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# Rational Principles for Modulating Fluorescence Properties of Fluorescein

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***Abstract:*** Rational design strategies based on practical fluorescence modulation mechanisms would enable us to rapidly develop novel fluorescence probes for target molecules. Here, we present a practical and general principle for modulating the fluorescence properties of fluorescein. We hypothesized that (a) the fluorescein molecule can be divided into two moieties, i.e., the xanthene moiety as a fluorophore and the benzene moiety as a fluorescence-controlling moiety, even though there is no obvious linker structure between them, and (b) the fluorescence properties can be modulated via a photoinduced electron transfer (PeT) process from the excited fluorophore to a reducible benzene moiety (donor-excited PeT; d-PeT). To evaluate the relationship between the reduction potential of the benzene moiety and the fluorescence properties, we designed and synthesized various derivatives in which the reduction potential of the benzene moiety was fine tuned by introducing electron-withdrawing groups onto the benzene moiety. Our results clearly show that the fluorescence properties of fluorescein derivatives were indeed finely modulated depending upon the reduction potential of the benzene moiety. This information provides a basis for a practical strategy for rational design of novel functional fluorescence probes.

## Introduction

Fluorescence probes are excellent tools to analyze and clarify the roles of biomolecules in living cells.[1] Many fluorescence probes for detecting biomolecules have been developed,[2−7] though most of them were obtained not rationally but empirically. Novel rational approaches are required for efficient development of practical fluorescence probes. An important but imperfectly realized goal is to establish a general strategy to create a wide variety of practical fluorescence probes for certain biomolecules. Recent efforts in our laboratory have been focused on establishment of the rational designing principles for functional fluorescence probes.

Fluorescein is widely employed as a platform for various fluorescence probes and fluorescence labels because of its high fluorescence quantum efficiency ($\Phi_{fl}$) in aqueous media.[1,4−7] Recently, we found that the fluorescein molecule could be understood as a directly linked donor−acceptor system (Figure 1A). The two parts are conjugatively uncoupled since they are orthogonal to each other, and their fluorescence properties can be modulated by intramolecular photoinduced electron transfer (PeT) from the benzene moiety to the acceptor fluorophore (acceptor-excited PeT; a-PeT).[8,9] These findings enabled us to design flexibly many kinds of functional fluorescence probes based on the change of the oxidation potential of the benzene moiety upon encountering a target molecule, such as DMAX for singlet oxygen.[8] In this way, establishment of a rational design strategy made it possible to develop novel fluorescence probes for target molecules with high efficiency.[6−8,10]

Here we report a novel principle for controlling the fluorescence properties of the fluorescein molecule based on electron

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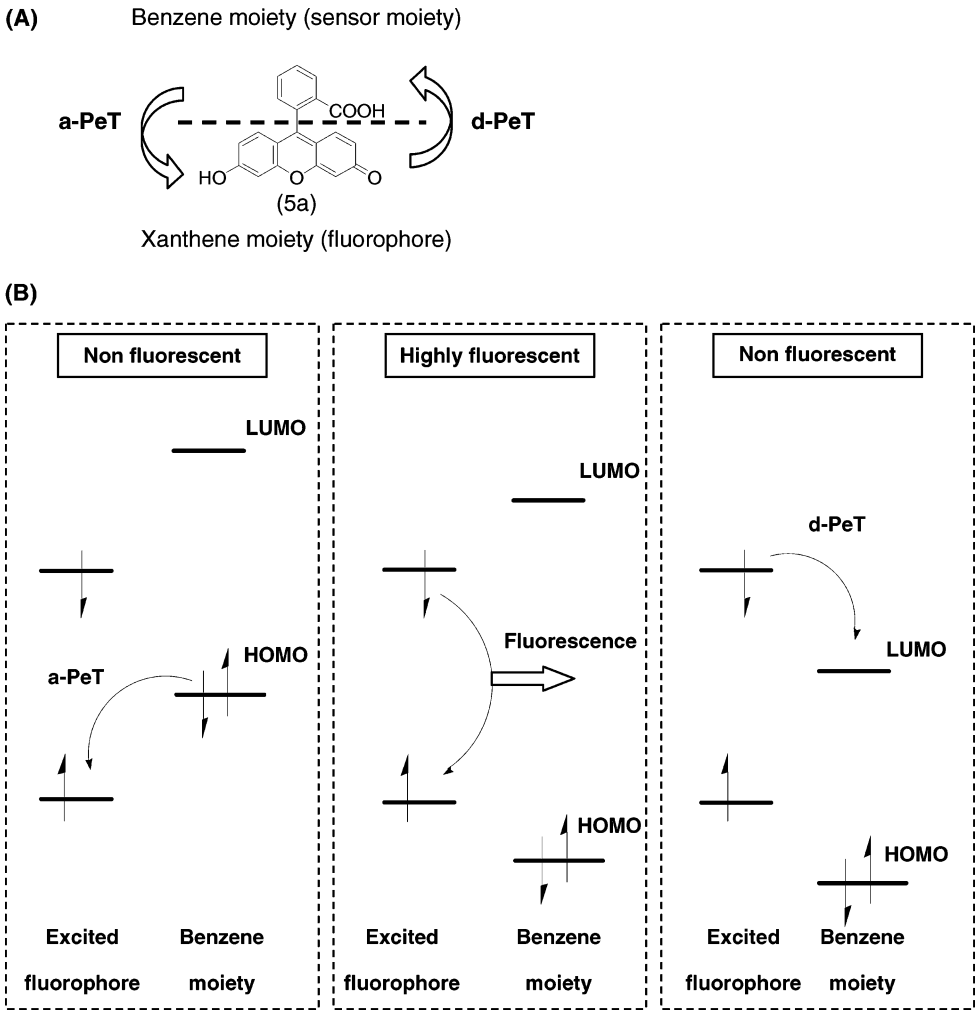


***Figure 1.*** (A) Fluorescein (**5a**) was divided into two parts, the benzene moiety and xanthene moiety. (B) Schematic molecular orbital diagram of the fluorescence off/on switch including the PeT process.

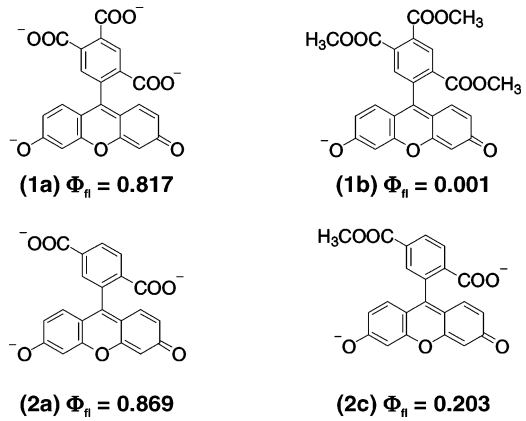


***Figure 2.*** Structures and fluorescence properties of carboxyfluorescein derivatives (**1a**, **1b**, **2a**, **2c**) measured in phosphate buffer pH 9.

transfer from the excited fluorophore to the benzene moiety (donor-excited PeT; d-PeT), i.e., the opposite direction to a-PeT (Figure 1A). These findings have allowed us to construct another widely applicable strategy for rational design of a novel class of functional fluorescence probes. The validity of this approach was confirmed by applying it to develop a novel fluorescence probe for reactive peroxometal species with an unique pattern of sensitivity.

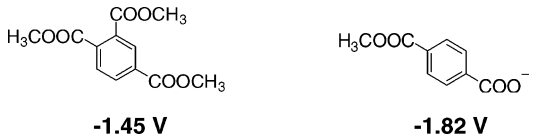


***Figure 3.*** Electrochemical properties of the benzene moiety of carboxyfluorescein derivatives (**1b**, **2c**) in aqueous media (V vs SCE).

## Results and Discussion

**Fluorescence Quenching by the Electron-Deficient Benzene Moiety.** We recently designed and synthesized dicarboxyfluorescein (**1a**) and its trimethyl ester derivative (**1b**) mainly in order to obtain probes with improbed water solubility. Owing to the presence of electron-withdrawing groups, the electron densities of the benzene moieties were lowered. According to our previous studies,[8,9] the oxidation potential of the benzene moiety is the most important factor determining the $\Phi_{fl}$ value of fluorescein. Therefore, we considered that **1a** and **1b** should be highly fluorescent (Figure 2). As anticipated, **1a** was highly fluorescent ($\Phi_{fl} = 0.817$), but unexpectedly, the fluorescence of **1b** was significantly quenched ($\Phi_{fl} = 0.001$). A similar phenomenon was also observed in the 6-carboxyfluorescein derivative (**2a**, **2c**). From the viewpoint of a-PeT, there are some apparent contradictions in these results.

To understand the mechanism underlying the fluorescence quenching further, we focused particularly on the reduction

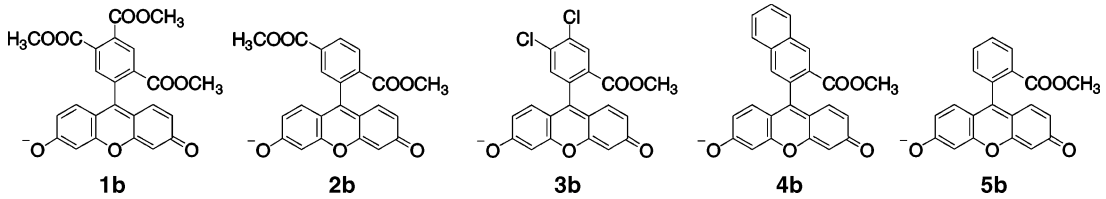


***Figure 4.*** Structures of benzene-moiety-substituted fluorescein derivative (**1b**−**5b**).

***Table 1.*** Photochemical Properties of Benzene-Moiety-Substituted Fluorescein Derivatives (**1b**−**5b**)

| | reduction potential of benzene moiety (V vs SCE)[a] | absorption maximum (nm)[b] | emission maximum (nm)[b] | $\Phi_{fl}$[b−d] | fluorescence lifetime[b] (ns) |
|---|---|---|---|---|---|
| **1b** | −1.65 | 501 | 525 | 0.001 | n.d.[e] |
| **2b** | −1.79 | 498 | 524 | 0.009 | 0.56 |
| **3b** | −1.84 | 499 | 527 | 0.213 | 2.55 |
| **4b** | −2.11 | 495 | 517 | 0.673 | 3.58 |
| **5b** | −2.36 | 494 | 518 | 0.761 | 3.93 |

[a] Measured in acetonitrile. [b] Measured in 0.1 M phosphate buffer (pH 9). [c] Excited at 490 nm. [d] Calculated by using fluorescein as a fluorescence standard ($\Phi_{fl} = 0.850$). [e] Below the detection limit.

potential of the benzene moiety. Our working hypothesis is that the fluorescence properties of fluorescein derivatives are influenced by not only the oxidation potential of the benzene moiety, but also the reduction potential of the benzene moiety. In principle, PeT is able to take place in both directions,[11−14] from the excited fluorophore to the lowest unoccupied molecular orbital (LUMO) of an electron acceptor (donor-excited PeT; d-PeT) as well as in the opposite direction, i.e., a-PeT (Figure 1B). If the reduction potential of the electron acceptor is high enough for electron transfer to occur thermodynamically, singlet excited energy will be lost as a consequence of electron transfer and the fluorophore will be in the off state. By taking advantage of the intramolecular donor−acceptor system, the fluorescein molecule might become useful as a platform not only for a-PeT probes, but also for d-PeT probes. To verify our hypothesis, we determined the reduction potential of the benzene moiety. The benzene moiety of **1b** has a 370 mV more positive reduction potential than that of **2c** in aqueous media (Figure 3). Moreover, the reduction potentials of the benzene moieties of **1a** and **2a**, highly fluorescent compounds, were not above −2.0 V. These results are consistent with our hypothesis.

**Relationship between the Reduction Potential of the Benzene Moiety and the $\Phi_{fl}$ Value.** Further, to appreciate fully the relationship between the reduction potential of the benzene moiety and the $\Phi_{fl}$ value, we designed and synthesized various fluorescein derivatives in which the benzene moieties are substituted with several electron-deficient aromatic rings. Their structures, the absorbance and fluorescence properties, the reduction potentials of their benzene moieties, and the relative quantum efficiencies of fluorescence ($\Phi_{fl}$) in basic aqueous media are summarized in Figure 4 and Table 1. The absorbance and emission maxima showed no significant change among these derivatives, and thus the ground-state interaction between the benzene moiety and the xanthene moiety was small in each derivative. On the other hand, the $\Phi_{fl}$ values varied greatly,

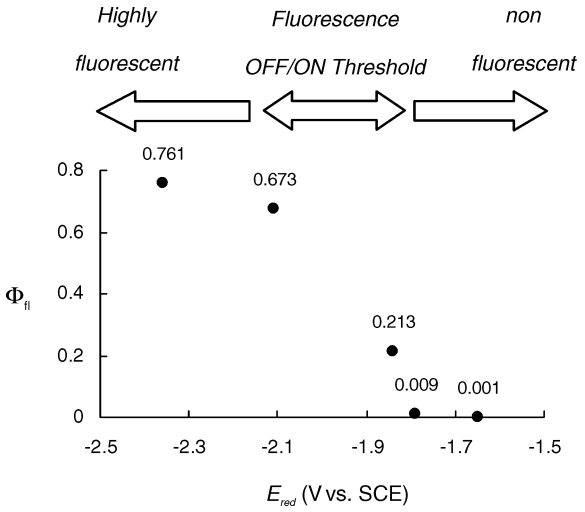


***Figure 5.*** Relationship between the fluorescence quantum efficiency ($\Phi_{fl}$) and the reduction potential ($E_{red}$).

depending on the reduction potential of the benzene moiety. In general, the feasibility of electron transfer between an excited-state sensitizer and quencher can be judged from the change in free energy ($\Delta G_{eT}$). The $\Delta G_{eT}$ value could be calculated from the Rehm−Weller equation[15]

$$\Delta G_{eT} = E_{ox} - E_{red} - \Delta E_{0,0} - w_p$$

where $E_{ox}$ and $E_{red}$ are the oxidation and reduction potentials of electron donor and acceptor, $\Delta E_{0,0}$ is the singlet excited energy, and $w_p$ is the work term for the charge separation state.[15] Owing to the similarity of their structures and the alteration in charge which accompanies electron transfer, fluorescein derivatives (**1b**−**5b**) have almost the same values of $E_{ox}$, $E_{0,0}$, and $w_p$. Therefore, in this study the $E_{red}$ value plays a primary role in deciding the feasibility of electron transfer. Indeed, the $\Phi_{fl}$ values of **1b**−**5b** were strongly modulated by changes in the $E_{red}$ value. In the case of derivatives in which the reduction potential of benzene moiety is more negative than −2.1 V (vs SCE), very high $\Phi_{fl}$ values were observed. For reduction potentials more positive than −2.1 V, the $\Phi_{fl}$ values dropped sharply, finally reaching $\Phi_{fl} \approx 0$ in the case of **1b**. From these results we determined that the threshold level between fluorescence off and on lies from around −1.80 to −2.15 V (Figure 5). Despite the widespread use of a-PeT as a fluorescence modulating principle of current PeT probes,[3−10,16−18] to our knowledge there are few functional fluorescence probes which

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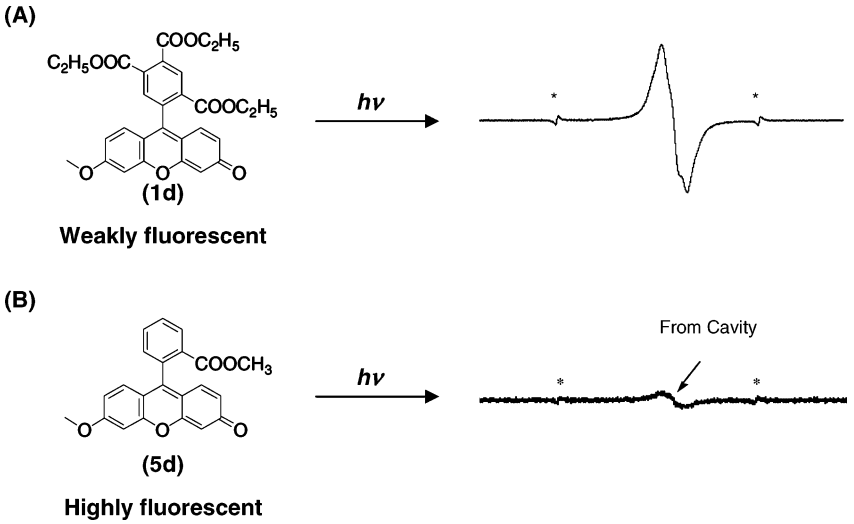


***Figure 6.*** (A) Structure and ESR spectrum measured at 143 K of **1d** ($1.0 \times 10^{-3}$ M) under irradiation with light from a high-pressure mercury lamp in frozen acetonitrile. (B) Structure and ESR spectrum measured at 143 K of **5d** ($1.0 \times 10^{-3}$ M) under irradiation with light from a high-pressure mercury lamp in frozen acetonitrile. Asterisk denotes $Mn^{2+}$ marker.

utilize the d-PeT concept. If such a d-PeT-based fluorescence modulation mechanism is applicable as a rational design principle for PeT probes, it would allow the development of novel and useful functional fluorescence probes for other kinds of biomolecules that could not be visualized with so-far developed a-PeT-based probes.

**Direct Evidence that Fluorescence Quenching Is via Electron Transfer.** To confirm the role of electron transfer, we tried to detect the production of radical species as a consequence of electron transfer in weakly fluorescent fluorescein derivatives. As previously reported by us, the formation of the radical ion pair state of a-PeT-based fluorescein derivatives under photoirradiation at 143 K could be observed with ESR. The ESR spectrum consisted of two characteristic signals of which one was attributable to the radical cation of the electron donor moiety and the other to the radical anion of the fluorophore.[9]

As expected, the weakly fluorescent fluorescein derivative **1d** exhibited strong ESR signals under photoirradiation at 143 K in acetonitrile (Figure 6A). Unfortunately, we could not assign this spectrum to two radical species due to signal broadening. However, the fact that no ESR signal could be observed under the same irradiation conditions with the strongly fluorescent fluorescein derivative **5d** led us to conclude that the d-PeT-based emission of weakly fluorescent fluorescein derivative was quenched via an electron transfer process.

**Rational Design of a Novel Fluorescent Probe To Detect Reactive Oxygen Species.** To confirm the potential of this principle, we then moved on to the development of a novel type of fluorescence probe, namely, MTM-CF, for reactive oxygen species.

MTM-CF contains a methylthiomethoxycarbonyl group, i.e., MTM ester, on the benzene moiety. MTM group is a protective group for the carboxyl group and can be easily removed with oxidizing agents.[19,20] A scheme showing reaction of MTM-CF with ROS is given in Figure 7. As expected, the fluorescence of MTM-CF was strongly extinguished but was

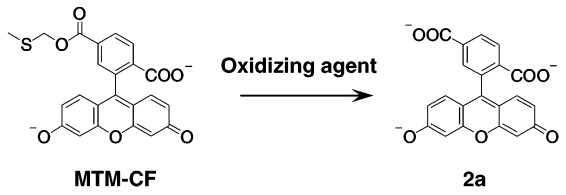


***Figure 7.*** Reaction of MTM-CF with ROS. The fluorescence of MTM-CF was extinguished before reaction, and then oxidation of the MTM group by an oxidizing agent triggers elimination of the carboxyl group, yielding a highly fluorescent compound (**2a**).

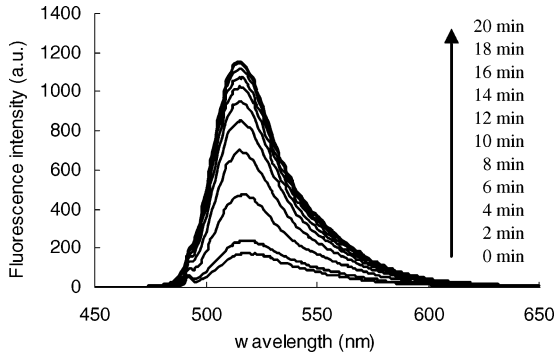


***Figure 8.*** Emission spectra (excited at 490 nm) of MTM-CF (1 $\mu$M, 0.1% DMF) in 0.1 M sodium phosphate buffer (pH 7.4) 0, 2, 4, 6, 8, 10, 12, 14, 16, 18, and 20 min after reaction with a mixture of $H_2O_2$ and molybdate, permolybdate.

dramatically enhanced by adding a mixture of $H_2O_2$ and $MoO_4^{2-}$, permolybdate (Figure 8). Combination of transition metal and $H_2O_2$ was reported to generate potent oxidizing agents, namely, peroxometal species.[21−27]

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***Table 2.*** Fluorescence Increase of MTM-CF after Reaction with Several ROS[a]

| reactant | MTM-CF | APF |
|---|---|---|
| permolybdate[b] | 302 | 94 |
| •OH[c] | <1 | 201 |
| $ClO^-$ [d] | <1 | 1000 |
| blank | 1 | <1 |

[a] MTM-CF or APF (final $1.0 \times 10^{-6}$ M, 0.1% DMF) was added to 0.1 M sodium phosphate buffer (pH 7.4). The fluorescence intensity was determined at 516 nm. [b] $Na_2MoO_4$ ($1.0 \times 10^{-2}$ M) and $H_2O_2$ ($5.0 \times 10^{-1}$ M) were added, and stirred for 3 min. [c] Ferrous perchlorate ($1.0 \times 10^{-4}$ M) and $H_2O_2$ ($1.0 \times 10^{-3}$ M) were added and stirred for 3 min. [d] $ClO^-$ ($1.0 \times 10^{-6}$ M) was added and stirred for 3 min.

The $\Phi_{fl}$ value of MTM-CF was lower than that of **2c**, although both derivatives have one carbonate ester group in the same position. This observation can be explained thermodynamically in terms of the d-PeT process. Compared with the methyl ester, MTM ester is more potently electron-withdrawing. As a consequence, the benzene moiety of MTM-CF should have a more positive reduction potential than that of **2c**, and thus, MTM-CF would be quenched more efficiently via the d-PeT process.

MTM-CF is potentially useful as a tool to study the role of peroxometal species in many biological and chemical applications. While APF, one of the a-PeT-based ROS probes,[7] reacts with several highly reactive oxygen species, including permolybdate, MTM-CF has an unique sensitivity pattern to ROS owing to the moderate reactivity of the MTM group (Table 2). The difference in reactivity between these probes can be mainly ascribed to the distinct properties of the sensor moiety. In the case of a-PeT-based fluorescence probes, a highly oxidizable moiety is essential for modulating fluorescence, so target molecule recognition is limited to species that can interact with the highly oxidizable sensor. By contrast, a highly oxidizable moiety is not needed for d-PeT-based fluorescence probes. Therefore, a d-PeT-based design strategy should allow us to develop novel types of probes with a characteristic reactivity pattern which is different from those of currently known PeT probes.

The basic principle for modulating the fluorescence of MTM-CF is the change of reduction potential which is caused by conversion of the carbonyl group. In short, the key feature for modulating fluorescence is not the MTM group but the carbonyl group. Therefore, we can easily obtain probes with altered sensitivity and selectivity by converting the MTM group into another protecting group. This principle is not limited to benzoate ester derivatives and could be extended to general electron acceptors. We believe that this design approach may serve as a core strategy to create a novel class of functional fluorescence probes, e.g., for esterases, peptidases, and oxidoreductases.

## Conclusion

In conclusion, we report herein a novel type of fluorescence modulation mechanism, d-PeT, i.e., fluorescence quenching based on electron transfer from the xanthene moiety to the benzene moiety of fluorescein derivatives. The value of this principle was confirmed by using it to develop a novel type of fluorescence probe, MTM-CF, for ROS. On the basis of this designing strategy, it should be possible to develop a novel class of PeT probes for a wide range of targets by introducing an appropriate sensor moiety. Now, we can choose either an oxidizable sensor moiety or a reducible moiety as the sensor moiety, and alteration of the redox potential is available as a fluorescence modulation switch. Thus, the fluorescence properties of fluorescein can be controlled by either a-PeT or d-PeT process, and this information provides the basis for a practical strategy for rational design of functional fluorescence probes to flexibly detect various biological events.

## Experimental Section

**Materials and General Instruments.** General chemicals were of the best grade available, supplied by Tokyo Chemical Industries, Wako Pure Chemical, or Aldrich Chemical Co., and used without further purification. Other chemicals used were dimethyl sulfoxide (DMSO, fluorometric grade, Dojindo), *N*,*N*-dimethylformamide (DMF, fluorometric grade, Dojindo), and tetrabutylammonium perchlorate (TBAP, electrochemical grade, Fluka). Acetonitrile, acetone, DMF, methanol, and ethanol were used after appropriate distillation or purification. NMR spectra were recorded on a JNM-LA300 (JEOL) instrument at 300 MHz for $^1$H NMR and at 75 MHz for $^{13}$C NMR. Mass spectra (MS) were measured with a JMS-DX300(JEOL) for EI and a JMS-T100LC AccuTOF(JEOL) for ESI. All experiments were carried out at 298 K unless otherwise specified.

**Synthesis and Characterization of Fluorescein Derivatives (1a−5a).** 9-[1-(2,5-Dicarboxy)phenyl]-6-hydroxy-3*H*-xanthen-3-one (2,5-diCOOHPh X, **2a**)[28] and 9-[2-(3-carboxy)naphthyl]-6-hydroxy-3*H*-xanthen-3-one (2-COOHNaph X, **4a**)[29] were prepared according to the literature. Fluorescein derivatives (**1a**, **3a**) were synthesized by means of the following procedure: to pyromellitic anhydride (**1a**) or 4,5-dichlorophthalic anhydride (**3a**) (1 equiv) was added to a solution of resorcinol (2 equiv) in methanesulfonic acid. The resulting mixture was heated under dry Ar at 85 °C for 20−36 h. The cooled mixture was poured onto ice, followed by filtration. The residue, containing the fluorescein derivative, was dried in vacuo to constant weight. The crude product was purified by silica gel chromatography.

**9-[1-(2,4,5-Tricarboxy)phenyl]-6-hydroxy-3*H*-xanthen-3-one (2,4,5-triCOOHPh X, dicarboxyfluorescein, 1a).** $^1$H NMR (300 MHz, $CD_3OD$): $\delta$ 6.56 (dd, 2H, $J = 2.2$, 8.6 Hz); 6.64 (d, 2H, $J = 8.6$ Hz); 6.70 (d, 2H, $J = 2.2$ Hz); 7.44 (s, 1H); 8.37 (s, 1H). $^{13}$C NMR (75 MHz, DMSO-$d_6$): $\delta$ 102.3, 108.5, 112.7, 123.7, 125.3, 127.5, 129.7, 133.7, 140.4, 151.8, 159.7, 167.0, 167.3, 167.8. HRMS (ESI$^+$): calcd for $M^+ + 1$, 421.0559; found, 421.0528.

**9-[1-(4,5-Dichloro 2-carboxy)phenyl]-6-hydroxy-3*H*-xanthen-3-one (4,5-diCl 2-COOHPh X, 3a).** $^1$H NMR (300 MHz, DMSO-$d_6$): $\delta$ 6.55 (dd, 2H, $J = 2.4$, 8.6 Hz); 6.66 (d, 2H, $J = 2.4$ Hz); 6.68 (d, 2H, $J = 8.6$ Hz); 7.74 (s, 1H); 8.26 (s, 1H); 10.17 (s, 2H). $^{13}$C NMR (75 Hz, DMSO-$d_6$): $\delta$ 83.3, 102.4, 108.6, 126.4, 126.6, 126.8, 129.3, 133.5, 138.7, 151.9, 159.9, 166.6. HRMS (ESI$^+$): calcd for $M^+ + 1$, 400.9983; found, 400.9961. Mp: 360 °C (dec). Anal. Calcd for $C_{20}H_{10}Cl_2O_5$: N, 0; C, 59.87; H, 2.51. Found: N, 0; C; 59.65; H, 2.70.

**Synthesis of Fluorescein Methyl Ester Derivatives (1b−5b).** Fluorescein was purchased from Tokyo Chemical Industries. 9-[1-(2,5-Dimethoxycarbonyl)phenyl]-6-hydroxy-3*H*-xanthen-3-one (2,5-diCOOMe-Ph X, 2b)[30] was prepared according to the literature. Fluorescein derivatives (**1b**, **3b**−**5b**) were synthesized by means of the following procedure: to a suspension of a fluorescein or fluorescein derivative (**1a**, **3a**−**5a**) in methanol was added a few drops of sulfuric acid. The reaction mixture was refluxed for 20 h; then it was poured onto ice and extracted with AcOEt. The organic layer was dried over anhydrous $Na_2SO_4$ and evaporated. The crude product was purified by silica gel

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chromatography. The product was recrystallized from methanol/$CH_2Cl_2$ to afford fluorescein methyl ester derivatives (**1b**, **3b**−**5b**).

**9-[1-(2,4,5-Trimethoxycarbonyl)phenyl]-6-hydroxy-3*H*-xanthen-3-one (2,4,5-triCOOMePh X, 1b).** $^1$H NMR (300 MHz, $CDCl_3$): $\delta$ 3.66 (s, 3H); 3.91 (s, 3H); 3.99 (s, 3H); 6.67 (br, 4H); 6.93 (d, 2H, $J$ = 9.3 Hz); 7.78 (s, 1H); 8.61 (s, 1H). $^{13}$C NMR (75 Hz, $CDCl_3$ + $CD_3OD$): $\delta$ 53.3, 53.6, 78.6, 104.4, 115.5, 130.8, 131.6, 132.5, 133.4, 133.7, 136.6, 138.3, 164.9, 167.2, 167.4. HRMS (ESI$^+$): calcd for [M + Na]$^+$, 458.0848; found, 458.0819. Mp: 270 °C (dec). Anal. Calcd for $C_{25}H_{18}O_9$·$CH_3OH$: N, 0; C, 63.16; H, 4.48. Found: N, 0; C, 64.94; H, 3.92.

**9-[1-(3,4-Dichloro-2-methoxycarbonyl)phenyl]-6-hydroxy-3*H*-xanthen-3-one (4.5-diCl 2-COOMePh X, 3b).** $^1$H NMR (300 MHz, DMSO-$d_6$): $\delta$ 3.59 (s, 3H); 6.52 (br, 4H); 6.90 (d, 2H, $J$ = 9.4 Hz); 7.91 (s, 1H); 8.33 (s,1H); 11.07 (br, 1H). $^{13}$C NMR (300 MHz, DMSO-$d_6$): $\delta$ 53.8, 104.6, 105.1, 108.1, 131.1, 131.5, 133.3, 134.3, 165.3. HRMS (ESI$^+$): calcd for M$^+$ + 1, 421.0559; found, 421.0528. Mp: 330 °C (dec). Anal. Calcd for $C_{21}H_{12}Cl_2O_5$·$0.25H_2O$: N, 0; C, 60.09; H, 3.00. Found: N, 0; C, 60.21; H, 3.21.

**9-[2-(3-Methoxycarbonyl)naphthyl]-6-hydroxy-3*H*-xanthen-3-one (2-COOMeNaph X, 4b).** $^1$H NMR (300 MHz, DMSO-$d_6$): $\delta$ 3.63 (s, 3H); 6.30 (br, 4H); 6.84 (m, 4H); 7.76 (m, 2H); 8.06 (m, 2H); 8.30 (m, 1H); 8.89 (s, 1H); 11.04 (br, 1H). HRMS (ESI$^+$): calcd for M$^+$ + 1, 397.107; found, 397.105. Mp: 325 °C (dec). Anal. Calcd for $C_{25}H_{18}O_6$·$0.75H_2O$: N, 0; C, 73.25; H, 4.30. Found: N, 0; C, 73.43; H, 4.37.

**9-[1-(2-Methoxycarbonyl)phenyl]-6-hydroxy-3*H*-xanthen-3-one-(2-COOMePh X, 5b).** $^1$H NMR (300 MHz, DMSO-$d_6$): $\delta$ 3.63 (s, 3H); 6.23−6.33 (br, 2H); 6.70−6.87 (m, 4H); 7.77 (m, 2H); 8.06 (m, 2H); 8.31 (m, 1H); 8.89 (s, 1H); 11.04 (br, 1H). $^{13}$C NMR (75 MHz, DMSO-$d_6$ + $CD_3OD$): $\delta$ 52.6, 103.0, 103.9, 115.6, 129.8, 130.7, 131.0, 131.3, 131.5, 133.6, 134.9, 153.7, 166.1. HRMS (ESI$^+$): calcd for M$^+$ + 1, 347.0919; found, 347.0889. Mp: 285 °C (dec). Anal. Calcd for $C_{21}H_{14}O_5$: N, 0; C, 72.83; H, 4.07. Found: N, 0; C, 72.58; H, 4.33.

**Synthesis of Compound 2c.** 6-Carboxyfluorescein diacetate (6-CF-DA)[31] was prepared according to the literature. 6-CF-DA (50 mg, 0.11 mmol) was dissolved in DMF (5 mL); iodomethane (19 mg, 0.13 mmol) and cesium carbonate (43 mg, 0.13 mmol) were added. The mixture was stirred at room temperature for 2 h, water was added, and reaction mixture was extracted with AcOEt (3 × 30 mL). The organic layer was dried over anhydrous $Na_2SO_4$ and concentrated in vacuo. The crude product was dissolved in methanol (10 mL). To this solution sodium methoxide (47 mg, 0.87 mmol) was added. The mixture was stirred at 0 °C for 1 h, then poured into 1 N HCl (aq), and extracted with AcOEt (3 × 50 mL). The organic layer was dried over anhydrous $Na_2SO_4$ and evaporated. The crude product was purified by silica gel chromatography to afford **2c** (7 mg, yield 16%).

**9-[1-(5-Methoxycarbonyl 2-carboxy)phenyl]-6-hydroxy-3*H*-xanthen-3-one (5-COOMe 2-COOHPh X, 2c).** $^1$H NMR (300 MHz, $CD_3OD$): $\delta$ 3.86 (s, 3H); 6.52 (dd, $J$ = 2.2, 8.6 Hz, 2H); 6.56 (d, $J$ = 8.6 Hz, 2H); 6.69 (d, $J$ = 2.2 Hz, 2H); 7.75 (d, $J$ = 1.3 Hz, 1H); 8.09 (d, $J$ = 7.9 Hz, 1H); 8.30 (dd, $J$ = 1.3, 7.9 Hz, 1H). $^{13}$C NMR (75 MHz, $CD_3OD$): 53.1, 103.6, 110.8, 113.8, 126.3, 130.2, 132.0, 137.8, 154.1, 166.9, 170.3. HRMS (ESI$^+$): calcd for M$^+$ + 1, 391.0817; found, 391.0795. Anal. Calcd for $C_{22}H_{14}O_7$·$H_2O$: N, 0; C, 64.71; O, 3.95. Found: N, 0; C, 64.81; H, 3.85.

**Synthesis and Characterization of 1d and 5d.** 9-[1-(2-Methoxycarbonyl)phenyl]-6-methoxy-3*H*-xanthen-3-one (**5d**) was prepared according to the literature.[9] 9-[1-(2,4,5-Triethoxycarbonyl)phenyl]-6-hydroxy-3*H*-xanthen-3-one (**1d**) was prepared via the following procedure: to a suspension of **1a** (500 mg, 1.19 mmol) in ethanol (500 mL) was added a few drops of sulfuric acid. The reaction mixture was refluxed for 20 h and then concentrated in vacuo. The residue was poured onto ice, and the whole mixture was extracted with AcOEt. The organic layer was dried over $Na_2SO_4$ and concentrated in vacuo to afford a crude product. The crude product (100 mg) was dissolved in DMF (5 mL); then iodomethane (42 mg, 0.30 mmol) and cesium carbonate (97 mg, 0.3 mmol) were added. The mixture was stirred at room temperature for 12 h, water (50 mL) was added, and the reaction mixture was extracted with AcOEt (3 × 60 mL). The combined organic layers were dried over anhydrous $Na_2SO_4$ and concentrated in vacuo. The crude product was purified by silica gel chromatography to afford **1d** in 28% yield in two steps.

**9-[1-(2,4,5-Triethoxycarbonyl)phenyl]-6-methoxy-3*H*-xanthen-3-one (1d).** $^1$H NMR (300 MHz, $CDCl_3$): $\delta$ 0.88 (t, 3H, $J$ = 3.7 Hz); 1.37 (t, 3H, $J$ = 3.7 Hz); 1.44 (t, 3H, $J$ = 3.7 Hz); 3.93 (s, 3H, b); 4.09 (m, 2H); 4.09 (q, 2H, $J$ = 3.7 Hz); 4.40 (q, 2H, $J$ = 3.7); 6.45 (d, 1H, $J$ = 0.9 Hz); 6.55 (dd, 1H, $J$ = 1.1, 4.5 Hz); 6.81 (d, 1H, $J$ = 4.5 Hz); 6.83 (d, 1H, $J$ = 4.5 Hz); 6.96 (d, 1H, $J$ = 1.1 Hz); 8.02 (s, 1H); 8.56 (s, 1H). $^{13}$C NMR (75 MHz, $CDCl_3$): $\delta$ 13.5, 13.9, 14.0, 56.0, 62.0, 62.3, 62.4, 100.4, 105.9, 113.5, 114.1, 117.6, 128.5, 129.7, 130.2, 130.8, 131.6, 133.0, 135.6, 136.8, 147.7, 154.1, 158.5, 163.8, 164.3, 165.7, 165.9, 185.5. HRMS (ESI$^+$): calcd for [M + Na]$^+$, 541.1474; found, 543.1436. $abs_{max}$ = 460, 483 nm. $em_{max}$ = 527 nm. $\Phi_{fl}$ = 0.148.

**Synthesis and Characterization of MTM-CF.** 6-CF-DA (150 mg, 0.32 mmol) was dissolved in DMF (5 mL). To this solution, chloromethyl methyl thioether (81 mg, 0.86 mmol) and cesium carbonate (309 mg, 0.65 mmol) were added. The mixture was stirred for 6 h at room temperature, water was added, and the whole mixture was extracted with 3 × 50 mL of AcOEt. The organic layer was dried and concentrated in vacuo. The crude product was purified by silica gel chromatography to afford MTM-CF DA (49 mg, yield 30%). MTM-CF DA (10 mg, 0.02 mmol) was dissolved in 0.1 M sodium phosphate buffer (50 mL) (2% DMF cosolvent). To this solution porcine liver esterase (10 mg) was added. The mixture was incubated for 25 min at 25 °C. After the reaction, reaction mixture was diluted with 1 M HCl (aq, 50 mL) and whole mixture was extracted with AcOEt (3 × 50 mL). The organic layer was dried over anhydrous $Na_2SO_4$ and concentrated in vacuo. The crude product was purified by silica gel chromatography to afford MTM-CF (1.3 mg, yield 13%).

**MTM-CF-DA.** $^1$H NMR (300 MHz, $CDCl_3$): $\delta$ 2.27 (s, 3H); 2,32 (s, 6H); 5.38 (s, 2H); 6.80 (d, 2H, $J$ = 4.3 Hz); 6.84 (dd, 2H, $J$ = 1.8, 4.3 Hz); 7.12 (d, 2H, $J$ = 1.8 Hz); 7.83 (d, 1H, $J$ = 1.3 Hz); 8.12 (d, 1H, $J$ = 8.1 Hz); 8.33 (dd, 1H, $J$ = 1.3, 8.1 Hz). $^{13}$C NMR (75 Hz, $CDCl_3$): $\delta$ 15.7, 21.0, 69.9, 81.9, 110.4, 115.5, 117.8, 125.2, 125.3, 128.7, 129.7, 131.4, 136.2, 151.4, 152.1, 152.8, 164.4, 167.8, 168.6. HRMS (ESI$^+$): calcd for [M + Na]$^+$, 543.07257; found, 543.07687.

**MTM-CF.** $^1$H NMR ($CD_3OD$): $\delta$ 2.29 (s, 3H); 5.38 (s, 2H); 6.53 (dd, 2H, $J$ = 2.3, 8.6 Hz); 6.60 (d, 2H, $J$ = 8.6 Hz); 6.70 (d, 2H, $J$ = 2.3 Hz); 7.76 (d, 1H, $J$ = 1.5 Hz); 8.11 (d, 1H, $J$ = 7.9 Hz); 8.33 (dd, 1H, $J$ = 1.5, 7.9 Hz). HRMS (ESI$^-$): calcd for [M − 1]$^-$, 435.05385; found, 435.05384.

**Preparation of Benzene Moieties of Fluorescein Derivatives.** All the benzene moieties of methylated fluorescein derivatives, i.e., substituted methyl benzoates, are commercially available except 3,4-dichlorobenzoic acid methyl ester.

**3,4-Dichlorobenzoic Acid Methyl Ester.** 3,4-Dichlorobenzoic acid (100 mg, 1.05 mmol) was dissolved in DMF (10 mL), and iodomethane (177 mg, 1.26 mmol) and cesium carbonate (408 mg, 1.26 mmol) were added. The mixture was stirred at room temperature for 12 h, water (50 mL) was added, and reaction mixture was extracted with AcOEt (3 × 60 mL). The organic layers were dried over anhydrous $Na_2SO_4$ and concentrated in vacuo. The crude product was purified by silica gel chromatography and recrystallized from methanol to afford 3,4-dichlorobenzoic acid methyl ester (145 mg, yield 68%). $^1$H NMR (300 MHz, $CDCl_3$): 3.93 (s, 3H); 7.52 (d, $J$ = 8.4 Hz, 1H); 7.86 (dd, $J$ = 2.0, 8.4 Hz, 1H); 8.12 (d, $J$ = 2.0 Hz, 1H). $^{13}$C NMR (75 MHz, $CDCl_3$): 52.4, 128.5, 129.8, 130.4, 131.4, 132.8, 137.4, 165.0. HRMS

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($EI^+$): calcd for $M^+$, 203.97448; found, 203.9767. Mp: 46.4−47.3 °C. Anal. Calcd for $C_8H_6Cl_2O_2$: N, 0; C, 46.86; H, 2.95. Found: N, 0; C, 46.66; H, 3.15.

**Fluorescence Properties and Quantum Efficiency of Fluorescence.** Steady-state fluorescence spectroscopic studies were performed on an F 4500 (Hitachi). UV−vis spectra were obtained on a UV-1650PC (Shimazu) with 0.1 M sodium phosphate buffer (pH 9) and 0.1 M NaOH. Each solution contained up to 0.2% (v/v) DMSO as a cosolvent. For determination of the quantum efficiency of fluorescence ($\Phi_{fl}$), fluorescein in 0.1 M NaOH ($\Phi_{fl}$) was used as a fluorescence standard. The quantum efficiencies of fluorescence were obtained with the following equation ($F$ denotes fluorescence intensity at each wavelength and $\Sigma[F]$ was calculated by summation of fluorescence intensity)

$$\Phi_{fl}^{sample} = \Phi_{fl}^{standard} Abs^{standard} \Sigma[F^{sample}]/Abs^{sample} \Sigma[F^{standard}]$$

Fluorescence decay of samples was recorded with a C4780 system (Hamamatsu Photonics). The solution of samples was prepared to be $5.0 \times 10^{-6}$ M in 0.1 M sodium phosphate buffer (pH 9) containing 0.1% DMSO as a cosolvent. It was excited with an $N_2$:Coumarin 102 pulse laser. The obtained data were appropriately deconvoluted and fitted to monoexponential decay curves to determine the fluorescence lifetime ($\tau$) of samples.

**Cyclic Voltammetry.** Cyclic voltammetry was performed on a 600A electrochemical analyzer (ALS).

**(a) In Aqueous Media.** A three-electrode arrangement in a single cell was used for the measurement: a Pt wire as the auxiliary electrode, a GC electrode as the working electrode, and an Ag/AgCl electrode as the reference electrode. The sample solution contained 0.1 M sodium phosphate pH 9 as a supporting electrolyte, and argon was bubbled for 2 min before each measurement.

**(b) In Acetonitrile.** A three-electrode arrangement in a single cell was used for the measurement: a Pt wire as the auxiliary electrode, GC electrode as the working electrode, and an $Ag/Ag^+$ electrode as the reference electrode. The sample solution contained 0.1 M tetrabutylammonium perchlorate as a supporting electrolyte in acetonitrile, and argon was bubbled for 2 min before each measurement.

**ESR Measurements.** The ESR measurements of the photoexcited compound were carried out with a JES−RE1XE X-band spectrometer (JEOL) equipped with a variable-temperature apparatus to detect the transient radical species in a solution of sample ($1.0 \times 10^{-3}$) M in frozen acetonitrile at 143 K under irradiation with light from a high-pressure mercury lamp (USH-1005D, Ushio). A quartz ESR tube containing a deaerated acetonitrile solution of sample was irradiated with the high-pressure Hg lamp through an aqueous filter.

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