A Novel Design Method of Ratiometric Fluorescent Probes Based on Fluorescence Resonance Energy Transfer Switching by Spectral Overlap Integral

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Abstract: A ratiometric measurement, namely, simultaneous recording of the fluorescence intensities at two wavelengths and calculation of their ratio, allows greater precision than measurements at a single wavelength, and is suitable for cellular imaging studies. Here we describe a novel method of designing probes for ratiometric measurement of hydrolytic enzyme activity based on switching of fluorescence resonance energy transfer (FRET). This method employs fluorescent probes with a 3′-*O*,6′-*O*-protected fluorescein acceptor linked to a coumarin donor through a linker moiety. As there is no spectral overlap integral between the coumarin emission and fluorescein absorption, the fluorescein moiety cannot accept the excitation energy of the donor moiety and the donor fluorescence can be observed. After cleavage of the protective groups by hydrolytic enzymes, the fluorescein moiety shows a strong absorption in the coumarin emission region, and then acceptor fluorescence due to FRET is observed. Based on this mechanism, we have developed novel ratiometric fluorescent probes (**1**–**3**) for protein tyrosine phosphatase (PTP) activity. They exhibit a large shift in their emission wavelength after reaction with PTPs. The fluorescence quenching problem that usually occurs with FRET probes is overcome by using the coumarin–cyclohexane–fluorescein FRET cassette moiety, in which close contact of the two dyes is hindered. After study of their chemical and kinetic properties, we have concluded that compounds **1** and **2** bearing a rigid cyclohexane linker are practically useful for the ratiometric measurement of PTPs activity. The design concept described in this paper, using FRET switching by spectral overlap integral and a rigid link that prevents close contact of the two dyes, should also be applicable to other hydrolytic enzymes by introducing other appropriate enzyme-cleavable groups into the fluorescein acceptor.

Keywords: fluorescent probes · fluorescence resonance energy transfer · protein tyrosine phosphatase · ratiometric measurement

Introduction

In recent years, many fluorescent probes have been developed to study biological phenomena in living cells.[1] A fluorescent probe is advantageous due to its high sensitivity; however, the fluorescence measurement can be influenced by many factors, such as the localization of the probe, changes of environment around the probe (e.g., pH, polarity, temperature) and changes in the excitation intensity. To reduce the influence of such factors, a ratiometric measurement is to be utilized, namely, simultaneous recording of the fluorescence intensities at two wavelengths and calculation of their ratio. This technique provides greater precision than measurement at a single wavelength, and is suitable for cellular imaging studies. To carry out a ratiometric measurement, the probe must exhibit a large shift in its emission or excitation spectrum after it reacts (or binds) with the target molecule. Fluorescence resonance energy transfer[2] (FRET) is one mechanism used as a basis to obtain a large shift in the spectral peak. FRET is an interaction between the electronic excited states of two fluorophores, in which excitation energy is transferred from a donor to an acceptor without emission of a photon. The efficiency of FRET depends on the donor–acceptor distance, the relative orientation of the donor and acceptor transition dipoles, the extent of spectral overlap of the emission spectrum of the donor with the absorption spectrum of the acceptor (spectral overlap integral), and other factors. Recently, several ratiometric fluorescent probes using FRET have been developed,[3] such as the β-lactamase probe CCF2[4]

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and the phosphodiesterase probe CPF4.[5] They are all based on the same principle, that is, that FRET efficiency is dependent upon the donor–acceptor distance. To design such probes, it is necessary that the donor and acceptor should be located on opposite sides of an enzyme-cleavable structure. It is difficult to apply this design method widely, because of the fluorescence quenching problem due to dye-to-dye close contact[6] and a decrease in affinity for the target enzymes.[5] To expand the range of application of FRET-based fluorescent probes, we have utilized a novel approach to design probes whose absorption characteristics are altered by enzymatic reaction, but which do not suffer from the above problems.

Results and Discussion

Detection mechanism: FRET efficiency is dependent on the spectral overlap integral between the donor emission and acceptor absorption. We thought that if the acceptor absorption could be dramatically changed by hydrolytic enzymes, FRET switching by spectral overlap integral would be possible. It was anticipated that fluorescein would be appropriate as such an acceptor, because it has two conformations (lactone form and quinoid form) with distinctly different absorption properties. Fluorescein in the quinoid form has strong absorption at around 490 nm, whereas the lactone form has absorption only in the UV region. If a coumarin derivative is chosen as the donor, there is a significant difference in the spectral overlap between the two forms of fluorescein (Figure 1). So, we propose a novel ratiometric detection method

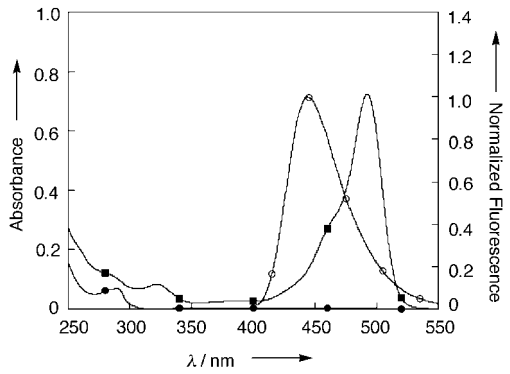
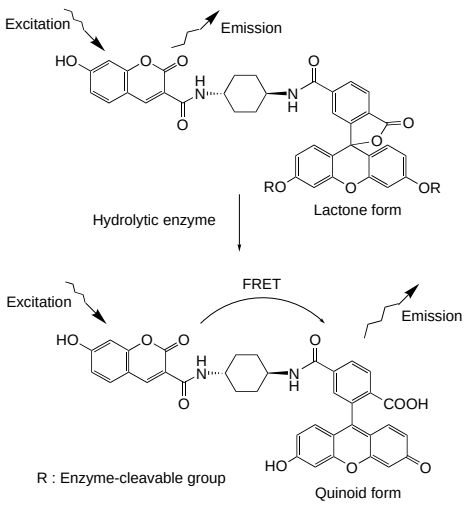


Figure 1. Spectral overlap of the coumarin emission with the fluorescein absorption. ○: normalized emission spectrum of 7-hydroxycoumarin-3-carboxylic acid, $\lambda_{ex} = 400$ nm; ●: absorption spectrum of 10 μM 6-carboxyfluorescein diacetate (lactone form); ■: absorption spectrum of 10 μM 6-carboxyfluorescein (quinoid form).

for hydrolytic enzyme activity based on resonance energy transfer switching by spectral overlap integral (Scheme 1). This method employs fluorescent probes with 3′-*O*,6′-*O*-protected fluorescein linked to coumarin through an appropriate linker. As fluorescein is locked in the lactone form, it has no absorption band in the wavelength region of the coumarin emission, so there will be no overlap integral between the coumarin emission and fluorescein absorption. Thus, the fluorescein moiety cannot accept the excitation



Scheme 1. The FRET-based detection mechanism.

energy of the donor moiety, and the donor fluorescence can be observed. After cleavage of the protective groups by hydrolytic enzymes, the fluorescein structure changes to the quinoid form, which has a strong absorption in the coumarin emission region. Then, acceptor fluorescence due to FRET will occur, and so the probes will exhibit a large red shift in their emission spectrum after the reaction with the target enzymes. To validate the proposed strategy, we designed and synthesized novel fluorescent compounds bearing phosphate groups in the fluorescein moiety.

Design and synthesis of the ratiometric fluorescent probes: As shown in Figure 2, three compounds which have a coumarin moiety as the donor and a phosphorylated fluorescein moiety as the acceptor were designed and synthesized as candidate probes for protein tyrosine phosphatase (PTP). PTPs are involved in many biologically important processes,[7] including cell differentiation, synaptic function, cytoskeletal function

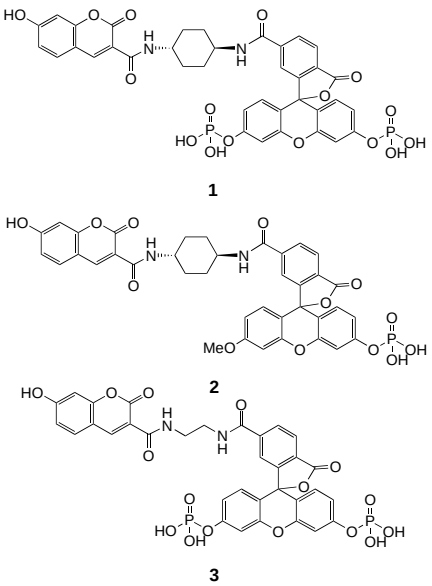


Figure 2. The structures of compounds **1**–**3**.

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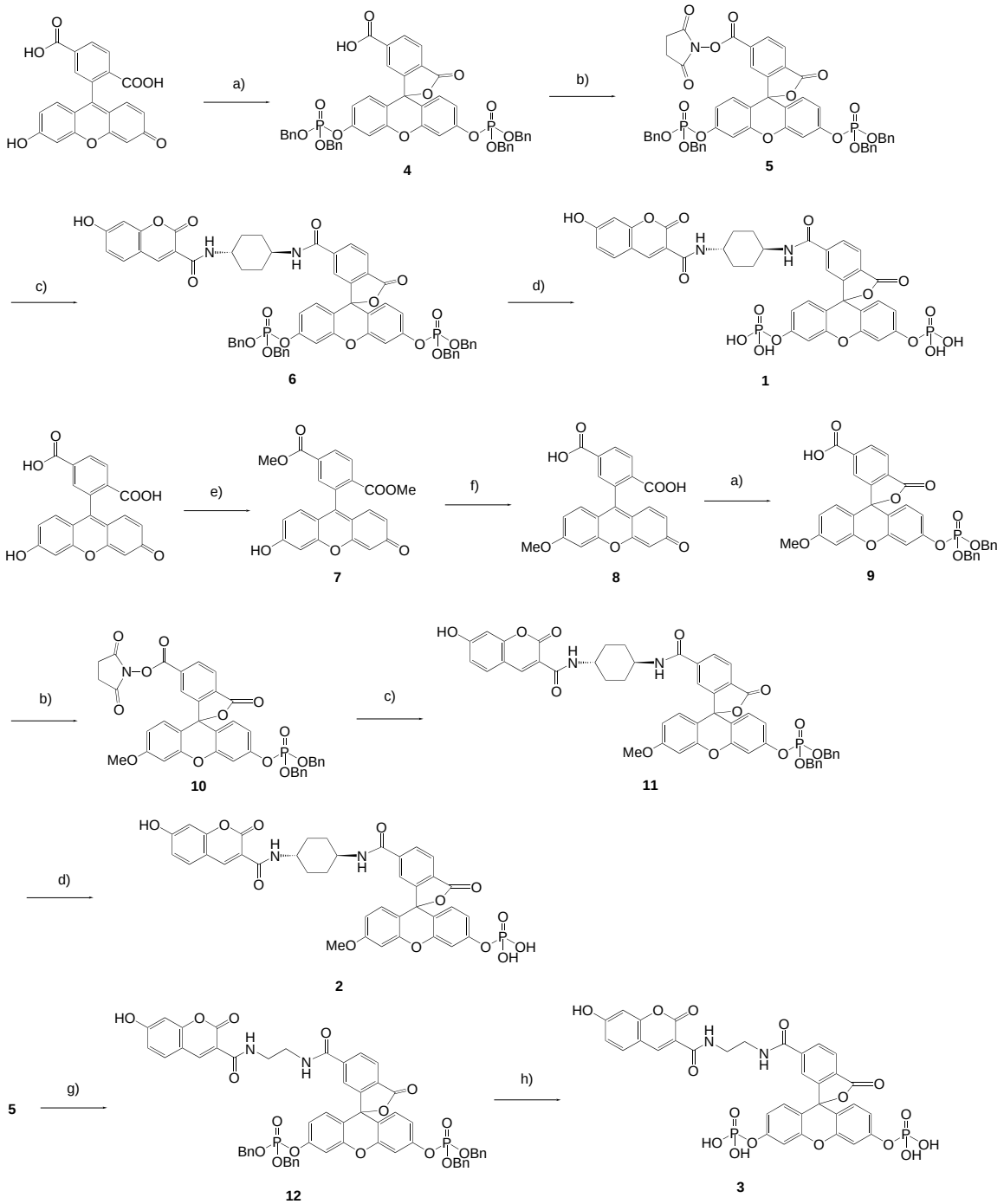
and immune responses, and also in human diseases[8] such as cancers and diabetes. However, there is little effective real-time method to monitor their activity in living cells. In compound **1**, two phosphate residues are introduced into the fluorescein moiety as enzyme-cleavable groups, and this moiety is linked to the coumarin donor through a cyclohexane moiety as a rigid linker. In compound **2**, the second phosphate group was replaced by a methyl residue in order to eliminate the complication of two dephosphorylation steps. To investigate the effect of the linker structure on the fluorescence properties and the affinity for PTPs, an ethylene moiety was introduced as a flexible linker in compound **3**. The synthetic schemes are shown in Scheme 2.

Spectroscopic properties of 1–3: The emission spectra of **1–3** in an aqueous buffer excited at the excitation wavelength of the coumarin donor exhibited the emission of the coumarin donor at around 450 nm before the enzymatic reaction. Addition of PTP1B, an intracellular PTP,[9] to aqueous solutions of **1–3** resulted in a decrease in the donor



Scheme 2. Synthesis of compounds **1–3**: a) $(BnO)_2PN(iPr)_2$, 1*H*-tetrazole, NEt_3, *m*CPBA, $CHCl_3$; b) *N*-hydroxysuccinimide, EDC·HCl, $CHCl_3$; c) 7-hydroxycoumarin-3-carboxylic succinimidyl ester, *trans*-1,4-cyclohexanediamine, DMF; d) Pd/C, H_2, MeOH; e) H_2SO_4, MeOH; f) CH_3I, Cs_2CO_3, DMF, aq NaOH; g) 7-hydroxycoumarin-3-carboxylic succinimidyl ester, ethylenediamine, DMF; h) Pd/C, H_2, MeOH/$CHCl_3$.

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fluorescence at around 450 nm and an increase in the acceptor fluorescence at around 515 nm, as shown in Figure 3. The same spectral changes were also observed upon the addition of CD45, a receptor-like PTP.[10] These observations demonstrate that the proposed FRET switching by spectral overlap integral worked as expected. As the time courses of the changes in the emission spectra show, the enhancement of the acceptor fluorescence was slower than the decrease of the donor fluorescence in the cases of **1** and **3**; on the other hand, they occurred simultaneously in the emission spectrum of **2**.

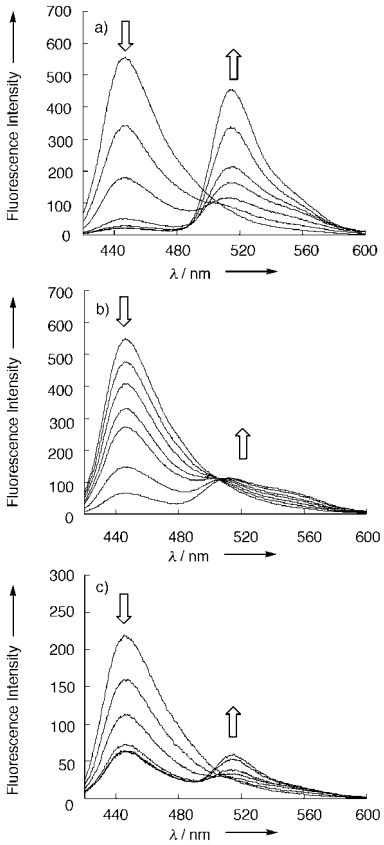

Figure 3. The emission spectra of compounds **1**–**3** (1.0 μM) after the addition of PTP1B (10 μg mL^{-1}) in 0.1M HEPES buffer (pH 7.4) containing 1.0 mM DTT and EDTA at 30 °C. a) Emission spectra of **1** measured at 1, 5, 10, 20, 30, 60, and 120 min after the addition, $\lambda_{ex} = 402$ nm. b) Emission spectra of **2** measured at 1, 5, 10, 20, 30, 60, and 180 min after the addition, $\lambda_{ex} = 402$ nm. c) Emission spectra of **3** measured at 1, 5, 10, 30, 60, 120, and 180 min after the addition, $\lambda_{ex} = 407$ nm.

The absorption spectra of **1**–**3** measured in an aqueous buffer exhibited the donor absorption peak at around 400 nm (Figure 4). After the addition of PTP1B or CD45, the absorption peaks of the fluorescein moiety appeared and increased with time. In the cases of **1** and **3**, the absorption at around 450 nm was initially enhanced, then the absorption at around 500 nm was enhanced with a slight decrease of the absorption at around 450 nm. These observations in the fluorescence and absorption spectra indicate that the two phosphate groups of **1** and **3** are hydrolyzed in two steps through the monophosphate intermediates, whereas **2** is hydrolyzed in one step. The fluorescence and absorption properties of compounds **1**–**3** before and after hydrolysis by the enzymes are summarized in Table 1. Compound **3** with the flexible ethylene linker exhibited weaker fluorescence and longer wavelength of the absorption maxima compared to **1** and **2** with the rigid

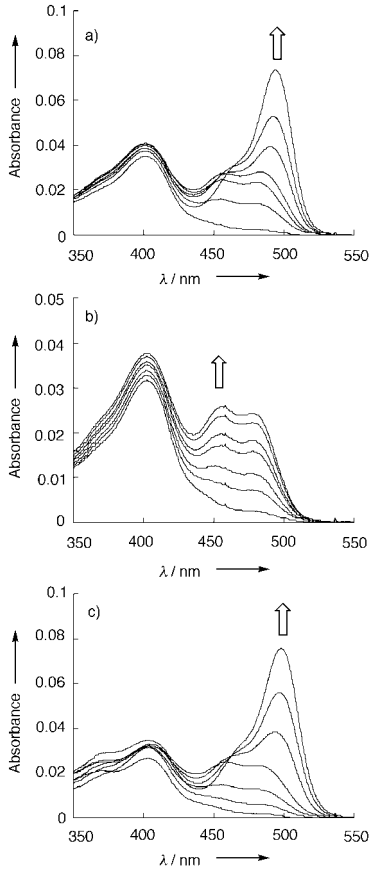

Figure 4. The absorption spectra of compounds **1**–**3** (1.0 μM) after the addition of PTP1B (10 μg mL^{-1}) in 0.1M HEPES buffer (pH 7.4) containing 1.0 mM DTT and EDTA at 30 °C. a) Absorption spectra of **1** measured at 1, 5, 10, 20, 45, 60, and 120 min after the addition. b) Absorption spectra of **2** measured at 1, 5, 10, 20, 30, 60, and 120 min after the addition. c) Absorption spectra of **3** measured at 1, 5, 10, 20, 120, 240, and 360 min after the addition.

Table 1. Chemical Properties of Compounds **1**–**3** before and after enzymatic hydrolysis.

Compound		Absorption max [nm][a]	Emission max [nm][a]	Quantum efficiency[a]	FRET efficiency [%][b]
1	before hydrolysis	402	445	0.53	0
	after hydrolysis	402	515	0.32	96
		494	515	0.76	
2	before hydrolysis	402	445	0.36	0
	after hydrolysis	402	515	0.11	96
		458	515	0.20	
3	before hydrolysis	404	445	0.11	0
	after hydrolysis	407	515	0.033	–
		498	520	0.088	

[a] Data were measured in HEPES buffer (0.1M, pH 7.4). [b] Data were obtained from the fluorescence quantum efficiency of the donor in the presence and in the absence of acceptor.

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cyclohexane linker. These observations indicate that fluorescence quenching due to dye-to-dye close contact[6] occurred in **3**. In other words, the cyclohexane linker is sufficiently rigid to prevent dye-to-dye close contact. After hydrolysis by PTPs, efficient FRET could be observed in **1** and **2**. Although compound **2** after enzymatic cleavage shows weaker fluorescence intensity in the acceptor emission than compound **1** due to the effect of the methyl group incorporated into fluorescein acceptor, the fluorescence quantum yield of the fluorescein moiety in compound **2** is equivalent to that of some widely used fluorescent indicators, and so, the fluorescence of **2** should be sufficient to be detected in living cells.

Kinetic studies: Kinetic parameters such as K_m and k_{cat} were determined by direct fitting of the initial velocity versus substrate concentration data to the Michaelis–Menten equation (Table 2). As a standard, K_m and k_{cat} of 4-nitrophenyl phosphate were also measured under the same conditions. Compounds **1** and **3** had significantly lower K_m values than compound **2** and 4-nitrophenyl phosphate, especially with CD45. The higher affinity of **1** and **3** would be due to the presence of the second phosphate group with its negative charges, that is, electrostatic interaction between the second phosphate group and PTPs is important for the enhanced binding.[11] A comparison of the K_m values of **1** and **3** shows that there is no significant difference in their affinities for PTP1B or CD45. This indicates that the rigid and bulky structure of the cyclohexane linker does not affect substrate binding.

Table 2. Kinetic parameters of compounds **1**–**3** with PTPs.[a]

Compound	Enzyme	K_m [μM]	k_{cat} [s^{-1}]	k_{cat}/K_m [$mM^{-1}s^{-1}$]
1	PTP1B	67	0.8	12
	CD45	12	3.7	300
2	PTP1B	87	1.6	18
	CD45	1000	4.1	4.1
3	PTP1B	27	0.33	12
	CD45	14	6.7	480
4-nitrophenyl phosphate	PTP1B	1100	13	12
	CD45	6500	63	10

[a] Data were measured at 30 °C in HEPES buffer (0.1M, pH 7.4), containing DTT (1.0 mM) and EDTA (1.0 mM).

Development of membrane-permeable derivative: Since compounds **1**–**3** have low membrane permeability due to their negative charges, we derivatized compound **1** to the membrane-permeable **1-AM**, which bears acetoxymethyl residues on the phosphate and hydroxyl groups (Scheme 3). Compound **1-AM** is more lipophilic, and should thus permeate better into the cells, where it should be hydrolyzed to compound **1** by esterase in the cytosol. In order to determine whether **1-AM** can detect intracellular PTPs activity, it was used for ratiometric imaging in human umbilical vein endothelial cells (HUVEC). The fluorescence ratio of the acceptor and donor within the cells was monitored, in the presence and absence of sodium orthovanadate, a membrane-permeable PTP inhibitor, as shown in Figure 5. We observed a significant difference in the increase of the fluorescence ratio between inhibitor-treated and untreated cells. Thus, **1-AM** should be practically useful for the ratiometric measurement of intracellular PTP activity.

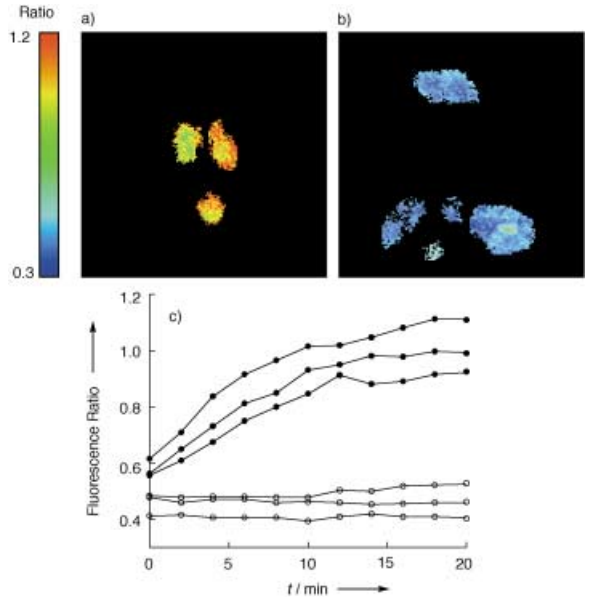
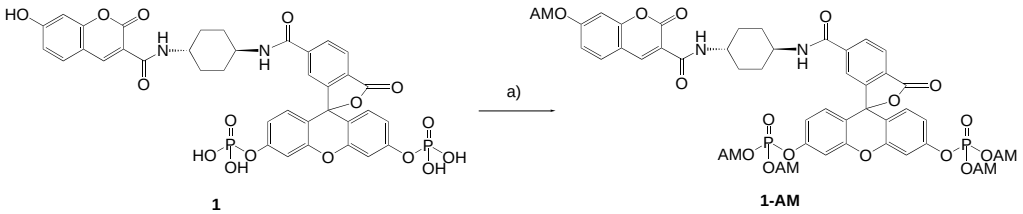


Figure 5. Ratiometric imaging of PTPs activity in HUVEC by using **1-AM**. The fluorescence ratio (acceptor/donor) is shown in pseudo color. a) Ratiometric image of the inhibitor-untreated cells 20 min after the probe labeling. b) Ratiometric image of cells in the presence of 1.0 mM sodium orthovanadate 20 min after the probe labeling. c) Time course of the changes in the fluorescence ratio. ●: the fluorescence ratio at randomly selected regions in HUVEC in the absence of sodium orthovanadate; ○: the fluorescence ratio at randomly selected regions in HUVEC in the presence of 1.0 mM sodium orthovanadate.

Conclusion

Our results indicate that FRET switching by spectral overlap integral is feasible for practical use. It should be possible to develop novel ratiometric fluorescent probes for various hydrolytic enzymes by introducing other appropriate enzyme-cleavable groups into the fluorescein acceptor. The fluorescence quenching problem that usually arises in developing



Scheme 3. Synthesis of compound **1-AM**: a) acetoxymethyl bromide, diisopropylethylamine, DMF; AM = CH_3COOCH_2.

FRET probes can be overcome by using the coumarin-cyclohexane–fluorescein FRET cassette moiety. In addition to fluorescein, it should be possible to use rhodamine as the acceptor, because rhodamine also exhibits a large shift in its absorption spectrum. Consequently, this study has provided important information about the design and synthesis of FRET-based fluorescent probes.

Furthermore, by using the above design approach we have succeeded in developing the PTP probes **1**–**3**. Based on their chemical and kinetic properties, we anticipate that compounds **1** and **2** bearing the rigid cyclohexane linker are practically useful for the ratiometric measurement of PTPs activity. Although compound **1** has the two dephosphorylation sites, it exhibits PTP activity-dependent increase in the ratio value of the acceptor and donor fluorescence with a large dynamic range. So, it should be useful for monitoring the PTP activity with high sensitivity, especially for comparing the PTP activity between the different regions in cells or tissues. Meanwhile, compound **2** does have the advantage of only one dephosphorylation step, and this could be especially important for quantitative detection. The range of biological applications could be extended by selecting a suitable probe according to the experimental aim. In summary, chemically synthesized FRET-based probes for ratiometric measurement of PTP activity have been developed for the first time by using spectral overlap integral switching, with a rigid linker moiety to prevent close contact of the two dyes. These probes should allow precise, quantitative detection of PTPs activity, and so should be useful for studies on the biological functions of PTPs and for studies aimed at developing effective therapeutics for PTP-related diseases.

Experimental Section

Materials and general methods: PTP1B and CD45 were purchased from BIOMOL Research Laboratories Inc. (Plymouth Meeting, PA). Fluorescence spectra were measured using an F4500 spectrometer (Hitachi, Tokyo, Japan). Slit width was 2.5 nm for both excitation and emission. The photomultiplier voltage was 950 V. Absorption spectra were measured using a UV-1600 spectrometer (Hitachi, Japan). Compounds were dissolved in DMSO to make stock solutions, which were diluted to the required concentration for measurement. ^{1}H NMR and ^{13}C NMR spectra were recorded on a JEOL JNM-LA300 instrument; δ values are given relative to tetramethylsilane. Fast atom bombardment mass spectroscopy (FAB-MS) was conducted with a JEOL SX-102A mass spectrometer. High-resolution mass spectroscopy (HRMS) was done with a JEOL JMS-700T mass spectrometer. Silica gel column chromatography was performed using BW-300 (Fuji Silysia Chemical Ltd.).

Preparation of 4: Dibenzyl diisopropylphosphoramidite (1.8 g, 5.2 mmol) was added to a solution of 6-carboxyfluorescein (500 mg, 1.3 mmol), triethylamine (TEA) (1.3 g, 13 mmol) and 1*H*-tetrazole (930 mg, 13 mmol) in chloroform (20 mL). The reaction mixture was stirred for 30 min at room temperature and then cooled to 0 °C. The phosphite intermediate was oxidized by adding *m*CPBA (2.0 g, 12 mmol) in portions and the mixture was stirred for 30 min at room temperature. The mixture was diluted with chloroform, then washed with 1.5 M Na_2SO_3, 2 N HCl and brine, and dried over sodium sulfate. After evaporation of the chloroform, the residue was purified by chromatography on silica gel to afford **4** (960 mg, 81 %). ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 5.14$ (d, $J = 9.2$ Hz, 8H), 6.66 (d, $J = 8.8$ Hz, 2H), 6.81 (dd, $J = 2.5$, 8.8 Hz, 2H), 7.02 (d, $J = 2.5$ Hz, 2H), 7.31 (m, 20H), 7.79 (d, $J = 1.3$ Hz, 1H), 8.09 (d, $J = 8.1$ Hz, 1H), 8.34 (dd, $J = 1.3$, 8.1 Hz, 1H); MS (FAB): m/z: 897 $[M^+ + H]$.

Preparation of 5: Compound **4** (600 mg, 0.67 mmol) was added to a solution of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC·HCl) (190 mg, 0.99 mmol) and *N*-hydroxysuccinimide (120 mg, 1.0 mmol) in chloroform. The reaction mixture was stirred for 2 h at room temperature, washed with an aqueous solution of citric acid and brine, and dried over sodium sulfate. After evaporation of the chloroform, the residue was purified by chromatography on silica gel to afford **5** (650 mg, 98 %). ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 2.89$ (s, 4H), 5.14 (d, $J = 9.0$ Hz, 8H), 6.66 (d, $J = 8.8$ Hz, 2H), 6.82 (dd, $J = 2.4$, 8.8 Hz, 2H), 7.08 (d, $J = 2.4$ Hz, 2H), 7.32 (m, 20H), 7.86 (d, $J = 1.3$ Hz, 1H), 8.18 (d, $J = 8.1$ Hz, 1H), 8.40 (dd, $J = 1.3$, 8.1 Hz, 1H); MS (FAB): m/z: 994 $[M^+ + H]$.

Preparation of 6: *trans*-1,4-Cyclohexanediamine (82 mg, 0.73 mmol) was added to a solution of **5** (600 mg, 0.60 mmol) and the *N*-hydroxysuccinimidyl ester of 7-hydroxycoumarin-3-carboxylic acid (190 mg, 0.63 mmol) in DMF, and the mixture was stirred for 1 h at 0 °C. Then it was extracted with ethyl acetate, and the extract was washed with an aqueous solution of citric acid and brine, and dried over sodium sulfate. After evaporation of the ethyl acetate, the residue was purified by chromatography on silica gel to afford **6** (240 mg, 34 %). ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 1.45$ (m, 4H), 2.12 (m, 4H), 3.90 (m, 2H), 5.15 (d, $J = 9.0$ Hz, 8H), 6.22 (d, $J = 7.7$ Hz, 1H), 6.68 (d, $J = 8.8$ Hz, 2H), 6.80 (dd, $J = 2.2$, 8.8 Hz, 2H), 6.85 (dd, $J = 2.4$, 8.2 Hz, 1H), 6.87 (d, $J = 2.4$ Hz, 1H), 7.02 (d, $J = 2.2$ Hz, 2H), 7.33 (m, 20H), 7.44 (s, 1H), 7.47 (d, $J = 8.2$ Hz, 1H), 8.08 (s, 2H), 8.65 (d, $J = 7.9$ Hz, 1H), 8.75 (s, 1H); MS (FAB): m/z: 1181 $[M^+ + H]$.

Preparation of 1: 10 % Pd/C (10 mg) was added to a solution of **6** (35 mg, 30 μmol) in methanol, and the reaction mixture was stirred for 30 min under a slight positive pressure of H_2 gas using a balloon. The progress of the reduction was followed by reverse-phase HPLC. When no more starting material was evident and only the product peak was observed, the reaction mixture was filtered and the filtrate was evaporated to give **1** (20 mg, 82 %). ^{1}H NMR (300 MHz, CD_3OD): $\delta = 1.48$ (m, 4H), 2.00 (m, 4H), 3.75 (m, 2H), 6.74 (d, $J = 2.0$ Hz, 1H), 6.86 (d, $J = 8.8$ Hz, 2H), 6.87 (dd, $J = 2.0$, 8.6 Hz, 1H), 7.00 (dd, $J = 2.4$, 8.8 Hz, 2H), 7.25 (d, $J = 2.4$ Hz, 2H), 7.63 (d, $J = 8.6$ Hz, 1H), 7.63 (d, $J = 1.3$ Hz, 1H), 8.10 (d, $J = 8.1$ Hz, 1H), 8.16 (dd, $J = 1.3$, 8.1 Hz, 1H), 8.71 (s, 1H); ^{13}C NMR (125 MHz, CD_3OD): $\delta = 32.0$, 32.4, 49.8, 49.9, 83.5, 103.1, 109.7, 112.8, 114.4, 115.7, 115.9, 118.1, 123.9, 126.4, 129.5, 130.5, 130.9, 132.9, 142.7, 149.6, 153.0, 154.6, 154.7, 158.2, 163.2, 163.7, 165.7, 167.3, 170.3; HRMS (FAB +): m/z: calcd for $[M^+ + H]$: 821.1149; found: 821.1166.

Preparation of 7: A solution of 6-carboxyfluorescein (600 mg, 1.6 mmol) and H_2SO_4 (1.0 mL) in methanol (30 mL) was heated under reflux overnight. The reaction mixture was concentrated and diluted with dichloromethane, then washed with sodium phosphate buffer (pH 7.0) and brine, and dried over sodium sulfate. After evaporation of the dichloromethane, compound **7** (330 mg, 52 %) was obtained. ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 3.64$ (s, 3H), 3.94 (s, 3H), 6.79 (dd, $J = 2.0$, 9.2 Hz, 2H), 6.90 (d, $J = 2.0$ Hz, 1H), 6.91 (d, $J = 9.2$ Hz, 2H), 7.98 (s, 1H), 8.31 (s, 2H); MS (FAB): m/z: 405 $[M^+ + H]$.

Preparation of 8: MeI (100 mg, 0.70 mmol) was added to a solution of **7** (130 mg, 0.32 mmol) and cesium carbonate (130 mg, 0.4 mmol) in DMF (5.0 mL). The reaction mixture was stirred for 20 min at room temperature and then diluted with ethyl acetate. The mixture was washed with an aqueous solution of citric acid and brine, and dried over sodium sulfate. The organic phase was concentrated and diluted in methanol. To the methanol solution, 2 N NaOH (5.0 mL) was added and the mixture was stirred for 1 h at room temperature. The methanol was evaporated and the aqueous residue was acidified with 2 N HCl. The resulting precipitate was collected by filtration and dried to afford **8** (110 mg, 90 %). ^{1}H NMR (300 MHz, CD_3OD): $\delta = 3.95$ (s, 3H), 6.78–7.14 (m, 6H), 7.84 (s, 1H), 8.22 (d, $J = 8.4$ Hz, 1H), 8.37 (d, $J = 8.4$ Hz, 1H); MS (FAB): m/z: 391 $[M^+ + H]$.

Preparation of 9: Dibenzyl diisopropylphosphoramidite (510 mg, 1.5 mmol) was added to a solution of **8** (290 mg, 0.74 mmol), TEA (730 mg, 7.2 mmol) and 1*H*-tetrazole (530 mg, 7.6 mmol) in chloroform (10 mL). The reaction mixture was stirred for 30 min at room temperature and then cooled to 0 °C. The phosphite intermediate was oxidized by adding *m*CPBA (1.5 g, 8.7 mmol) in portions and the mixture was stirred for 30 min at room temperature. Then it was diluted with chloroform, washed with 1.5 M Na_2SO_3, 2 N HCl and brine, and dried over sodium sulfate. After evaporation of the chloroform, the residue was purified by chromatography on silica gel to afford **9** (400 mg, 83 %). ^{1}H NMR

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(300 MHz, $CDCl_3$): $\delta = 3.81$ (s, 3H), 5.14 (d, $J = 9.0$ Hz, 4H), 6.61–7.06 (m, 6H), 7.29 (m, 5H), 7.83 (d, $J = 1.1$ Hz, 1H), 8.08 (d, $J = 7.9$ Hz, 1H), 8.31 (dd, $J = 1.1$, 7.9 Hz, 1H); MS (FAB): m/z: 651 [M^++H].

Preparation of 10: Compound **9** (300 mg, 0.46 mmol) was added to a solution of EDC·HCl (130 mg, 0.68 mmol) and *N*-hydroxysuccinimide (80 mg, 0.70 mmol) in chloroform and the mixture was stirred for 2 h at room temperature. Subsequently the reaction mixture was washed with an aqueous solution of citric acid and brine, and dried over sodium sulfate. After evaporation of the chloroform, the residue was purified by chromatography on silica gel to afford **10** (330 mg, 96%). ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 2.82$ (s, 4H), 3.81 (s, 3H), 5.14 (d, $J = 9.2$ Hz, 4H), 6.66–7.12 (m, 6H), 7.31 (m, 10H), 7.91 (d, $J = 1.3$ Hz, 1H), 8.16 (d, $J = 8.0$ Hz, 1H), 8.36 (dd, $J = 1.3$, 8.0 Hz, 1H); MS (FAB): m/z: 748 [M^++H].

Preparation of 11: *trans*-1,4-Cyclohexanediamine (64 mg, 0.56 mmol) was added to a solution of **10** (350 mg, 0.47 mmol) and the *N*-hydroxysuccinimidyl ester of 7-hydroxycoumarin-3-carboxylic acid (140 mg, 0.46 mmol) in DMF, and the mixture was stirred for 1 h at room temperature. The reaction mixture was then extracted with ethyl acetate and the extract was washed with an aqueous solution of citric acid and brine, and dried over sodium sulfate. After evaporation of the ethyl acetate, the residue was purified by chromatography on silica gel to afford **11** (100 mg, 24%). ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 1.42$ (m, 4H), 2.04 (m, 4H), 3.81 (s, 3H), 3.94 (m, 2H), 5.14 (dd, $J = 5.1$, 9.0 Hz, 4H), 6.57–7.03 (m, 6H), 6.68 (d, $J = 2.2$ Hz, 1H), 6.73 (dd, $J = 2.2$, 8.4 Hz, 1H), 7.33 (m, 10H), 7.47 (d, $J = 8.4$ Hz, 1H), 7.60 (s, 1H), 8.03 (d, $J = 8.0$ Hz, 1H), 8.12 (d, $J = 8.0$ Hz, 1H), 8.71 (s, 1H); MS (FAB): m/z: 935 [M^++H].

Preparation of 2: 10% Pd/C (10 mg) was added to a solution of **11** (20 mg, 21 μmol) in methanol, and the reaction mixture was stirred for 30 min under a slight positive pressure of H_2 gas using a balloon. The progress of the reduction was followed by reverse-phase HPLC. When no more starting material was evident and only the product peak was observed, the reaction mixture was filtered and the filtrate was evaporated to give **2** (12 mg, 75%). ^{1}H NMR (300 MHz, CD_3OD): $\delta = 1.44$ (m, 4H), 2.00 (m, 4H), 3.77 (s, 3H), 3.78 (m, 2H), 6.56 (dd, $J = 2.6$, 8.6 Hz, 1H), 6.69 (d, $J = 2.6$ Hz, 1H), 6.75 (d, $J = 2.4$ Hz, 1H), 6.82 (d, $J = 8.4$ Hz, 1H), 6.86 (dd, $J = 2.4$, 8.6 Hz, 1H), 6.90 (d, $J = 8.6$ Hz, 1H), 7.00 (s, 1H), 7.03 (d, $J = 8.6$ Hz, 1H), 7.49 (d, $J = 1.5$ Hz, 1H), 7.62 (dd, $J = 1.5$, 8.2 Hz, 1H), 7.65 (d, $J = 8.4$ Hz, 1H), 7.90 (d, $J = 8.2$ Hz, 1H), 8.73 (s, 1H); ^{13}C NMR (125 MHz, CD_3OD): $\delta = 31.9$, 32.4, 49.3, 49.9, 56.2, 79.5, 102.0, 103.1, 103.5, 109.7, 111.6, 112.8, 113.3, 114.4, 115.7, 116.3, 117.8, 123.9, 126.2, 129.8, 130.2, 130.5, 130.8, 132.9, 142.7, 149.6, 153.3, 153.6, 154.5, 158.2, 163.2, 163.4, 163.7, 165.7, 167.4, 170.2; HRMS (FAB+): m/z: calcd for [M^++H]: 755.1642; found: 755.1638.

Preparation of 12: Ethylenediamine (60 mg, 1.0 mmol) was added to a solution of **5** (800 mg, 0.81 mmol) and the *N*-hydroxysuccinimidyl ester of 7-hydroxycoumarin-3-carboxylic acid (240 mg, 0.79 mmol) in DMF, and the mixture was stirred for 30 min at 0 °C. The reaction mixture was extracted with ethyl acetate and the extract was washed with an aqueous solution of citric acid and brine, and dried over sodium sulfate. After evaporation of the ethyl acetate, the residue was purified by chromatography on silica gel to afford **12** (180 mg, 20%). ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 3.61$ (m, 4H), 5.13 (d, $J = 9.0$ Hz, 8H), 6.58 (d, $J = 8.8$ Hz, 2H), 6.65 (dd, $J = 2.2$, 7.3 Hz, 1H), 6.67 (d, $J = 2.2$ Hz, 1H), 6.72 (dd, $J = 1.3$, 8.8 Hz, 2H), 6.94 (d, $J = 1.3$ Hz, 2H), 7.27–7.36 (m, 21H), 7.73 (s, 1H), 8.15 (s, 2H), 8.41 (m, 1H), 8.63 (s, 1H), 9.12 (m, 1H); MS (FAB): m/z: 1127 [M^++H].

Preparation of 3: 10% Pd/C (15 mg) was added to a solution of **12** (35 mg, 31 μmol) in methanol/chloroform 1:1 (5 mL), and the reaction mixture was stirred for 10 min under a slight positive pressure of H_2 gas using a balloon. The progress of the reduction was followed by reverse-phase HPLC. When no more starting material was evident and only the product peak was observed, the reaction mixture was filtered and the filtrate was evaporated to give **3** (21 mg, 90%). ^{1}H NMR (300 MHz, CD_3OD): $\delta = 3.50–3.65$ (m, 4H), 6.55 (dd, $J = 2.4$, 8.8 Hz, 1H), 6.63 (d, $J = 8.8$ Hz, 1H), 6.71 (d, $J = 2.4$ Hz, 1H), 6.75 (d, $J = 2.4$ Hz, 1H), 6.79 (d, $J = 8.8$ Hz, 1H), 6.87 (dd, $J = 2.4$, 8.8 Hz, 1H), 6.93 (dd, $J = 2.4$, 8.7 Hz, 1H), 7.20 (d, $J = 2.4$ Hz, 1H), 7.62 (d, $J = 1.3$ Hz, 1H), 7.63 (d, $J = 8.7$ Hz, 1H), 8.07 (d, $J = 8.6$ Hz, 1H), 8.12 (dd, $J = 1.3$, 8.6 Hz, 1H), 8.62 (s, 1H); ^{13}C NMR (75 MHz, CD_3OD): $\delta = 40.0$, 40.9, 83.6, 103.1, 103.5; 109.6, 112.7, 114.4, 115.7, 116.0, 118.1, 124.0, 126.4, 129.6, 130.4, 133.0, 142.8, 149.5, 153.1, 154.5, 154.7, 158.2, 161.0, 162.9, 165.0, 165.7, 170.0; HRMS (FAB+): m/z: calcd for [M^++H]: 765.0523; found: 765.0507.

Preparation of 1-AM: Acetoxymethyl bromide (30 μL, 300 μmol) and diisopropylethylamine (100 μL, 600 μmol) were added to a solution of **1** (5.0 mg, 6.1 μmol) in DMF, and the reaction mixture was stirred for 2 h at room temperature under Ar. Subsequently the mixture was extracted with ethyl acetate, and the extract was washed with HEPES buffer (pH 7.4), and dried over sodium sulfate. After evaporation of ethyl acetate, the residue was purified by chromatography on silica gel to afford **1-AM** (3.2 mg, 45%). ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 1.44$ (m, 4H), 2.07 (m, 4H), 2.11 (s, 12H), 2.14 (s, 3H), 3.95 (m, 2H), 5.68–5.78 (m, 8H), 5.83 (s, 2H), 6.08 (d, $J = 8.3$ Hz, 1H), 6.81 (d, $J = 8.8$ Hz, 2H), 6.97 (dd, $J = 2.2$, 8.8 Hz, 2H), 7.04 (dd, $J = 2.3$, 8.1 Hz, 1H), 7.06 (d, $J = 2.3$ Hz, 1H), 7.23 (d, $J = 2.2$ Hz, 1H), 7.41 (s, 1H), 7.62 (d, $J = 8.1$ Hz, 1H), 8.05 (dd, $J = 1.5$, 7.5 Hz, 1H), 8.10 (d, $J = 7.5$ Hz, 1H), 8.65 (d, $J = 7.9$ Hz, 1H), 8.81 (s, 1H); MS (FAB): m/z: 1181 [M^++H].

Ratiometric imaging: HUVEC were cultured in EGM-2 medium (Sanko-Junyaku, Japan). The cells were incubated with PBS containing 5.0 μM **1-AM** for 10 min, in the presence or absence of 1.0 mM sodium orthovanadate (a PTP inhibitor), and the fluorescence ratio was measured every 2 min. The imaging system comprised an Olympus IX-70 inverted fluorescence microscope, a Hamamatsu Photonics ICCD camera C2400, a Hamamatsu Photonics Argus 50 image processor, and a Hamamatsu Photonics W-VIEW system A4313. The microscope was equipped with a xenon lamp, an objective lens X 40, a 400DF15 excitation filter (OMEGA), a 420DCLP dichroic mirror (OMEGA), and a 435ALP emission filter (OMEGA).

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