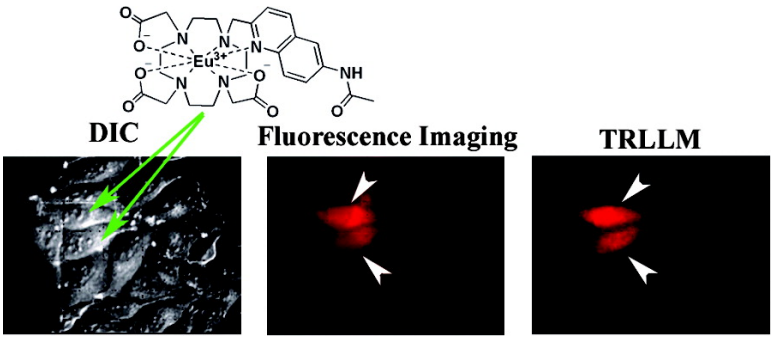
Article

Time-Resolved Long-Lived Luminescence Imaging Method Employing Luminescent Lanthanide Probes with a New Microscopy System

Kenjiro Hanaoka, Kazuya Kikuchi, Shigeru Kobayashi, and Tetsuo Nagano

J. Am. Chem. Soc., **2007**, 129 (44), 13502-13509• DOI: 10.1021/ja073392j • Publication Date (Web): 10 October 2007



More About This Article

Additional resources and features associated with this article are available within the HTML version:

- Supporting Information
- Links to the 9 articles that cite this article, as of the time of this article download
- Access to high resolution figures
- Links to articles and content related to this article
- Copyright permission to reproduce figures and/or text from this article

View the Full Text HTML

J|A|C|S
ARTICLES
Published on Web 10/10/2007

Time-Resolved Long-Lived Luminescence Imaging Method Employing Luminescent Lanthanide Probes with a New Microscopy System

Kenjiro Hanaoka,[†] Kazuya Kikuchi,[‡] Shigeru Kobayashi,[§] and Tetsuo Nagano*[,†]

Contribution from the Graduate School of Pharmaceutical Sciences, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Department of Materials and Life Sciences, Graduate School of Engineering, Osaka University, 2-1 Yamada-oka, Suita City, Osaka 565-0871, and Products Development Department 2, Micro-Imaging Systems Division, OLYMPUS CORPORATION, 2951 Ishikawa-cho, Hachioji-shi, Tokyo 192-8507, Japan

Received May 13, 2007; E-mail: tlong@mol.f.u-tokyo.ac.jp

Abstract: Superior fluorescence imaging methods are needed for detailed studies on biological phenomena, and one approach that permits precise analyses is time-resolved fluorescence measurement, which offers a high signal-to-noise ratio. Herein, we describe a new fluorescence imaging system to visualize biomolecules within living biological samples by means of time-resolved, long-lived luminescence microscopy (TRLLM). In TRLLM, short-lived background fluorescence and scattered light are gated out, allowing the long-lived luminescence to be selectively imaged. Usual time-resolved fluorescence microscopy provides fluorescence images with nanosecond resolution and has been used to image interactions between proteins, protein phosphorylation, the local pH, the refractive index, ion or oxygen concentrations, etc. Luminescent lanthanide complexes (especially europium and terbium trivalent ions (Eu^{3+} and Tb^{3+})), in contrast, have long luminescence lifetimes on the order of milliseconds. We have designed and synthesized new luminescent Eu^{3+} complexes for TRLLM and also developed a new TRLLM system using a conventional fluorescence microscope with an image intensifier unit for gated signal acquisition and a xenon flash lamp as the excitation source. When the newly developed luminescent Eu^{3+} complexes were applied to living cells, clear fluorescence images were acquired with the TRLLM system, and short-lived fluorescence was completely excluded. By using Eu^{3+} and Tb^{3+} luminescent complexes in combination, time-resolved dual-color imaging was also possible. Furthermore, we monitored changes of intracellular ionic zinc (Zn^{2+}) concentration by using a Zn^{2+}-selective luminescent Eu^{3+} chemosensor, [Eu-7]. This new imaging technique should facilitate investigations of biological functions with fluorescence microscopy, complementing other fluorescence imaging methodologies.

Introduction

Fluorescence imaging is a powerful tool for the visualization of biomolecules in various biological environments and is important for elucidating biological functions.[1,2] Further, the development of new fluorescent dyes with improved photophysical properties combined with technical progress in optical devices for fluorescence microscopy will open the way to superior fluorescence imaging technologies. Usual time-resolved fluorescence microscopy exploits differences in fluorescence lifetimes on the order of nanoseconds to monitor target fluorescence, and this technique has been used to image interactions between proteins or protein phosphorylation by detecting fluorescence resonance energy transfer (FRET) and to report on the local environment of fluorophores, for example, the local pH, the refractive index, ion or oxygen concentrations, etc.[3] In this paper, we present a new system for time-resolved long-lived luminescence microscopy (TRLLM), which utilizes the extremely long luminescence lifetimes of luminescent lanthanide complexes, on the order of milliseconds. This approach is expected to allow imaging with complete elimination of the short-lived background fluorescence,[4−13] providing high signal-to-noise ratios, by the introduction of an appropriate delay

[†] The University of Tokyo.
[‡] Osaka University.
[§] OLYMPUS CORPORATION.

(1) Nalbant, P.; Hodgson, L.; Kraynov, V.; Toutchkine, A.; Hahn, K. M. *Science* **2004**, *305*, 1615−1619.
(2) Wang, X.; Weisleder, N.; Collet, C.; Zhou, J.; Chu, Y.; Hirata, Y.; Zhao, X.; Pan, Z.; Brotto, M.; Cheng, H.; Ma, J. *Nat. Cell Biol.* **2005**, *7*, 525−530.
(3) Suhling, K.; French, P. M. W.; Phillips, D. *Photochem. Photobiol. Sci.* **2005**, *4*, 13−22.
(4) Weibel, N.; Charbonnière, L. J.; Guardigli, M.; Roda, A.; Ziessel, R. *J. Am. Chem. Soc.* **2004**, *126*, 4888−4896.
(5) Marriott, G.; Heidecker, M.; Diamandis, E. P.; Marriott, Y. Y. *Biophys. J.* **1994**, *67*, 957−965.
(6) Marriott, G.; Clegg, R. M.; Arndt-Jovin, D. J.; Jovin, T. M. *Biophys. J.* **1991**, *60*, 1374−1387.
(7) Beeby, A.; Botchway, S. W.; Clarkson, I. M.; Faulkner, S.; Parker, A. W.; Parker, D.; Williams, J. A. G. *J. Photochem. Photobiol., B* **2000**, *57*, 83−90.
(8) Phimphivong, S.; Saavedra, S. S. *Bioconjugate Chem.* **1998**, *9*, 350−357.
(9) de Haas, R. R.; van Gijlswijk, R. P. M.; van der Tol, E. B.; Veuskens, J.; van Gijssel, H. E.; Tijdens, R. B.; Bonnet, J.; Verwoerd, N. P.; Tanke, H. J. *J. Histochem. Cytochem.* **1999**, *47*, 183−196.
(10) Vereb, G.; Jares-Erijman, E.; Selvin, P. R.; Jovin, T. M. *Biophys. J.* **1998**, *74*, 2210−2222.

10.1021/ja073392j CCC: $37.00

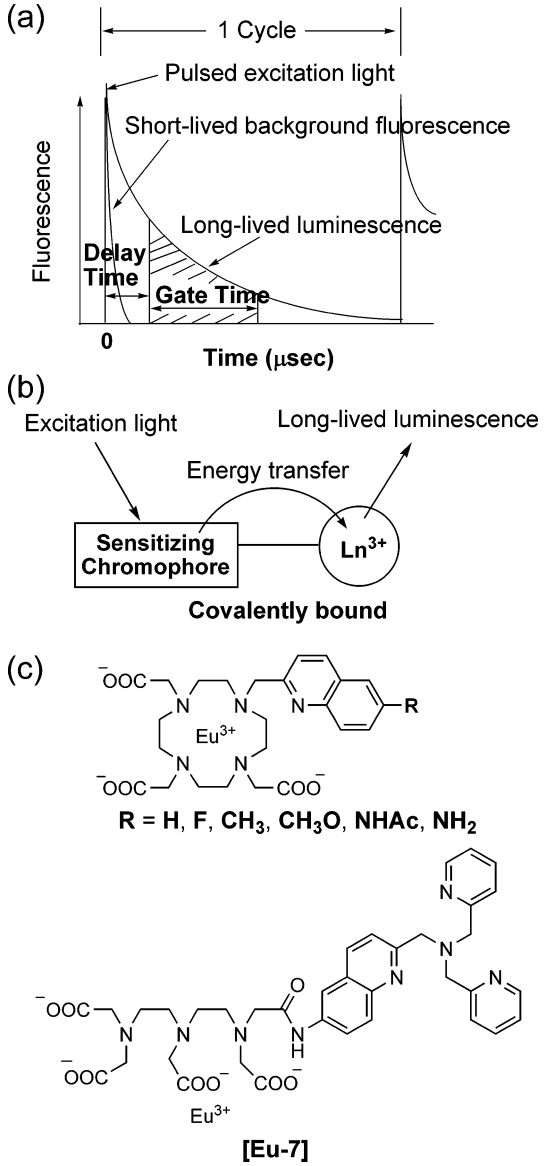


Figure 1. Design of luminescent lanthanide complexes. (a) Principle of time-resolved long-lived luminescence measurements. The short-lived background fluorescence decays to negligible levels during an appropriate *delay time* between a pulse of excitation light and the measurement of the long-lived luminescence. The period for which the fluorescence is measured is the *gate time*. The time-resolved long-lived luminescence measurement obviates the short-lived background fluorescence. (b) Schematic view of a sensitizing chromophore incorporated into a lanthanide emitter. Efficient intramolecular energy transfer should occur from the excited chromophore to the proximate lanthanide ion after excitation of the sensitizing chromophore, and the metal ion becomes excited to the emission state. (c) Structure of the synthesized Eu^{3+}−polyaminocarboxylate complexes incorporating a covalently bound sensitizing chromophore. Eu^{3+} complexes possess various substituents, H, F, CH_3, CH_3O, NHAc, and NH_2, at the 6-position of quinoline (=R) as a light-harvesting moiety for efficient Eu^{3+} sensitization. Also shown is the structure of the Zn^{2+}-selective luminescent Eu^{3+} chemosensor [Eu-7]. Reprinted from ref 29. Copyright 2004 American Chemical Society.

time between the pulsed excitation light and measurement of the long-lived luminescence of the dyes (Figure 1a). Thus, this TRLLM imaging has characteristics very different from those of conventional time-resolved fluorescence imaging.

New fluorescent dyes with a long fluorescence lifetime are needed for the TRLLM-based microscopic method. Typical organic phosphorescent dyes, which possess high triplet quantum yields, such as eosin or erythrosine or metal complexes showing MLCT luminescence are considered to be good candidates for the TRLLM-based microscopic method,[9,14] because phosphorescence lifetimes are relatively long. However, these compounds often transfer the energy of the triplet excited state to surrounding biomolecules in cells and tissues through singlet oxygen, acting as photosensitizers.[14,15] Such light-activated photosensitizing severely limits the use of these phosphorescent dyes in TRLLM, resulting in cell death via apoptosis and/or necrosis and photobleaching of the dyes. Luminescent lanthanide complexes, in particular complexes of europium and terbium trivalent ions (Eu^{3+} and Tb^{3+}), possess extremely long luminescence lifetimes on the order of milliseconds, whereas typical organic fluorescent compounds possess short fluorescence lifetimes in the nanosecond region.[16] Further, luminescent lanthanide complexes are thought to be relatively insensitive to photobleaching and the generation of singlet oxygen compared with phosphorescent dyes.[5] For these reasons, luminescent lanthanide complexes have been recently exploited in various bioassays with time-gating in the fields of medicine, biotechnology, and biological science.[17,18] The lanthanide f−f transitions have low absorbance, so a sensitizing chromophore covalently bound to the ligand is desirable for high luminescence.[16] Absorption by the chromophore results in effective population of its triplet level, and efficient intramolecular energy transfer conveys the absorbance energy of the excited chromophore to a chelated lanthanide metal ion, which becomes excited to the emission state (Figure 1b). Therefore, a proper chromophore design should afford luminescent lanthanide complexes which possess appropriate photochemical properties for TRLLM.[16]

Despite the tremendous amount of work on luminescent lanthanide complexes, only a few complexes have yet been employed for TRLLM,[4,5,7,8,10−13,19−21] and most complexes are used as labeling reagents for proteins. There are numerous requirements to be fulfilled by luminescent lanthanide complexes for usage in molecular imaging with TRLLM, and these include kinetic stability in water and a sensitizing chromophore unit that generates an efficient antenna effect. Further, it would be desirable to move the excitation wavelength toward the visible region to reduce the phototoxicity of the excitation light for biological samples and to ensure compatibility with the optics of standard fluorescence microscopes. Here, we report the design and synthesis of new luminescent Eu^{3+} complexes for use as long-lived luminescent dyes for TRLLM and the development of a new TRLLM system, based on conventional fluorescence

(11) Seveus, L.; Väisälä, M.; Syrjänen, S.; Sandberg, M.; Kuusisto, A.; Harju, R.; Salo, J.; Hemmilä, I.; Kojola, H.; Soini, E. *Cytometry* **1992**, *13*, 329−338.
(12) Sevéus, L.; Väisälä, M.; Hemmilä, I.; Kojola, H.; Roomans, G. M.; Soini, E. *Microsc. Res. Tech.* **1994**, *28*, 149−154.
(13) Connally, R.; Veal, D.; Piper, J. *Microsc. Res. Tech.* **2004**, *64*, 312−322.
(14) Redmond, R. W.; Gamlin, J. N. *Photochem. Photobiol.* **1999**, *70*, 391−475.
(15) Gorman, A.; Killoran, J.; O'Shea, C.; Kenna, T.; Gallagher, W. M.; O'Shea, D. F. *J. Am. Chem. Soc.* **2004**, *126*, 10619−10631.
(16) Parker, D.; Williams, J. A. G. *J. Chem. Soc., Dalton Trans.* **1996**, 3613−3628.
(17) Hemmilä, I.; Webb, S. *Drug Discovery Today* **1997**, *2*, 373−381.
(18) Petoud, S.; Cohen, S. M.; Bünzli, J. C. G.; Raymond, K. N. *J. Am. Chem. Soc.* **2003**, *125*, 13324−13325.
(19) Poole, R. A.; Bobba, G.; Cann, M. J.; Frias, J. C.; Parker, D.; Peacock, R. D. *Org. Biomol. Chem.* **2005**, *3*, 1013−1024.
(20) Song, B.; Wang, G.; Tan, M.; Yuan, J. *J. Am. Chem. Soc.* **2006**, *128*, 13442−13450.
(21) Pandya, S.; Yu, J.; Parker, D. *Dalton Trans.* **2006**, 2757−2766.

microscopy, for molecular imaging in living biological samples. We also confirmed the usefulness of this TRLLM method in cultured living cells.

Results and Discussion

Synthesis of Long-Lived Luminescent Dyes for TRLLM. Various luminescent Eu^{3+} complexes (R = H, F, CH_3, CH_3O, NHAc, and NH_2 in the 6-position of the quinoline) (Figure 1c), which were developed as candidate long-lived luminescent dyes for TRLLM, were synthesized via similar routes to examine their suitability for microscopic measurement, which requires strong luminescence and an excitation wavelength longer than 350 nm (because the optics of standard fluorescence microscopes have only marginal transmittance below 350 nm). The synthetic schemes and detailed descriptions of the synthetic procedures for these Eu^{3+} complexes are shown in the Supporting Information.

Luminescence and Chemical Properties of Synthesized Long-Lived Luminescent Dyes. Quinolines with F, CH_3, and CH_3O in the 6-position (=R) have been reported as efficient sensitizing chromophores for Eu^{3+},[22−24] but the photophysical properties of these Eu^{3+} complexes have not been analyzed well. The integrity of the Eu^{3+} complexes must be maintained in vivo to ensure nontoxicity, because the free metal ion and unchelated chelators are generally more toxic than the complex itself.[25] Chelators such as DTPA (diethylenetriamine-*N*,*N*′,*N*″,*N*‴-pentaacetic acid) and DOTA (1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid) have very high stability constant values for lanthanide ion,[25] so we expected that our Eu^{3+} complexes would not have serious toxicity. First, the time-delayed luminescence spectra and UV−vis absorption spectra of these Eu^{3+} complexes were measured in 100 mM HEPES buffer (pH 7.4). When time-delayed luminescence spectra were measured with a delay time of 50 μs and a gate time of 1.00 ms, these Eu^{3+} complexes (except R = NH_2) were highly luminescent (Figure 2a). The luminescence spectra displayed six bands, arising from transitions from the emissive $^5D_0$ level to the $^7F_0$, $^7F_1$, $^7F_2$, $^7F_3$, $^7F_4$, and $^7F_5$ levels of the ground states, respectively.[16] Indeed, aqueous solutions of Eu^{3+} complexes except R = NH_2 showed bright pink luminescence upon excitation with a TLC plate reader lamp (312 nm) (see the Supporting Information). The UV−vis absorption spectra of these Eu^{3+} complexes showed intense absorption bands due to the quinolyl substituent, which is the sensitizing chromophore (Figure 2b,c). Figure 2c shows the normalized absorption spectra of Eu^{3+} complexes (R = H, F, CH_3, CH_3O, NHAc, NH_2) between 280 and 450 nm on the basis of Figure 2b, indicating the comparison between the dominant absorption bands of Eu^{3+} complexes at a concentration of 50 μM in HEPES buffer (pH 7.4). The absorption spectra of Eu^{3+} complexes with R = H, F, and CH_3 showed a peak around 320 nm, and introduction of a CH_3O or NHAc substituent (=R) resulted in an absorption peak at close to 330 nm, tailing out to 370 nm. The absorption spectra of the Eu^{3+} complex with R = NH_2 showed an absorption peak at 352 nm, tailing out to 420 nm. Although the Eu^{3+} complex with R = NH_2 showed the longest absorption wavelength among the synthesized Eu^{3+} complexes, this Eu^{3+} complex did not luminesce at all. The luminescence and chemical properties of Eu^{3+} complexes are listed in Table 1. These Eu^{3+} complexes were dissolved even at 10 mM, so they were highly water-soluble. The luminescence quantum yields (ϕ) of the Eu^{3+} complexes except R = NH_2 are sufficiently large for luminescence detection in fluorescence microscopy.[4,7] The luminescence lifetimes of the above Eu^{3+} complexes except R = NH_2 were approximately 0.60 ms in H_2O and 2.00 ms in D_2O. These values indicate that the number of coordinated water molecules (q value)[27] at the Eu^{3+} ion was

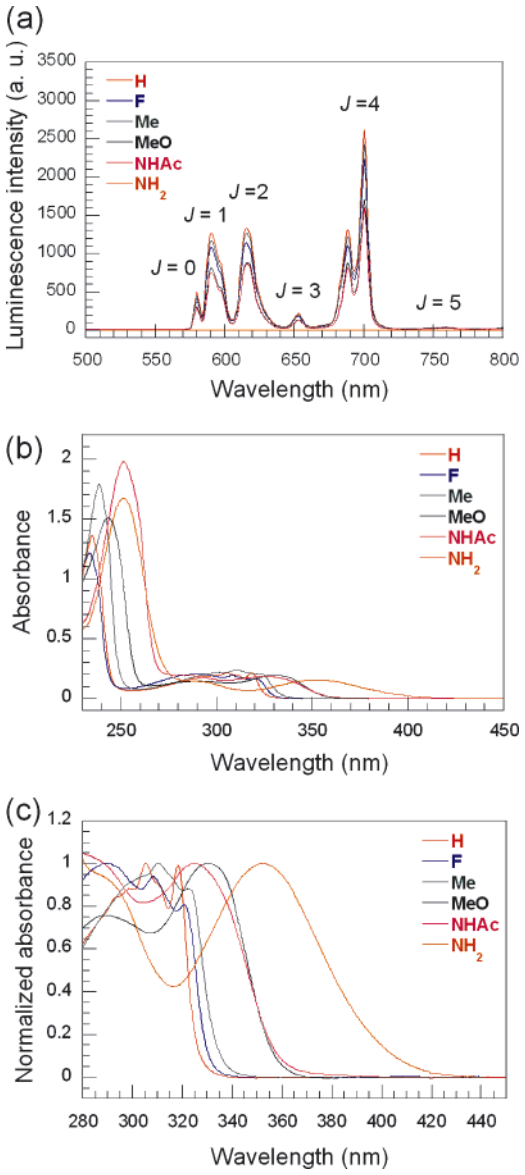


Figure 2. Time-resolved luminescence spectra and absorbance spectra of newly synthesized Eu^{3+} complexes. (a) Time-resolved luminescence spectra of Eu^{3+} complexes (H, red; F, blue; CH_3, green; CH_3O, black; NHAc, pink; NH_2, orange) in HEPES buffer at pH 7.4. The bands arise from $^5D_0 \rightarrow {}^7F_J$ transitions; the J values of the bands are labeled. (b, c) Absorbance spectra of the Eu^{3+} complexes in HEPES buffer at pH 7.4. (b) Absorbance spectra and (c) normalized absorbance spectra over 280 nm. R = H, red; F, blue; CH_3, green; CH_3O, black; NHAc, pink; NH_2, orange.

(22) Griffin, J. M. M.; Skwierawska, A. M.; Manning, H. C.; Marx, J. N.; Bornhop, D. J. *Tetrahedron Lett.* **2001**, *42*, 3823−3825.
(23) Manning, H. C.; Goebel, T.; Marx, J. N.; Bornhop, D. J. *Org. Lett.* **2002**, *4*, 1075−1078.
(24) Kiefer, G. E.; Bornhop, D. J. PCT Int. Appl. WO03/035655 A1, 2003.
(25) Caravan, P.; Ellison, J. J.; McMurry, T. J.; Lauffer, R. B. *Chem. Rev.* **1999**, *99*, 2293−2352.
(26) Nakamaru, K. *Bull. Chem. Soc. Jpn.* **1982**, *55*, 2697−2705.
(27) Beeby, A.; Clarkson, I. M.; Dickins, R. S.; Faulkner, S.; Parker, D.; Royle, L.; de Sousa, A. S.; Williams, J. A. G.; Woods, M. *J. Chem. Soc., Perkin Trans. 2* **1999**, 493−503.

Table 1. Luminescence and Chemical Properties

R	ϕ^a (%)	$\tau_{H_2O}{}^b$ (ms)	$\tau_{D_2O}{}^c$ (ms)	q^d
H	5.9	0.59	2.01	1.14
F	4.2	0.60	1.99	1.10
Me	4.2	0.60	2.00	1.10
MeO	2.4	0.59	1.88	1.10
NHAc	2.6	0.60	1.96	1.09
NH_2	NL[e]	NL	NL	NL

[a] Quantum yields were calculated using $[Ru(bipy)_3]Cl_2$ (bipy = 2,2′-bipyridine; $\phi = 0.028$ in water) as a standard and measured in 100 mM HEPES buffer at pH 7.4, 25 °C.[26] [b] The luminescence lifetimes were measured in H_2O. [c] The luminescence lifetimes were measured in D_2O. [d] q values were estimated using the equation $q^{Eu} = 1.2\,(\tau_{H_2O}{}^{-1} - \tau_{D_2O}{}^{-1} - 0.25)$, which allows for the contribution of unbound water molecules.[27] [e] NL = no luminescence.

approximately 1.10. Thus, the lanthanide hydration state was hardly affected by the introduction of various substituents at the 6-position of the quinolyl sensitizing chromophore of the Eu^{3+} complexes. These results demonstrate that it is feasible to modulate the photophysical properties of Eu^{3+} complexes, such as the intensity of luminescence and the excitation wavelength, and also indicate that the Eu^{3+} complexes with R = NHAc or CH_3O are good candidates for long-lived luminescence dyes for TRLLM because they have a sufficiently long excitation wavelength of over 350 nm.

Time-Resolved Long-Lived Luminescence Imaging in Living Cells. A schematic of the TRLLM system is shown in Figure 3a. This new TRLLM system utilizes a conventional fluorescence microscopy system equipped with an image intensifier (I.I.) unit, a xenon flash lamp, and a timing controller. Applications of mechanical choppers in TRLLM with the use of luminescent lanthanide complexes have been reported by some groups,[4−6,8−12] and some groups have employed an I.I. unit for the time resolution.[7,13] In this study, we used an I.I. unit for the time resolution for the following reasons. First, the gate time (10 μs to 100 ms) and delay time (10 μs to 100 ms) can be easily controlled with the I.I. unit. Second, the I.I. unit improves the uniformity of the emission light, which can be unsatisfactory with mechanical choppers. Third, the gain of the luminescence signal can be adjusted easily. As regards the excitation source, a powerful excitation light is required for time-resolved long-lived luminescence measurements, so a xenon flash lamp was selected. A laser might be preferable, but its wavelength cannot be changed easily.

We examined the application of two Eu^{3+} complexes with R = NHAc or CH_3O to conventional microscopy or TRLLM of cultured living cells (HeLa cells). The fluorescence microscope had an optical window centered at 617 ± 37 nm for the emission due to Eu^{3+}-based luminescence upon excitation at 360 ± 40 nm. The Eu^{3+} complex with R = NHAc or CH_3O was injected into the cultured HeLa cells (Figure 3b). In the prompt fluorescence images, the Eu^{3+} complex-injected cells were seen, together with the Eu^{3+} complex-noninjected cells, which appeared owing to their weak autofluorescence. In the TRLLM images, the autofluorescence from the cells, which is short-lived, was gated out, allowing the Eu^{3+} complex-injected cells to be clearly distinguished.

We next investigated the usefulness of the luminescent Eu^{3+} complexes as long-lived luminescent dyes, since they were expected to have advantages for biological applications. Unlike triplet-state phosphorescent dyes, Eu^{3+} complexes are thought to be insensitive to photobleaching and inefficient generators of singlet oxygen.[5] Reactive oxygen species such as singlet oxygen cause significant cellular damage. First, to evaluate the photostability of Eu^{3+} complexes (R = NHAc or CH_3O), photobleaching experiments with Eu^{3+} complexes and fluorescein were performed in 100 mM HEPES buffer at pH 7.4 (Figure S2, Supporting Information). Fluorescein is often used as a fluorescent dye for fluorescence microscopy. The Eu^{3+} complexes (40 μM) and fluorescein (1 μM) were irradiated with an external light at an appropriate wavelength, 325 nm (1.57 mW) for Eu^{3+} complexes and 492 nm (2.07 mW) for fluorescein. The luminescence intensity of Eu^{3+} complexes (R = NHAc and CH_3O) without time-gating reached 114% and 111% of the initial intensity after 420 min, respectively. The fluorescence intensity of fluorescein was decreased to 48% of the initial intensity after 420 min. The Eu^{3+} complexes appear to be more insensitive to photobleaching than fluorescein. Furthermore, the ability of Rose Bengal, fluorescein, and Eu^{3+} complexes (R = NHAc or CH_3O) to produce singlet oxygen was tested by trapping with 1,3-diphenylisobenzofuran (DPBF), an efficient quencher of singlet oxygen.[15] Rose Bengal is commonly exploited as a photosensitizer and possesses a high triplet quantum yield as a phosphorescent dye.[14] Fluorescein was employed here as a reference sensitizer. The generation of singlet oxygen was evaluated by following the disappearance of the 410 nm absorbance of DPBF at the initial concentration of 20 μM. We used a xenon lamp as a light source and an irradiation wavelength of 555 nm for Rose Bengal, 492 nm for fluorescein, and 325 nm for Eu^{3+} complexes. The values of the relative rate of singlet oxygen generation were 0.89, 0.51, 1, and 18 for Eu^{3+} complexes (R = NHAc and R = CH_3O), fluorescein, and Rose Bengal, respectively (details in Figure S3, Supporting Information). Thus, the Eu^{3+} complexes had relatively low singlet oxygen production, whereas Rose Bengal showed efficient production of singlet oxygen compared with a reference sensitizer, fluorescein.

Dual-Color Imaging with TRLLM. We investigated the feasibility of dual-color imaging with TRLLM using long-lived luminescent Eu^{3+} and Tb^{3+} complexes. Luminescent Tb^{3+} complexes also have advantages for TRLLM, such as long luminescence lifetimes on the order of milliseconds, narrow emission peaks, large Stokes shifts, high quantum yields, and excellent water solubility, like luminescent Eu^{3+} complexes. The main luminescence wavelength of Tb^{3+} complexes is between 480 and 630 nm,[16] and that of Eu^{3+} complexes is between 570 and 720 nm (Figure 2a). The newly synthesized Eu^{3+} complex (R = NHAc) is used as a red emitter, and the Tb^{3+} complex DTPA-cs124 with a chelated Tb^{3+} ion (Tb^{3+}−DTPA-cs124) is employed as a green emitter.[28] This DTPA-cs124 chelator was prepared using the reported procedure,[28] and the complexation of DTPA-cs124 with Tb^{3+} is described in the Supporting Information. The Eu^{3+} complex (R = NHAc) and Tb^{3+}−DTPA-cs124 were injected separately into cultured living HeLa cells. The fluorescence microscope had optical windows centered at 617 ± 37 nm for the Eu^{3+} emission (commercially available Cy3 filter) and at 528 ± 19 nm for the Tb^{3+} emission (commercially available FITC filter) with the same excitation wavelength at 360 ± 40 nm, which also utilized a commercially available filter. In the time-resolved long-lived luminescence

(28) Li, M.; Selvin, P. R. *J. Am. Chem. Soc.* **1995**, *117*, 8132−8138.

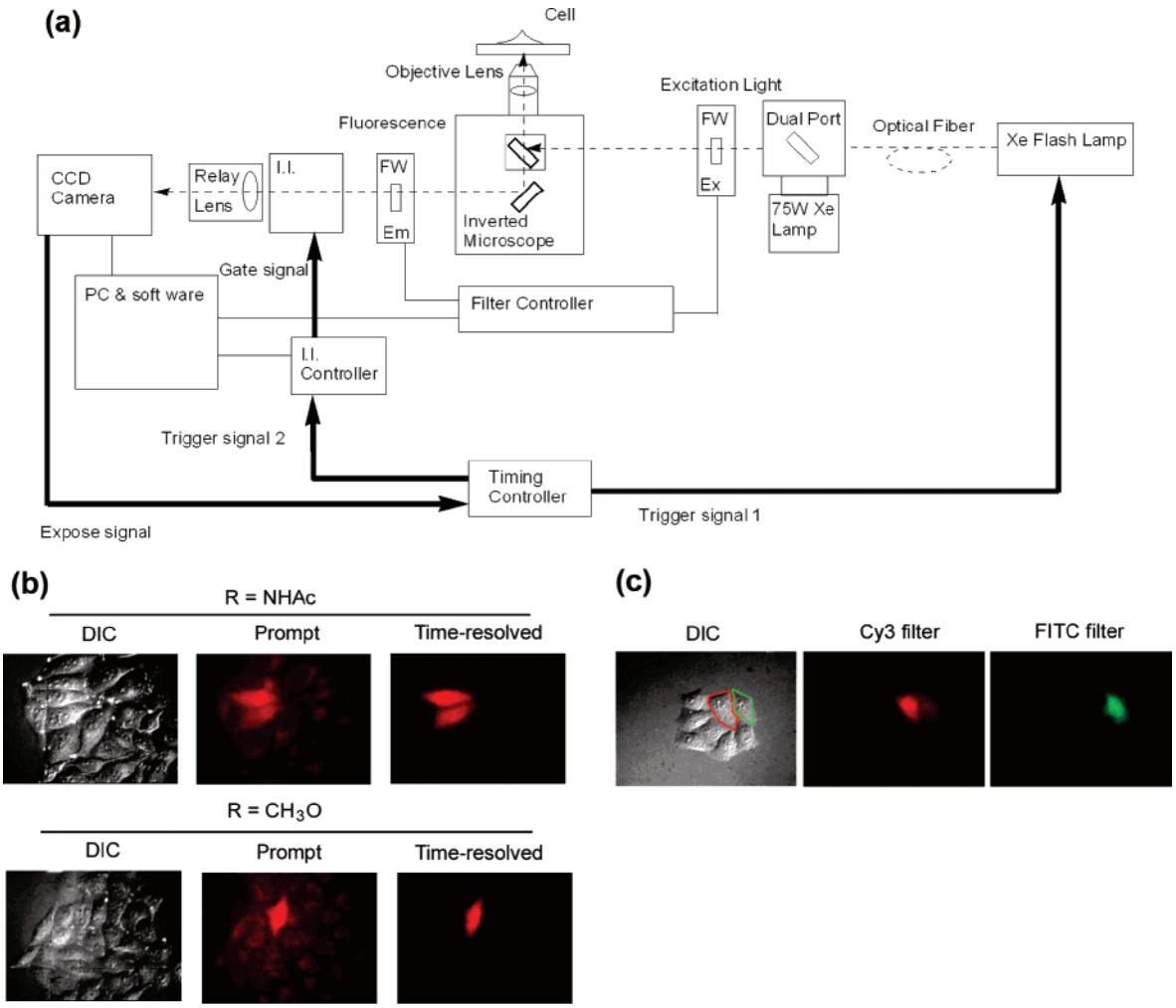


Figure 3. Time-resolved, long-lived luminescence images provided by our new TRLLM system. (a) Schematic diagram of the optical apparatus used for the TRLLM system. The excitation source is a xenon flash lamp. The excitation light passes through the excitation filter and is focused onto cells with dichroic mirrors. The emission light passes through the emission filter. The I.I. unit passes the long-lived luminescence to the CCD camera, controlling the delay time, the gate time, and the gain. The excitation and emission light can be easily selected by using appropriate excitation and emission filters. The image is recorded on the CCD camera and then transferred to the computer for further processing with MetaFluor 6.1 software. (b) One or two cells were injected with the Eu^{3+} complex (R = NHAc or CH_3O) in HBSS buffer. The fluorescence was measured at 617 ± 37 nm, which is the wavelength range of the Cy3 emission filter, with excitation at 360 ± 40 nm. Bright-field transmission images (DIC), prompt fluorescence images (Promp), and time-resolved long-lived luminescence images (Time-resolved) of living cells, including the Eu^{3+} complex-injected cells. (c) Time-resolved dual-color luminescence images of living HeLa cells. One cell was injected with the Eu^{3+} complex (R = NHAc) or Tb^{3+}−DTPA-cs124, respectively. The fluorescence emission at 617 ± 37 nm (the Cy3 emission filter) for Eu^{3+} or 528 ± 19 nm (the FITC emission filter) for Tb^{3+} was measured with excitation at 360 ± 40 nm. DIC: Bright-field transmission image. Red and green colors in DIC show Eu^{3+} complex-injected and Tb^{3+}−DTPA-cs124-injected cells, respectively. Cy3 filter: Time-resolved long-lived luminescence image of DIC with the Cy3 emission filter (617 ± 37 nm). FITC filter: Time-resolved long-lived luminescence image of DIC with the FITC emission filter (528 ± 19 nm).

image produced with the Cy3 filter, the luminescence of Eu^{3+} was clearly seen, whereas that of Tb^{3+} was only weakly detected due to the difference in the range of the emission wavelengths (Figure 3c). With the FITC filter, the Tb^{3+} luminescence was strongly detected, while the Eu^{3+} luminescence was not observed at all (Figure 3c). Thus, our TRLLM technique can provide dual-color detection with appropriate emission filters, in addition to discriminating against short-lived background fluorescence. To our knowledge, this is the first report of dual-color imaging in living cells with TRLLM.

Time-Resolved Long-Lived Luminescence Imaging of Zn^{2+} in Living Cells. We previously reported a Zn^{2+}-selective luminescent Eu^{3+} chemosensor, [Eu-7] (Figure 1c).[29] Zn^{2+} is the second most abundant heavy metal ion after iron in the human body, and chelatable Zn^{2+} plays an important role in various biological systems.[30] This compound, [Eu-7], has a sufficiently long excitation wavelength for fluorescence microscopy and is suitable for fluorescence microscopic measurements of the Zn^{2+} concentration in living cells. In this study, we examined the application of [Eu-7] to cultured living HeLa cells by using our TRLLM system (Figure 4). Optical parameters used for the fluorescence microscope were as follows: the fluorescence microscope had an optical window centered at 617 ± 37 nm for the emission due to Eu^{3+}-based luminescence excited at 360 ± 40 nm. The delay time, prior to initiation of counting, and the gate time, during which counting takes place, were set at 70 and 808 μs, respectively. Compound [Eu-7] was

(29) Hanaoka, K.; Kikuchi, K.; Kojima, H.; Urano, Y.; Nagano, T. *J. Am. Chem. Soc.* **2004**, *126*, 12470−12476.
(30) Vallee, B. L.; Falchuk, K. H. *Physiol. Rev.* **1993**, *73*, 79−118.

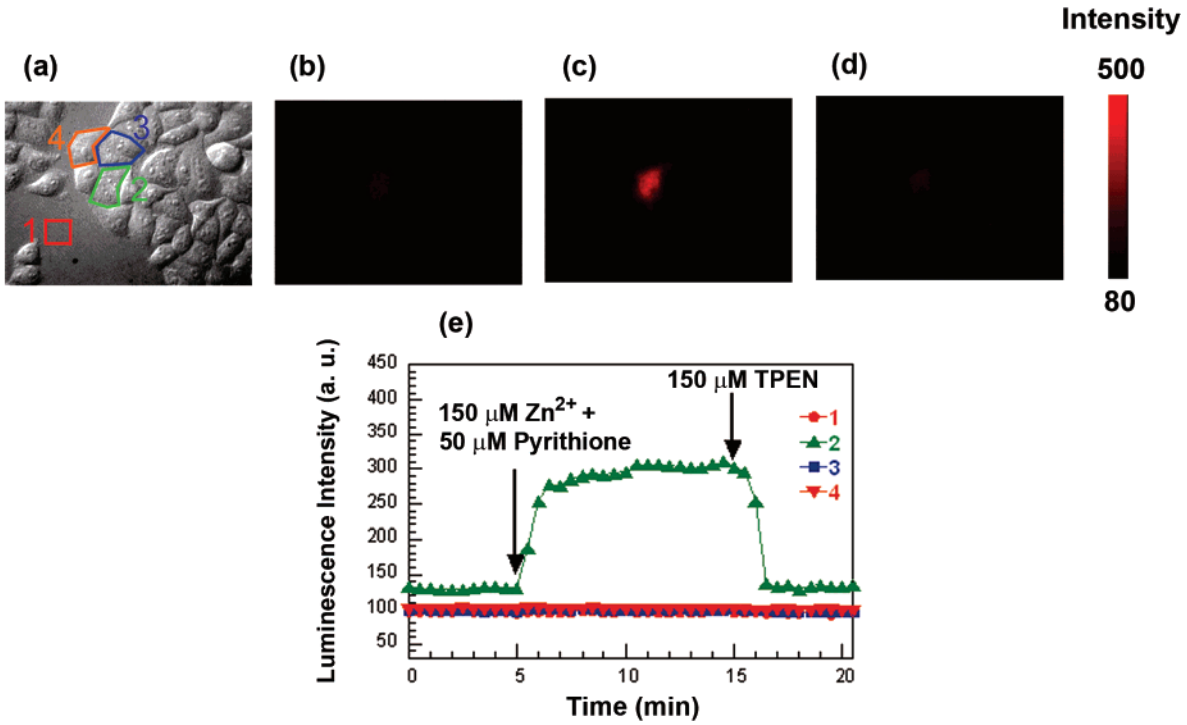


Figure 4. Time-resolved long-lived luminescence imaging of intracellular Zn^{2+} in living HeLa cells. The luminescence at 617 ± 37 nm, excited at 360 ± 40 nm, was measured at 30 s intervals. The time-resolved long-lived luminescence images were measured with our TRLLM system using a delay time of 70 μs and a gate time of 808 μs. The HeLa cells were injected in HBSS buffer with the [Eu-7] solution. (a) Bright-field transmission image (0 min). (b) Time-resolved long-lived luminescence image of (a) (0 min). (c) Time-resolved long-lived luminescence image (7 min) following addition of 5 μM pyrithione (zinc ionophore) and 50 μM $ZnSO_4$ to the medium at 5 min. (d) Time-resolved long-lived luminescence image (17 min) following addition of 100 μM TPEN to the medium at 15 min. Time-resolved long-lived luminescence images (b−d) correspond to the luminescence intensity data in (e), which shows the average intensity of the corresponding area or cell area in (a) (1, extracellular region; 2, intracellular region of the injected cell; 3, 4, intracellular regions of noninjected cells).

injected into a single cultured HeLa cell in the central part of the field of view in Figure 4a,b. A prompt increase of intracellular luminescence was induced when Zn^{2+} (50 μM) and pyrithione (2-mercaptopyridine *N*-oxide, 5 μM), which is a zinc-selective ionophore, were added to the medium at 5 min (Figure 4c). Further, the luminescence intensity decreased immediately upon the extracellular addition of the cell-membrane-permeable chelator TPEN (*N*,*N*,*N*′,*N*′-tetrakis(2-picolyl)ethylenediamine) (100 μM) at 15 min (Figure 4d). Clear images of the intracellular Zn^{2+} concentration changes were obtained with the TRLLM system (Figure 4e). To explore further the utility of TRLLM, we tested whether long-lived luminescence could be well distinguished from the short-lived fluorescence of rhodamine 6G as an artificial source of short-lived background fluorescence. Rhodamine 6G fluorescence directly interferes with Eu^{3+} luminescence because rhodamine 6G has excitation and emission wavelength ranges similar to those of [Eu-7]. As a result, the time-resolved long-lived luminescence images were not affected at all by rhodamine 6G fluorescence (Figure S4, Supporting Information); i.e., [Eu-7] could be used to monitor changes of the intracellular Zn^{2+} concentration even in cells stained with rhodamine 6G.

Conclusions

We have developed a new methodology, i.e., the TRLLM technique with our new luminescent lanthanide complexes, for fluorescence imaging in living biological samples such as live cells. Time-resolved fluorescence imaging has been achieved using other TRLLM systems and luminescent lanthanide complexes, but these complexes were mostly used as labeling reagents for biological molecules. We have shown here that the long-lived luminescence of luminescent lanthanide complexes can be clearly distinguished from the short-lived background fluorescence such as autofluorescence, present in most biological systems as well as scattered excitation light, and even the strong short-lived fluorescence of rhodamine 6G by using our TRLLM system.

By using both luminescent Eu^{3+} and luminescent Tb^{3+} complexes, dual-color imaging was possible with our microscope system. For example, it was possible to discriminate red and green images of luminescent Eu^{3+} and Tb^{3+} complexes with our microscope system using commercially available Cy3 and FITC filters, while excluding short-lived fluorescence. Moreover, we could monitor intracellular Zn^{2+} concentration changes in living cells by using the TRLLM system with our Zn^{2+}-sensitive luminescent lanthanide sensor probe [Eu-7]. This paper is the first to describe imaging of the concentration changes of intracellular metal ion, including Zn^{2+}, in living cells as time-resolved long-lived luminescence images obtained with a luminescent lanthanide sensor probe. The next step should be the development of a range of superior long-lived luminescent lanthanide sensor probes, which possess extremely high quantum yields, long excitation wavelengths over 400 nm, and cell permeability.[31,32] Efforts are under way to develop new long-lived luminescent lanthanide sensor probes which switch between weak and strong luminescence in the absence and the

(31) Yang, C.; Fu, L. M.; Wang, Y.; Zhang, J. P.; Wong, W. T.; Ai, X. C.; Qiao, Y. F.; Zou, B. S.; Gui, L. L. *Angew. Chem., Int. Ed.* **2004**, *43*, 5010−5013.
(32) Allen, M. J.; MacRenaris, K. W.; Venkatasubramanian, P. N.; Meade, T. J. *Chem. Biol.* **2004**, *11*, 301−307.

presence of specific biological molecules, in parallel with efforts to improve our TRLLM system. We consider that our TRLLM imaging technique using luminescent lanthanide sensor probes should be useful as a complement to, rather than a replacement for, existing fluorescence imaging methodologies.

Experimental Section

Materials. DTPA bisanhydride was purchased from Aldrich Chemical Co. Inc. (St. Louis, MO). All other reagents were purchased from either Tokyo Kasei Kogyo Co., Ltd. (Japan) or Wako Pure Chemical Industries, Ltd. (Japan). All solvents were used after distillation. Silica gel column chromatography was performed using BW-300 and Chromatorex-ODS (all from Fuji Silysia Chemical Ltd.).

Instruments. ^{1}H and ^{13}C NMR spectra were recorded on a JEOL JNM-LA300. Mass spectra were measured with a JEOL-T100LC AccuTOF (ESI$^+$ or ESI$^-$). HPLC purification was performed on a reversed-phase column (GL Sciences (Tokyo, Japan), Inertsil Prep-ODS 30 mm × 250 mm) fitted on a Jasco PU-1587 system. Time-resolved luminescence spectra were recorded on a Perkin-Elmer LS55 luminescence spectrometer (Beaconsfield, Buckinghamshire, England). The slit width was 5 nm for both excitation and emission. A delay time of 50 μs and a gate time of 1.00 ms were used. UV−vis spectra were obtained on a Shimadzu UV-1650PC (Tokyo, Japan) or an Agilent 8453 (Agilent Technologies, Waldbronn, Germany) UV−vis spectroscopy system. The fluorescence intensities without a delay time were also recorded on a Perkin-Elmer LS55 luminescence spectrometer (Beaconsfield). The slit width was 10 nm for both excitation and emission.

Time-Delayed Luminescence Spectral Measurements. The time-delayed luminescence spectra of Eu^{3+} complexes (50 μM) were measured in 100 mM HEPES buffer at pH 7.4, 24 °C (excitation at 318 nm (R = H), 320 nm (R = F), 323 nm (R = CH_3), 330 nm (R = CH_3O), 325 nm (R = NHAc), and 352 nm (R = NH_2)). The slit width was 5 nm for both excitation and emission. A delay time of 50 μs and a gate time of 1.00 ms were used.

UV−Vis Absorption Spectrum Measurements. The absorption spectra of Eu^{3+} complexes (50 μM) were measured at 24 °C in an aqueous solution buffered to pH 7.4 (100 mM HEPES buffer). UV−vis spectra in Figure 2b,c were recorded on a Shimadzu UV-1650PC.

Quantum Yield Measurements. The luminescence spectra were measured with a Hitachi F4500 spectrofluorometer (Tokyo, Japan). The slit width was 2.5 nm for both excitation and emission. The photomultiplier voltage was 950 V. The luminescence spectra of Eu^{3+} complexes (5 μM) were measured in 100 mM HEPES buffer at pH 7.4, 25 °C, with irradiation at 300 nm. The quantum yields of Eu^{3+} complexes were evaluated using a relative method with reference to a luminescence standard, $[Ru(bpy)_3]Cl_2$ (ϕ = 0.028 in air-equilibrated water).[26] The quantum yields of Eu^{3+} complexes can be expressed by eq 1,[33] where Φ is the quantum yield (subscript "st" stands for the reference and "x" for the sample), A is the absorbance at the excitation wavelength, n is the refractive index, and D is the area (on an energy scale) of the luminescence spectra. The samples and the reference were excited at the same wavelength (300 nm). The sample absorbance at the excitation wavelength was kept as low as possible to avoid fluorescence errors (A_{exc} < 0.03).

$$\Phi_x/\Phi_{st} = [A_{st}/A_x][n_x^2/n_{st}^2][D_x/D_{st}] \quad (1)$$

Luminescence Lifetime Measurements. The luminescence lifetimes of the Eu^{3+} complexes were recorded on a Perkin-Elmer LS-55 luminescence spectrometer (Beaconsfield). The data were collected with 10 μs resolution in H_2O and D_2O and fitted to a single-exponential curve obeying eq 2, where I_0 and I are the luminescence intensities at time t = 0 and time t, respectively, and τ is the luminescence emission lifetime. Lifetimes were obtained by monitoring the emission intensity at 614 nm (λ_{ex} = 318 nm (R = H), 320 nm (R = F), 323 nm (R = CH_3), 330 nm (R = CH_3O), 325 nm (R = NHAc)).

$$I = I_0 \exp(-t/\tau) \quad (2)$$

Determination of the Number of Water Molecules Bound to the Inner Coordination Sphere of Eu^{3+}. The number of coordinated water molecules (q value) at the Eu^{3+} ion is determined by the following equation:[27]

$$q^{Eu} = 1.2(\tau_{H_2O}^{-1} - \tau_{D_2O}^{-1} - 0.25) \quad (3)$$

where τ_{H_2O} or τ_{D_2O} is the luminescence lifetime of the complex in H_2O or D_2O, respectively.

Photobleaching of Eu^{3+} Complexes. Photobleaching of luminescent Eu^{3+} complexes (R = NHAc or CH_3O) (40 μM) and fluorescein (1 μM) was performed in 100 mM HEPES buffer (pH 7.4) at 24 °C by using a xenon lamp (UXL-500D-0, USHIO, Tokyo, Japan) as an excitation source in an SM-5 system (Bunkoh-Keiki Co., Ltd., Tokyo, Japan). The wavelength and the energy of the irradiation light were 325 nm and 1.57 mW for Eu^{3+} complexes and 492 nm and 2.07 mW for fluorescein. The absorbance values of Eu^{3+} complexes (R = NHAc or CH_3O) (40 μM) and fluorescein (1 μM) solution were 0.16 at 325 nm, 0.15 at 325 nm, and 0.08 at 492 nm, respectively. The fluorescence intensities at 618 nm (for Eu^{3+} complexes) and 516 nm (for fluorescein) were recorded on a Perkin-Elmer LS55 luminescence spectrometer (Beaconsfeld) and plotted.

Comparative Singlet Oxygen Generation Measurements. Reaction of 1,3-diphenylisobenzofuran (DPBF) with singlet oxygen was monitored in terms of the reduction in the intensity of the absorbance at 410 nm. An aerated solution of a photosensitizer and DPBF (20 μM) in 100 mM HEPES buffer (pH 7.4)/MeOH = 1/1 was irradiated with a xenon lamp (UXL-500D-0, USHIO) in an SM-5 system (Bunkoh-Keiki Co., Ltd.) at 24 °C. Rose Bengal (1 μM), fluorescein (1 μM), or a Eu^{3+} complex (R = NHAc or CH_3O) (100 μM) was used as a photosensitizer. The wavelength and the energy of the irradiation light were 555 nm and 3.0 mW for Rose Bengal, 492 nm and 3.09 mW for fluorescein, and 325 nm and 2.55 mW for Eu^{3+} complexes (R = NHAc or CH_3O). The UV−vis spectra were recorded on an Agilent 8453 UV−vis spectroscopy system (Agilent Technologies). The absorbance of a Rose Bengal solution (1 μM), a fluorescein solution (1 μM), and Eu^{3+} complex (R = NHAc and CH_3O) solutions without DPBF were 0.08 (at 555 nm), 0.07 (at 492 nm), 0.40 (at 325 nm), and 0.41 (at 325 nm), respectively. Irradiation at 555 or 492 nm of a 20 μM DPBF solution in 100 mM HEPES buffer (pH 7.4)/MeOH = 1/1 produced no change in the intensity of the 410 nm absorption band, which was derived from DPBF. No changes in the absorbance spectra of the photosensitizers were observed during the irradiation, indicating that the photosensitizers were not photobleached in these experiments. However, irradiation at 325 nm of a 20 μM DPBF solution without photosensitizer produced a reduction in the intensity of the 410 nm absorption band of DPBF. Therefore, the rates of oxygenation of a 20 μM DPBF solution without photosensitizer in 100 mM HEPES buffer (pH 7.4)/MeOH = 1/1 on irradiation at 325, 555, or 492 nm were also measured as background rates, and we subtracted the rates of oxygenation of a solution of DPBF (20 μM) only from the rates of oxygenation of a solution of the Eu^{3+} complex (R = NHAc or CH_3O), Rose Bengal, or fluorescein containing DPBF (20 μM) when irradiated at 325 nm (for Eu^{3+} complexes), 555 nm (for Rose Bengal), or 492 nm (for fluorescein). Details are shown in the Supporting Information.

Preparation of Cells. HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen Corp., Carlsbad, CA), supplemented with 10% fetal bovine serum (Invitrogen Corp.), 1% penicillin, and 1% streptomycin (Invitrogen Corp.) at 37 °C in a 5% CO_2/95% air incubator. The cells were grown on an uncoated 35 mm

(33) Chen, Q. Y.; Feng, C. J.; Luo, Q. H.; Duan, C. Y.; Yu, X. S.; Liu, D. J. *Eur. J. Inorg. Chem.* **2001**, 1063−1069.

diameter glass-bottomed dish (MatTek, Ashland, MA) and washed twice with Hank's balanced salt solution (HBSS) buffer (Invitrogen Corp.), and then the medium was replaced with HBSS buffer before imaging. The Eu^{3+} complexes (2 or 10 mM) were dissolved in microinjection buffer (HBSS buffer) and injected into cells with an Eppendorf injection system (Transjector 5246).

Prompt Fluorescence Microscopy and Imaging Methods. The imaging system consisted of an inverted microscope (IX71, Olympus) with a cooled CCD camera (Cool Snap HQ; Roper Scientific, Tucson, AZ). The microscope was equipped with a xenon lamp (AH2-RX, Olympus), a 40× objective lens (UApo 40× oil/340, NA 1.35; Olympus), and a dichroic mirror (420DCLP, Omega). The whole system was controlled using MetaFluor 6.1 software (Universal Imaging, Media, PA). The fluorescence microscope had an optical window centered at 617 ± 37 nm (S617/73m, Chroma) for the emission due to Eu^{3+}-based luminescence upon excitation at 360 ± 40 nm (D360/40×, Chroma).

TRLLM and Imaging Methods. For time-resolved long-lived luminescence imaging we used the above conventional fluorescence microscopy system with the following modifications, as shown in Figure 3a. The microscope was equipped with an auxiliary 50 Hz pulsed excitation source (60 W xenon flash lamp (L6784), Hamamatsu Photonics K. K., Shizuoka, Japan) triggered by a timing controller (model 555-2C, BNC (Berkeley Nucleonics Corp.)). The delay time and gate time were controlled by an I.I. and I.I. controller (C9016-02, Hamamatsu Photonics K. K.) located in the emission light pathway and synchronized with the xenon flash lamp using a timing controller. The excitation light was delivered into a dual port (U-DPLHA; Olympus, Tokyo, Japan) via an optical fiber (A7632, Hamamatsu Photonics K. K.). The excitation source was easily switched from a xenon flash lamp to a xenon CW lamp. The I.I. device can strongly amplify the fluorescence signal. The amplified emission signal was delivered via a relay lens (A4539, Hamamatsu Photonics K. K.) to a cooled CCD camera (Cool SNAP HQ, Roper Scientific). Time-resolved long-lived luminescence images were acquired with a 52 or 70 μs delay time between the application of a pulse of excitation light to the sample and the acquisition of the luminescence signal, while no delay was used for the acquisition of prompt fluorescence images; to obtain a good signal-to-noise ratio, the overall acquisition time for each image was 1 s. The gate time, which is the acquisition time of the luminescence signal, was 808 μs. Quantitative evaluation of the luminescence images was done using the MetaFluor imaging analysis software package (Universal Imaging Corp.).

Time-Resolved Dual-Color Imaging Methods. The synthesized luminescent Eu^{3+} complex (R = NHAc) (10 mM) or Tb^{3+}−DTPA-cs124 (2 mM) was dissolved in microinjection buffer (HBSS buffer) and injected into cultured living HeLa cells in HBSS buffer. A delay time of 70 μs and a gate time of 808 μs were used. An optical window centered at 528 ± 19 nm (S528/38m, Chroma) was employed for Tb^{3+}-based luminescence.

Time-Resolved Long-Lived Luminescence Imaging of Intracellular Zn^{2+} in Living Cultured HeLa Cells. A Zn^{2+}-selective luminescent Eu^{3+} chemosensor, [Eu-7] (2 mM), was dissolved in microinjection buffer (HBSS buffer) and injected into living HeLa cells. The time-resolved long-lived luminescence images were acquired every 30 s. A delay time of 70 μs and a gate time of 808 μs were used.

Rhodamine 6G Loading Conditions and Imaging Method. HeLa cells were grown on an uncoated 35 mm diameter glass-bottomed dish (MatTek, Ashland, MA) and washed twice with HBSS buffer (Invitrogen Corp.); then the cells were incubated with rhodamine 6G (4 μM) in HBSS buffer for dye loading for 30 min at room temperature, which was a sufficient time for intracellular accumulation of rhodamine 6G judging from the fluorescence seen in the intracellular regions (Figure S4b). The stained cells were washed twice with HBSS buffer, and the medium was replaced with HBSS buffer before imaging. Then [Eu-7] was injected into a cultured living HeLa cell in the same manner as the above experimental method of time-resolved long-lived luminescence imaging of intracellular Zn^{2+} in living cultured HeLa cells. The fluorescence microscope had an optical window centered at 617 ± 37 nm (S617/73m, Chroma) for the emission due to rhodamine 6G-based fluorescence upon excitation at 360 ± 40 nm (D360/40×, Chroma). In time-resolved long-lived luminescence imaging, images with a delay time of 70 μs were free of contributions from prompt fluorescence of rhodamine 6G, while a delay time of 52 μs was not sufficiently long to eliminate this prompt fluorescence from the time-resolved long-lived luminescence images.

Acknowledgment. This work was supported by the Ministry of Education, Culture, Sports, Science and Technology of Japan (Grants for The Advanced and Innovational Research Program in Life Sciences, 16370071 and 16659003 to T.N., 15681012, 17035019, 17036012, 017048006, and 17651119 to K.K.). T.N. was also supported by the Hoh-ansha Foundation. K.K. was also supported by the Sankyo Foundation, by the Kanagawa Academy of Science, and by the Suzuken Memorial Foundation. K.H. was the recipient of Research Fellowships of the Japan Society for the Promotion of Science for Young Scientists.

Supporting Information Available: Detailed descriptions of synthetic procedures for luminescent Eu^{3+} complexes and the luminescent Tb^{3+} complex, photograph of solutions of luminescent Eu^{3+} complexes, photobleaching profiles of solutions of Eu^{3+} complexes and fluorescein, comparative singlet oxygen generation plots of Rose Bengal, fluorescein, and Eu^{3+} complexes, and time-resolved long-lived luminescence images of intracellular Zn^{2+} in HeLa cells which were stained with rhodamine 6G. This material is available free of charge via the Internet at http://pubs.acs.org.

JA073392J