selected to avoid static fluorescence quenching due to close contact of donor and acceptor dyes in aqueous environment. This is particularly problematic but often overlooked in development of efficient energy transfer platforms intended for bioapplications. 5) A near perfect energy transfer

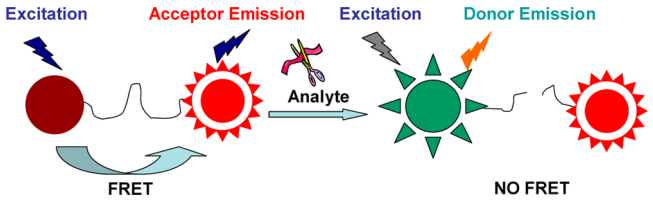


FIGURE 2. Ratiometric fluorescent probes based on analyte-induced cleavage of FRET dyads.

efficiency should be achieved in the energy transfer platform. These requirements render the selection of suitable energy donors, acceptors, and linkers very demanding.

With an appropriate energy transfer platform in hand, it is then necessary to tune the energy transfer efficiency in an analyte-dependent fashion to provide a FRET ratiometric fluorescent probe. From factors in eqs 1 and 2, there are three factors that can be modulated to influence energy transfer efficiency: k, R, and J. Because k^2 is typically assumed to be 2/3 in small-molecule FRET systems, R and J are the parameters of choice in regulating the energy transfer efficiency. Thus, modulating donor–acceptor distance and spectral overlap integral are often leveraged in design of FRET-based ratiometric fluorescent probes.

In this Account, we focus on discussion of the chemical and photophysical strategies involved in designing efficient FRET-based synthetic organic probes for biological imaging applications by using illustrative examples mainly from our group.

2. FRET-Based Ratiometric Fluorescent Probes by Tuning Donor–Acceptor Distance

FRET efficiency is distant-dependent, which serves as the basis for design of FRET probes with analyte-induced donor–acceptor distance changes. One type of such probes has the donor and acceptor positioned at the two ends and the reaction site for the analyte situated in the middle (Figure 2). Thus, in the absence of the analyte of interest, the free probe should display the emission of the acceptor upon excitation at the donor due to FRET. By contrast, when the probe is incubated with the analyte, the FRET dyad is cleaved. The distance between the donor and acceptor becomes infinite, and the FRET is switched off. Thus, upon excitation at the donor, only the emission of the donor is observed.

Inspired by the seminal work of Tsien's group[12] and the mechanism of native chemical ligation (NCL) reaction,[13] we designed NRFTP (Figure 3) as a new FRET-based ratiometric probe for thiols such as cysteine[14] by exploiting the unique

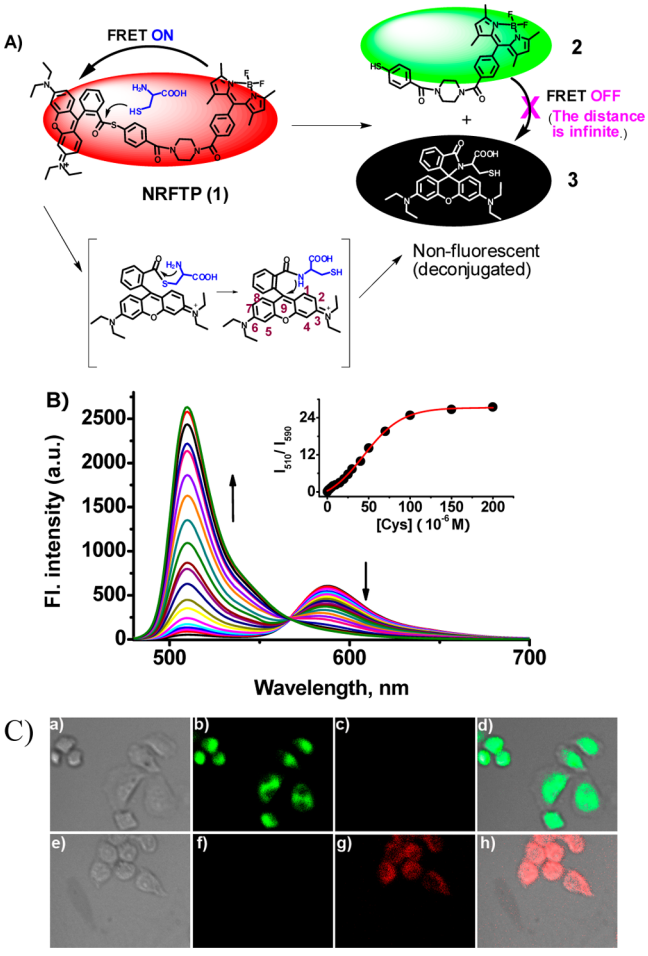


FIGURE 3. (A) The ratiometric sensing mechanism of NRFTP for cysteine. (B) The emission spectra of NRFTP incubated with varying concentrations of cysteine. (C) Confocal images of HeLa cells: (a–c) bright-field and fluorescence images of HeLa cells incubated with NRFTP; (d) overlay the images of panels a–c; (e–g) bright-field and fluorescence images of HeLa cells incubated with *N*-ethylmaleimide and then with NRFTP; (h) overlay the images of panels e–g. Reproduced with permission from ref 14. Copyright 2011 Royal Society of Chemistry.

ring opened/closed photophysical properties of rhodamine dyes.[15–17] NRFTP is composed of a rhodamine acceptor, a thioester group, a piperazyl moiety, and a Bodipy dye. The selection of Bodipy and rhodamine as the fluorophores is based on the consideration that the Bodipy emission has strong overlap with the rhodamine absorption. Furthermore, the emission windows of Bodipy and rhodamine are well-separated, which is favorable for measurement of the emission intensity and signal ratios with high precision. The piperazyl moiety was chosen as the rigid linker to facilitate the energy transfer between the Bodipy donor and rhodamine acceptor. The distance from the boron atom in the Bodipy dye to the oxygen atom in the xanthene ring of the rhodamine moiety was calculated to be about 23 Å. Thus,

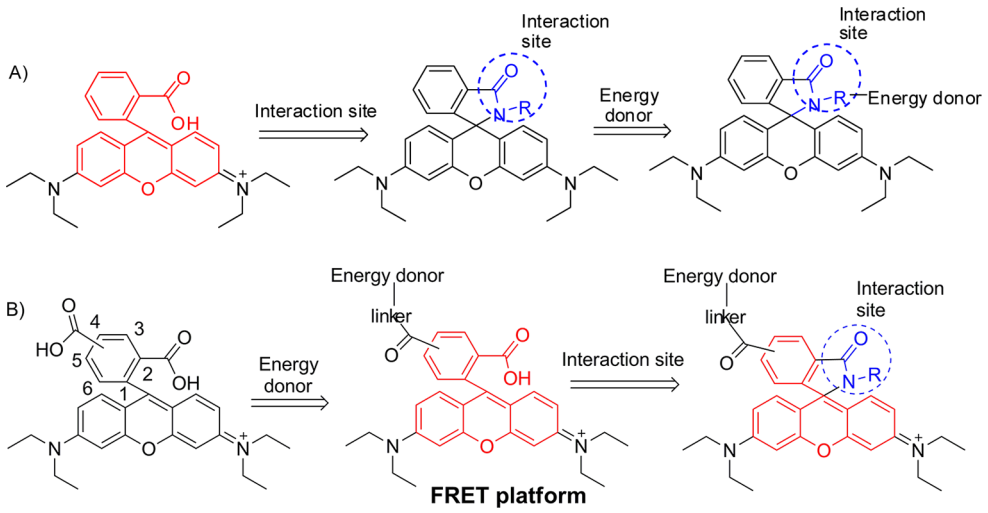


FIGURE 4. (A) A FRET strategy for development of the first-generation rhodamine-based ratiometric probes. (B) A robust FRET strategy for rational design of the second-generation rhodamine-based ratiometric probes.

based on these factors, we expected that the excited energy of the Bodipy donor could be efficiently transferred to the rhodamine acceptor. When the free probe is excited at the Bodipy absorption, we should observe the red emission of the rhodamine dye. To promote the NCL reaction, we judiciously selected a thiophenylester moiety as the reaction site, because it is a better leaving group than a thioalkylester group in physiological conditions.

As designed, incubation of NRFTP with cysteine results in a blue shift from 590 to 510 nm in the emission due to the cleavage of NRFTP and formation of Bodipy **2** and rhodamine **3** (Figure 3). Notably, the released acceptor in this case, rhodamine **3**, is nonfluorescent, because it is in the ring-closed form. Thus, the formation of nonemissive rhodamine **3** should be advantageous in avoiding the emission cross-talk problem often observed in FRET-based probes. The new probe has been successfully applied for ratiometric imaging of thiols in living cells (Figure 3C). Thus, we expect that NRFTP will be a useful molecular tool for diverse applications including determination of thiol levels in biological fluids, assaying enzymes with a thiol as a product for high-throughput enzyme inhibitor screening, and ratiometric fluorescence imaging of thiols in living cells. By employing a similar distance-changing strategy, we have engineered another FRET-based probe for thiol imaging in living cells.[18]

3. FRET Ratiometric Fluorescent Probes via Modulation of Acceptor Molar Absorption Coefficient

As shown in eqs 2 and 3, FRET efficiency also depends on the overlap integral (J_{DA}). This makes it possible to design FRET probes based on analyte-induced variation of the overlap integral. A method for tuning the J_{DA} is by shifting the acceptor absorption wavelength, which is often challenging to realize in practice. Alternatively, the J_{DA} can be modulated by changing the acceptor molar absorption coefficient[19] based on eq 3. Practically, this strategy is more facile for donor fluorophores such as rhodamine. We will summarize our recent progress in the development of FRET probes by changing the molar absorption coefficient of rhodamine dyes.

3.1. First-Generation Rhodamine-Based FRET Ratiometric Fluorescent Probes. A unique property of rhodamine is that it can exist in the ring-opened and -closed forms, which have distinct absorption profiles. Rhodamine in the ring-opened form displays strong absorption at around 550 nm, whereas the ring-closed form has essentially no absorption in the visible region. In addition, the ring-open and -close processes also render significant changes in the emission profiles of rhodamine. This characteristic has been widely exploited in design of turn-on type probes for a diverse array of analytes.[15−17] By contrast, only limited examples of rhodamine-based FRET probes have been developed based on the changes in the molar absorption coefficient during the ring-open and -close processes.

Although a number of rhodamine-based FRET ratiometric fluorescent probes have been developed via modulation of acceptor molar absorption coefficient (Figure 4 and Figure S1, Supporting Information),[20−24] they are associated with several drawbacks. First, because the energy donor is linked to the interaction site, a careful synthetic method for the introduction of the donor has to be selected to keep the interaction site intact. Second, they are not suitable for

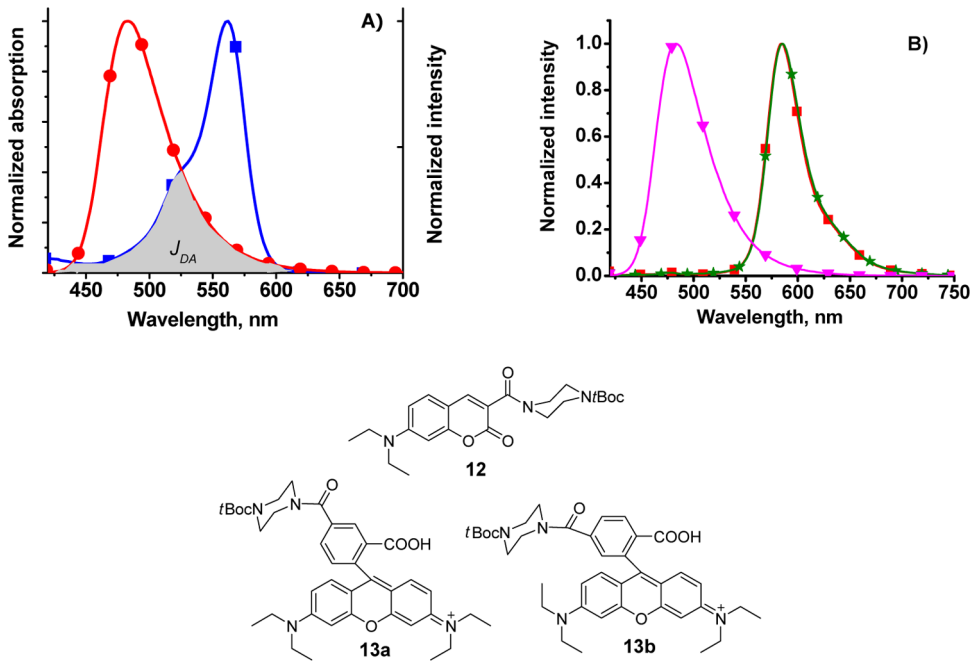


FIGURE 5. (A) The spectral overlap between coumarin **12** emission (red circle) and rhodamine **13a** absorption (blue square) in 25 mM PBS (pH 7.4, 1% EtOH). J_{DA} (3.6×10^{14} M^{-1} cm^{-1} nm^4) was calculated by eq 3. (B) Normalized emission spectra of the model energy donor **12** (pink triangle) and model rhodamine acceptors **13a** (red square) and **13b** (green star).

analytes that may induce the cleavage of the FRET dyad (e.g., Cu^{2+}-promoted hydrolysis of rhodamine-B hydrazide to rhodamine-B.[25]), because there is no variation of FRET efficiency before and after interaction with the target analyte (Figure S2, Supporting Information). Third, the sensing sensitivity of this type of probe may be affected, because the interaction site is surrounded by both the energy donor and the acceptor and thus becomes very crowded. For example, compound **11a** (Figure S3, Supporting Information) is a sensitive fluorescent probe for Au^{3+};[26] however, compound **11b** exhibits almost no response to Au^{3+} under the same assay conditions. These shortcomings indicate that strategies are needed to construct next generation FRET probes with improved properties.

3.2. Second-Generation Rhodamine-Based FRET Ratiometric Fluorescent Probes. To address the issues associated with the first-generation FRET probes, we formulated a strategy for development of second-generation rhodamine-based FRET probes (Figure 4B). By utilization of the distinct reactivity of the 4/5- and 2-position carboxylic acid groups, an energy donor is selectively incorporated into the 4/5-position carboxylic acid group through an appropriate linker to form a FRET platform. Then, an interaction site of interest is introduced into the 2-position carboxylic acid group to give the FRET probes. A similar strategy has been applied to develop fluorescein-based FRET probes.[27,28]

Compared with the existing approaches, our strategy has the following advantages: (a) The central theme of the strategy is to assemble the FRET platform prior to the interaction site. Thus, the complicated synthetic problems associated with the existing strategies may be alleviated. (b) The strategy renders FRET platforms with an intact 2-position carboxyl group, which can be readily functionalized to afford a diverse array of interaction sites of interest. This renders it robust and simple to employ. (c) The interaction site is far away from the donor. Therefore, unlike the first-generation ones, the sensing sensitivity should not be affected. (d) The strategy is suitable for target analytes that otherwise could induce the cleavage of the FRET dyad in the first-generation rhodamine-based FRET probes.

The key to the above strategy is to allow the FRET platform to be established before the interaction site. Thus, the rhodamine component (highlighted in red color in Figure 4) of the FRET platform is essentially the same as rhodamine B. In other words, the FRET platform is the FRET "version" of rhodamine dye. This implies that, in principle, any rhodamine spirolactam ring-opened interaction sites can be readily adapted to the FRET platform for construction of rhodamine-based FRET ratiometric sensors. Given the advantages of the strategy, we expect that it will be very useful for development of a wide variety of rhodamine-based FRET fluorescent probes.

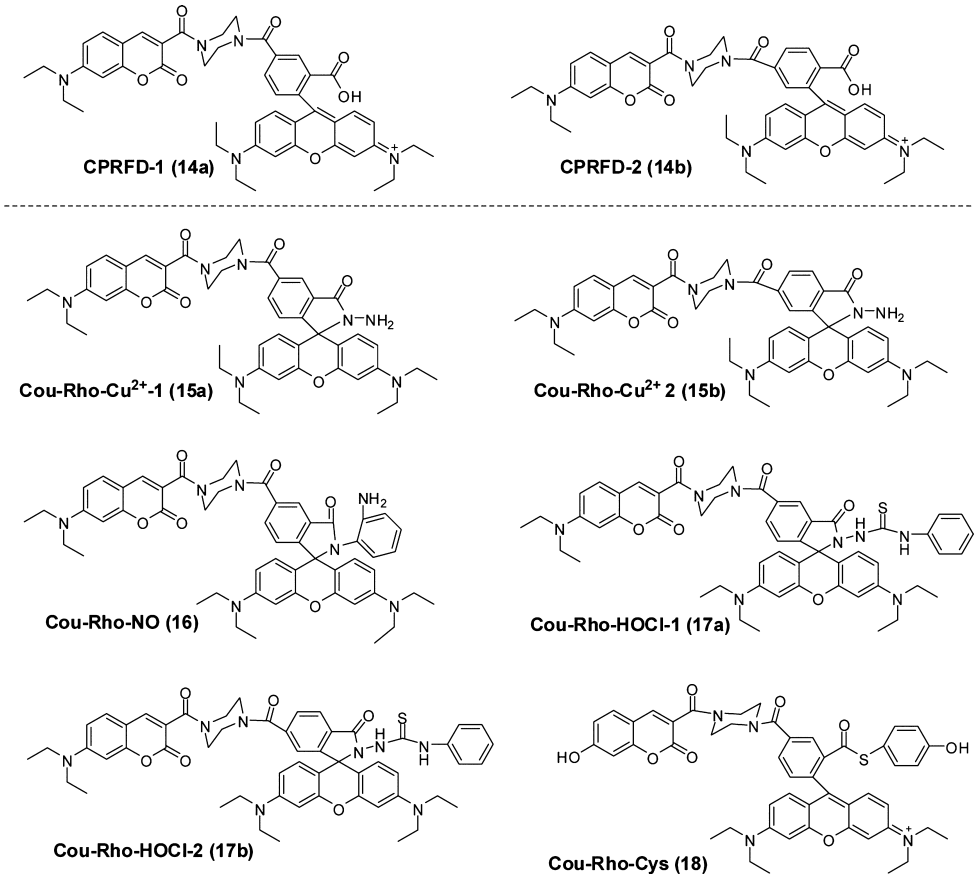


FIGURE 6. Structures of CPRFD-1/CPRFD-2 and the second-generation rhodamine-based FRET ratiometric fluorescent imaging probes developed based on these platforms.

To demonstrate the usefulness of the strategy, we should first establish an efficient rhodamine-based FRET platform. To this end, it is necessary to select an appropriate energy donor and a linker. 7-Diethylamino coumarin was chosen as the energy donor for several considerations, especially from the viewpoint of photophysical properties: (a) The emission spectrum of diethylamino coumarin has reasonable spectral overlap (J_{DA} (M^{-1} cm^{-1} nm^4) = 3.6×10^{14}) with the absorption spectrum of rhodamine B (Figure 5A). (b) The emission bands of diethylamino coumarin and rhodamine are almost completely resolved with a separation of around 110 nm (Figure 5B). This is constructive for high accuracy in the measurement of fluorescence intensity ratios.

Another key aspect for the establishment of an efficient FRET platform is the selection of a suitable linker to connect the energy donor with the acceptor. Appropriate rigid linkers should be judiciously chosen to avoid the static fluorescence quenching due to close contact of the donor and acceptor dyes in biological systems. This is very critical but often ignored in the development of efficient FRET platforms intended for bioapplications. Based on these considerations, we rationally designed CPRFD-1 and CPRFD-2 (Figure 6) as the FRET platforms. DFT calculations indicate that the distances between the coumarin donor and the rhodamine acceptor in CPRFD-1 and CPRFD-2 are 11.0 and 10.3 Å, respectively, (Figure S4, Supporting Information). The Förster radius (R_0) of the coumarin−rhodamine dyad was calculated as 36.0 Å, suggesting that the FRET efficiencies in CPRFD-1 and CPRFD-2 are potentially high based on eq 1. In addition, the HOMO and LUMO energy levels of the rhodamine acceptor locate within those of the coumarin donor (Figure S5, Supporting Information), which may ensure that the acceptor emission does not quench by PET under direct excitation at the acceptor,[11] further indicating that 7-diethylamino coumarin could be an appropriate energy donor. The excellent correspondence between the excitation and absorption spectra reinforces that CPRFD-1 and CPRFD-2 are efficient FRET platforms (Figure S6, Supporting Information). Furthermore, both the donor and acceptor undergo only a slight red shift in excitation and emission spectra with the increase of the solvent polarity (Figure S7, Supporting Information), and the changes of buffer strength have no

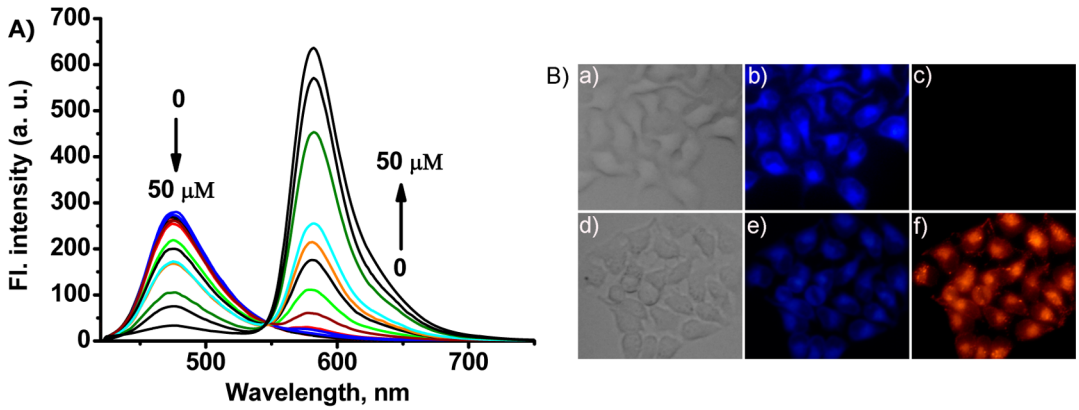


FIGURE 7. (A) Fluorescence response of Cou-Rho-Cu^{2+}-1 to Cu^{2+}. (B) Images of HeLa cells: (a−c) bright-field and fluorescence images of HeLa cells incubated with Cou-Rho-Cu^{2+}-1; (d−f) bright-field and fluorescence images of HeLa cells incubated with Cou-Rho-Cu^{2+}-1 and then with Cu^{2+}.

marked effect on the spectra (Figure S8, Supporting Information), indicating that both the solvent polarity and buffer strength have only minimal effect on the overlap integral. Thus, CPRFD-1 and CPRFD-2 appear to be suitable FRET platforms.

CPRFD-1 and CPRFD-2 have an intact 2-position carboxyl group available for further functionalization to afford interaction sites of interest. Thus, the modular nature of CPRFD-1 and CPRFD-2 may allow them to act as robust FRET platforms for development of a diverse array of FRET fluorescent probes. Next, we will briefly discuss the rational design of FRET ratiometric probes for Cu^{2+},[29] NO,[30] HOCl,[31] and cysteine[32] based on these two platforms.

The design strategies for the first-generation rhodamine-based FRET probes are not suitable for analytes that may induce the cleavage of the FRET dyad. Czarnik's group described a fluorescence turn-on copper probe based on the copper-promoted hydrolysis of rhodamine-B hydrazide.[27] We envisioned that this may provide a valuable testing ground to evaluate the effectiveness of FRET platforms CPRFD-1 and CPRFD-2 for development of ratiometric copper probes.[29] We prepared Cou-Rho-Cu^{2+}-1 and Cou-Rho-Cu^{2+}-2 (Figure 6) as novel FRET ratiometric copper probes based on the platforms CPRFD-1 and CPRFD-2. There is no FRET in the free Cou-Rho-Cu^{2+}-1, because the rhodamine acceptor is in the ring-closed form at different pH values (Figure S9, Supporting Information). Reaction of Cou-Rho-Cu^{2+}-1 with Cu^{2+} transforms rhodamine hydrazide into rhodamine acid with concomitant ring opening (Figure S10, Supporting Information) to result in a >238-fold variation in the emission ratios (I_{581}/I_{473}) (Figure 7A).[29] In addition, Cou-Rho-Cu^{2+}-1 is cell membrane permeable and could be employed to monitor fluctuations of Cu^{2+} in living HeLa cells by ratiometric fluorescence imaging (Figure 7B).

To further demonstrate the versatility of the strategy, we also designed Cou-Rho-NO (Figure 6) as a novel FRET-based ratiometric probe for nitric oxide (NO),[30] which is a secondary messenger in many physiological processes. The free probe displays the emission peak of the coumarin donor. However, addition of DEA/NONOate (NO donor) induces a significant ratiometric fluorescence response (Figure 8A) with a drastic change of emission color from green to orange-red (the inset in Figure 8A). Furthermore, Cou-Rho-NO can respond to NO over a wide pH range from 3 to 10 (Figure S13, Supporting Information). There is a ca. 420-fold variation in the fluorescence ratio (I_{583}/I_{473}). Moreover, Cou-Rho-NO could be employed to detect endogenously produced NO in macrophages (Figure 8B).

In addition to Cu^{2+} and NO, we also employed the platform to construct probes Cou-Rho-HClO-1 and Cou-Rho-HClO-2 for HClO (Figure 6) based on HOCl-promoted cyclization of rhodamine-thiosemicarbazides to rhodamine-oxadiazoles (Figure 8C).[31] The emission spectra of Cou-Rho-HClO-1 and Cou-Rho-HClO-2 are similar to that of Cou-Rho-NO. There is no FRET in the free Cou-Rho-HClO-1 at a wide pH range (Figure S14, Supporting Information). Introduction of HOCl to Cou-Rho-HClO-1 or Cou-Rho-HClO-2 triggers a significant red-shift in emission, as the FRET changes from the OFF to ON state. Furthermore, Cou-Rho-HClO-1 and Cou-Rho-HClO-2 can image endogenously produced HOCl in living cells in a ratiometric manner.

To further demonstrate the robust nature of our strategy, a FRET process switching from the ON to OFF state also was applied to develop ratiometric fluorescent probes.[32] Toward this end, we designed Cou-Rho-Cys (Figure 6) as a ratiometric fluorescent probe selective for aminothiols based on the NCL reaction (Figure 9A).[32] The free probe displays an emission band at 603 nm because the rhodamine is in the

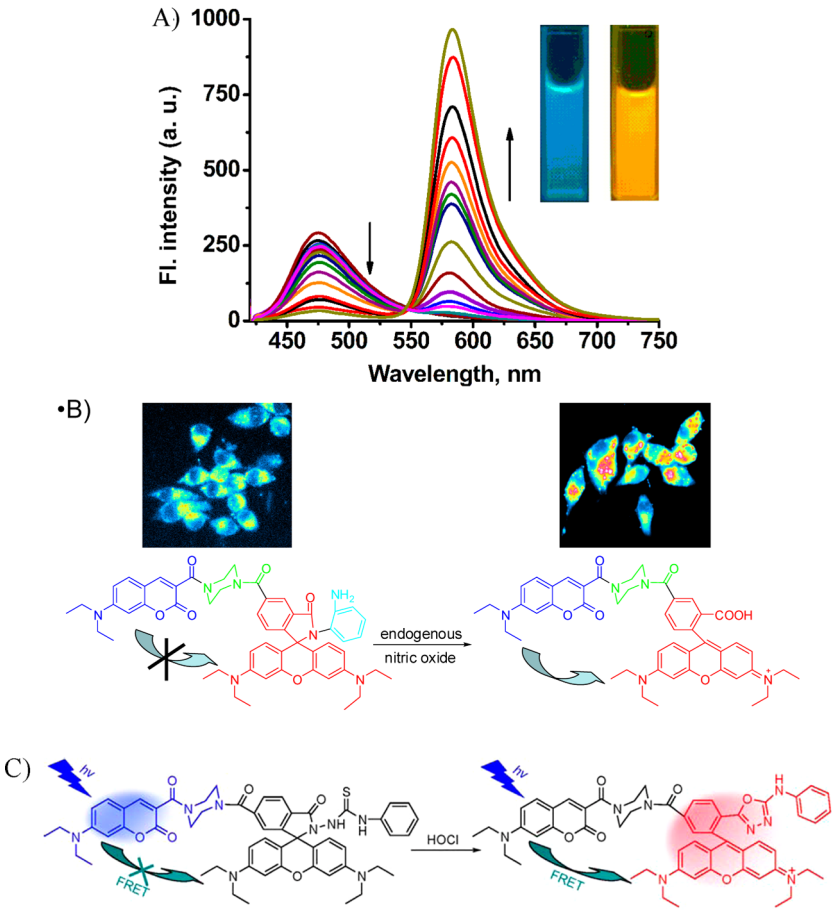


FIGURE 8. (A) Fluorescence response of Cou-Rho-NO to NO. (B) The sensing mechanism of Cou-Rho-NO to NO and fluorescence imaging of endogenously produced NO in RAW264.7 macrophage cells. (C) The sensing mechanism of Cou-Rho-HClO-2 to HOCl. Reproduced with permission from ref 30. Copyright 2011 Royal Society of Chemistry.

ring-opened form and the FRET is ON. However, treatment with cysteine induces a marked decrease of the emission intensity at 603 nm and concurrently a dramatic enhancement of the emission intensity at 458 nm (Figure 9B), because rhodamine exists in the ring-closed form. The emission ratio (I_{458}/I_{603}) shows a 160-fold enhancement (inset of Figure 9B). In contrast to NRFTP,[14] which is also developed based on the NCL reaction, Cou-Rho-Cys can selectively detect aminothiols over other biological thiols. In addition, Cou-Rho-Cys is cell membrane permeable and suitable for ratiometric imaging of aminothiols in the living cells (Figure 9C).

Hydrogen peroxide functions as a signaling molecule in many signaling transduction processes and an oxidative stress marker in aging and disease. However, excessive production of H_2O_2 is associated with a wide variety of diseases. On the other hand, nitric oxide serves as a secondary messenger inducing vascular smooth muscle relaxation. However, misregulation of NO production is implicated with various disorders. To disentangle the complicated inter-relationship between H_2O_2 and NO in the signal transduction and oxidative pathways, fluorescent reporters that are able to differentiate H_2O_2, NO, and H_2O_2/NO by distinct signals are highly desirable. By exploiting the unique photophysical properties of the coumarin–rhodamine platform, we also used the platform to design FP-H_2O_2-NO as the first single-fluorescent molecule that can respond to H_2O_2, NO, and H_2O_2/NO with three different sets of fluorescence signals (Figure 10).[33] The fluorescence signal pattern for the free probe is black–black–black. However, when the probe is incubated with H_2O_2, the boronate group of FP-H_2O_2-NO is removed to afford FP-NO, which contains 7-hydroxycoumarin and rhodamine in the spirocyclic form. FP-NO has intense emission at 460 nm when excited at 400 nm and exhibits no emission at 580 nm when excited at 400 or 550 nm. Thus, the fluorescence signal pattern for the probe in the presence of H_2O_2 is blue–black–black. Upon treatment of FP-H_2O_2-NO with NO, the phenylenediamine group of FP-H_2O_2-NO is eliminated to give FP-H_2O_2, which bears

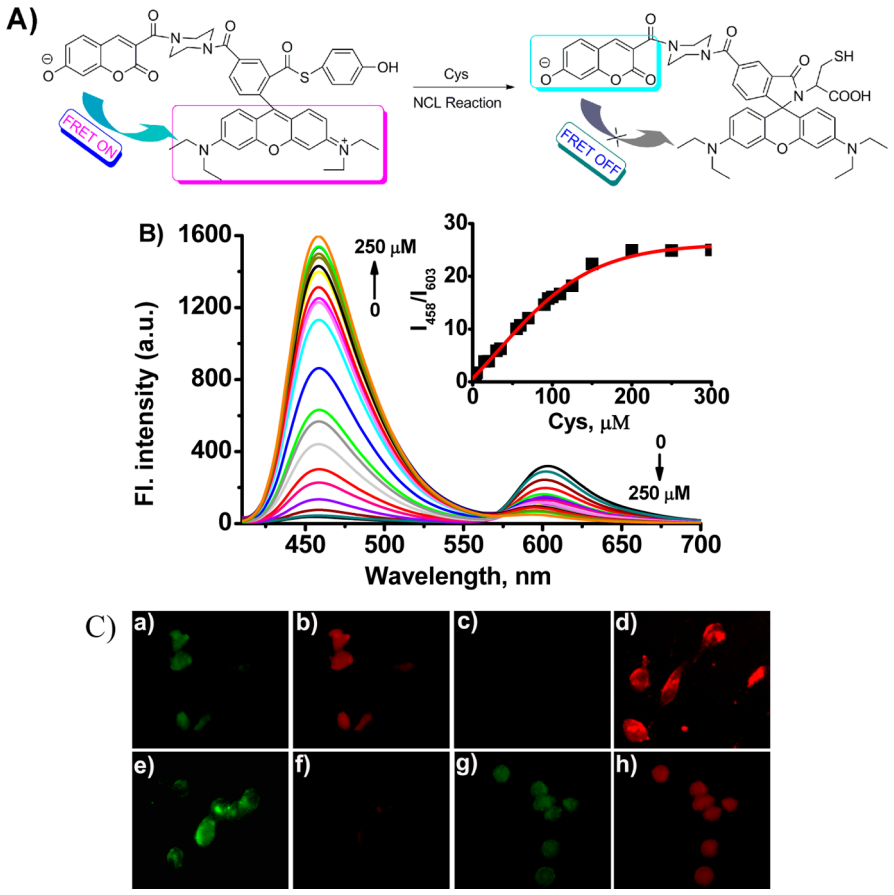
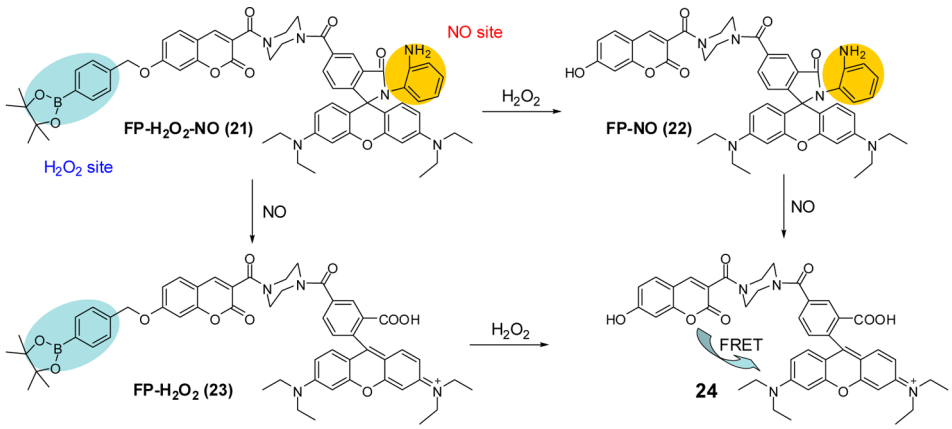


FIGURE 9. (A) The sensing mechanism of Cou-Rho-Cys for cysteine. (B) Fluorescence response of Cou-Rho-Cys to cysteine. The inset shows the changes of fluorescent intensity ratios at 458 and 603 nm (I_{458}/I_{603}) to increasing concentration of cysteine. (C) Fluorescence images of the living HepG2 cells treated with Cou-Rho-Cys in the green and red channels: (a, b) cells incubated with only Cou-Rho-Cys; (c, d) cells pretreated with *N*-ethylmaleimide and then incubated with Cou-Rho-Cys; (e, f) cells pretreated with *N*-acetylcysteine and then incubated with Cou-Rho-Cys; (g,h) The cells pretreated with α-lipolic acid and then incubated with Cou-Rho-Cys. Reproduced with permission from ref 32. Copyright 2012 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

7-alkoxycoumarin and rhodamine in the ring-opened form. FP-H_2O_2 has no emission at 460 and 580 nm when excited at 400 nm and exhibits strong emission at 580 nm when excited at 550 nm. Thereby, the fluorescence signal pattern for FP-H_2O_2-NO in the presence of NO is black−black−red. When FP-H_2O_2-NO is incubated with H_2O_2/NO, the boronate and phenylenediamine groups of FP-H_2O_2-NO are removed to eventually provide compound **24**, which contains 7-hydroxycoumarin and rhodamine in the ring-opened form. Compound **24** displays no emission at 460 nm when excited at 400 nm and displays strong emission at 580 nm when excited at 400 or 550 nm, as the emission of 7-hydroxycoumarin transfers to the rhodamine dye by FRET. Thus, the fluorescence signal pattern for the probe in the presence of H_2O_2/NO is black−red−red. Significantly, we have further demonstrated that FP-H_2O_2-NO is capable of simultaneously monitoring endogenously produced NO and H_2O_2 in living macrophage cells in multicolor imaging (Figure 11). We expect that FP-H_2O_2-NO will be a unique molecular tool to investigate the interplaying roles of H_2O_2 and NO in many important signaling and oxidative pathways. Further optimization of this type of probe may provide new opportunities in studies of complicated ROS and RNS biology. In addition, this work establishes a robust strategy for monitoring multiple ROS and RNS species (H_2O_2, NO, and H_2O_2/NO) using a single fluorescent probe, and the modularity of the strategy may allow it to be extended for other types of biomolecules by judicious selection of suitable reaction sites. Furthermore, because this type of fluorescent probe is capable of displaying multiple fluorescence signal patterns, it may have potential applications in flow cytometry.

4. FRET-Based Dual-Excitation Ratiometric Fluorescent Probes

Ratiometric fluorescent probes include two types: dual-emission and dual-excitation. However, compared with



	Ex. 400 nm		Ex. 550 nm	Fluorescence signal pattern
	Em. 460 nm	Em. 580 nm	Em.580 nm	
Free				black-black-black
H_2O_2				blue-black-black
NO				black-black-red
H_2O_2 + NO				black-red-red

FIGURE 10. The rational design strategy for FP-H_2O_2-NO, a unique type of a single fluorescent probe that can report H_2O_2, NO, and H_2O_2/NO with three different sets of fluorescence signals.

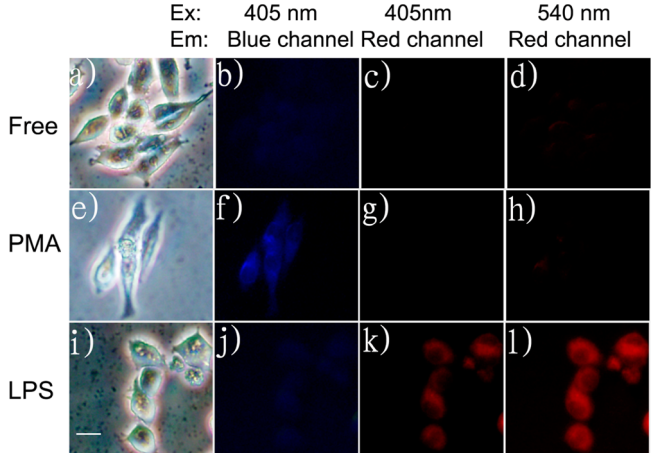


FIGURE 11. Representative images of RAW 264.7 macrophages treated with FP-H_2O_2-NO in the absence or presence of stimulants. (a–d) Images of RAW 264.7 macrophages incubated with only FP-H_2O_2-NO. (e–h) Images of RAW 264.7 macrophages coincubated with FP-H_2O_2-NO and PMA. (i–l) Images of RAW 264.7 macrophages incubated with LPS and then treated with FP-H_2O_2-NO.

FRET-based dual-emission ratiometric fluorescent probes, FRET-based dual-excitation ones are under-developed.[34] Our group also employed the coumarin–rhodamine platform to design dual-excitation ratiometric fluorescent probes. For instance, we created compounds **25a**,**b** as FRET-based dual-excitation ratiometric fluorescent pH probes (Figure 12A).[34] With the enhancement in pH values, the excitation peak at around 350 nm, attributed to the phenolic form of the hydroxycoumarin unit, significantly diminishes, and simultaneously a new red-shifted excitation band at around 400 nm, ascribed to the phenolate form of the hydroxycoumarin unit, is formed (Figure 12B). The red shift in the excitation spectrum of the coumarin unit with the increase of pH is due to ICT. By contrast, the excitation band centered at around 560 nm, attributed to the rhodamine moiety, is almost pH-insensitive. Thus, the ratios of fluorescence intensities (I_{400}/I_{560}) exhibits a 10-fold variation. It is noteworthy that the excitation separations between the donor and acceptor of the new dual-excitation ratiometric pH

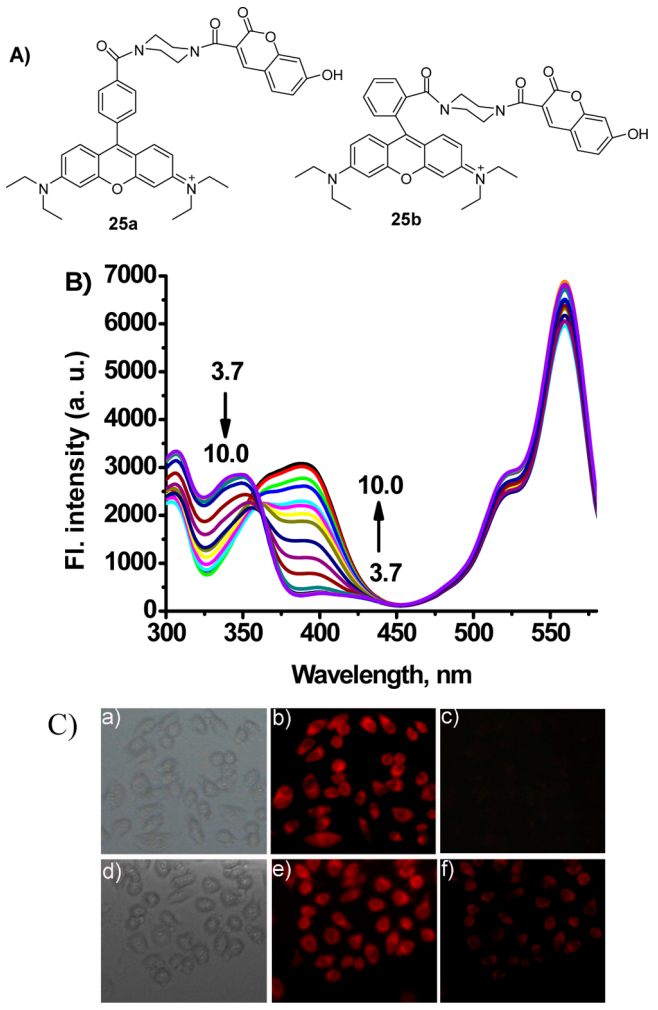


FIGURE 12. (A) Structure of FRET-based dual-excitation ratiometric fluorescent probes **25a**,**b**. (B) pH-dependence of the excitation intensity of **25a** monitored at 586 nm with the arrows indicating the change of the fluorescence intensities with pH enhancement from 3.7 to 10.0. (C) Bright field (a, d) and fluorescence images of Tca-8113 cells excited at around 540 nm (b, e) or 405 nm (c, f) treated with **25a** at pH 5.5 (a–c) or pH 7.4 (d–f). Reproduced with permission from ref 34. Copyright 2012 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

probes are remarkable, up to 160 nm, which are much larger than those of the single organic dye dual-excitation fluorescent probes (<70 nm). In addition, we have demonstrated that **25a** could be employed for ratiometric fluorescence imaging of pH fluctuations in the living cells (Figure 12C).

5. Concluding Remarks

In summary, we have discussed the chemical and photophysical strategies for rational design of synthetic FRET-based probes for biological imaging. Modulating donor–acceptor distance and spectral overlap integral are the two main methods to modulate the energy transfer efficiency in an analyte-dependent fashion for development of FRET probes. The unique changes in the absorption profiles of rhodamine dyes during the ring-open and -close processes have rendered them effective energy acceptors in designing FRET probes by tuning the spectral overlap integral, particularly via modulation of acceptor molar absorption coefficient. This leads to the construction of first-generation rhodamine-based FRET probes. Although limited success has been achieved in design of rhodamine-based FRET probes via modulation of acceptor molar absorption coefficient, further improvements in terms of versatility, sensitivity, and synthetic accessibility are needed. We have thus proposed a strategy for development of second-generation rhodamine-based FRET probes, which are advantageous over first-generation rhodamine-based ones in several aspects: (a) The central theme of the strategy is to assemble the FRET platform prior to the interaction site. (b) The strategy renders the FRET platforms with an intact 2-position carboxyl group, which can be readily functionalized to afford diverse array of interaction sites of interest. (c) The interaction site is far away from the energy donor, which may minimize the effect on the sensitivity. (d) The FRET strategy is suitable for target analytes such as copper ions to which the first-generation rhodamine-based FRET probes are not applicable. Thus, the strategy is robust, and we expect that it will be useful for development of a wide variety of rhodamine-based FRET fluorescent imaging agents for diverse biological targets. The parameters for the second-generation rhodamine-based FRET probes described herein and the representative fluorescent probes previously reported are summarized in Table S1–4, Supporting Information. However, there is a continuing need to improve the properties of second-generation rhodamine-based FRET probes. Although the coumarin–rhodamine FRET probes exhibit large shifts in the emission, the excitation wavelength of coumarin is relatively short. Thus, the development of new FRET probes with both large shifts in the emission and long excitation wavelengths remains a subject of high interest. In addition, the water solubility of the coumarin–rhodamine FRET probes can be further improved.

Supporting Information. Some spectra and chemical structures. This material is available free of charge via the Internet at http://pubs.acs.org.

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BIOGRAPHICAL INFORMATION

Lin Yuan received his B.S. degree from University of South China in 2006. Subsequently, he started his graduate studies under the supervision of Professor Weiying Lin in Hunan University, where he is currently an assistant professor. His research interests focus on the design and synthesis of novel functional fluorescent probes.

Weiying Lin received his Ph.D. from the University of Kansas in 2000. After completing postdoctoral research at Einstein College of Medicine and Massachusetts Institute of Technology, in 2005, he joined the faculty at Hunan University, where he is currently a professor. His research interests cover the interdisciplinary areas of molecular recognition, photochemistry, and chemical biology.

Kaibo Zheng received his master degree in organic chemistry from the Sichuan University in 2008. He started his doctoral work under the supervision of Professor Weiying Lin at Hunan University in 2011. His research interests focus on the design and synthesis of fluorescent probes.

Sasa Zhu obtained her B.S. degree at the Zhoukou Normal University in 2010. Currently, she is a graduate student under the supervision of Professor Weiying Lin in Hunan University. Her research interests focus on the design and synthesis of fluorescent probes.

FOOTNOTES

*To whom correspondence should be addressed. E-mail: weiyinglin@hnu.edu.cn.
The authors declare no competing financial interest.

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