Cell Imaging Technology

DOI: 10.1002/ange.201103775

Covalent Protein Labeling with a Lanthanide Complex and Its Application to Photoluminescence Lifetime-Based Multicolor Bioimaging**

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Long-lifetime photoluminescence (PL) enables the use of time-resolved (TR) measurements, which can eliminate short-lifetime luminescent signals.[1] Thus, lanthanide luminescence measurement has been utilized as a highly sensitive biochemical assay technique.[2] Recent progress in optical instrumentation has enabled the development of time-resolved luminescence (TRL) microscopy with pulse excitation techniques,[3] making lanthanide-based TRL imaging of biomolecules a promising technology for next-generation bioimaging. Recently, a TRL-based protein imaging technique was reported.[4] This system exploits a protein labeling system based on *Escherichia coli* dihydrofolate reductase and its specific inhibitors modified with a lanthanide complex. Because this system uses a noncovalent enzyme-inhibitor complex model,[5] dissociation of the labeled probe from the tag protein is a potential limitation, especially for long time-lapse imaging over several hours.

We recently developed a versatile protein labeling method using a mutant TEM-1 β-lactamase (BL-tag) and its fluorescent substrates.[6] By extending this covalent labeling technology, we aimed to develop a TRL imaging method for cell-surface proteins (Figure 1a). We designed and synthesized a novel luminescent europium(III) complex probe, TPA-Eu (Figure 1b and Scheme S1 in the Supporting Information). This compound consists of a terpyridinetetraacetate Eu^{3+} complex[7] connected to an ampicillin moiety, which covalently binds BL-tag.[6a] The wavelengths of excitation and emission maxima of TPA-Eu were 341.5 and 616.0 nm, respectively (Figure 1c). The emission spectrum was characteristic of Eu^{3+} luminescence. The luminescence lifetime was 1.25 ms (Figure S2 in the Supporting Information), which is long enough for TRL microscopy with a xenon flash lamp.

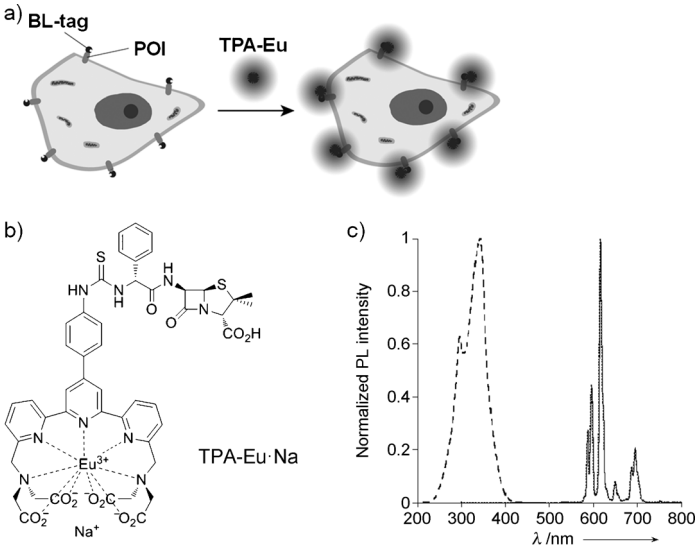

Figure 1. a) Covalent labeling of cell-surface protein of interest (POI) with a luminescent lanthanide complex for time-resolved photoluminescence imaging. b) Structure of TPA-Eu. c) Normalized steady-state excitation (– – –, λ_{em} = 616.0 nm), steady-state emission (——, λ_{ex} = 341.5 nm), and TR emission (-----, λ_{ex} = 341.5 nm, delay time: 60 μs, gate time: 2 ms) spectra of 10 μM TPA-Eu in 100 mM HEPES buffer (pH 7.4) at 25 °C; HEPES: 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid.

Next, the tag-labeling properties of TPA-Eu were investigated. TPA-Eu was incubated with BL-tag or wild-type TEM-1 (WT TEM) for 1 h, and the mixtures were analyzed by SDS-PAGE. Red PL of TPA-Eu labeled with the BL-tag was observed, but no labeling with WT TEM was seen (Figure 2a, b). The cell lysate did not interfere with labeling specificity (Figure 2c). Covalent labeling was also confirmed by MALDI-TOF MS. The mass spectrum of TPA-Eu-labeled BL-tag showed that TPA-Eu bound to the tag with a 1:1 stoichiometry (Table S1 in the Supporting Information). The PL spectra and lifetime of TPA-Eu labeled with BL-tag were measured, and both excitation and emission spectra were found to be almost identical to those of free TPA-Eu (data not shown), while the PL lifetime of the labeled TPA-Eu was also scarcely changed (1.27 ms, Figure S2 in the Supporting Information). These results indicated that TPA-Eu labeled with the tag protein maintained the photophysical properties of free TPA-Eu.

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[**] We thank Shigeru Kobayashi at Olympus Engineering Co. Ltd. for his technical help in constructing the TRL microscopy system. This work was supported by MEXT of Japan, by the Funding Program for World-Leading Innovative R&D on Science and Technology from JSPS, by CREST from JST, and by Asahi Glass Foundation.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201103775.

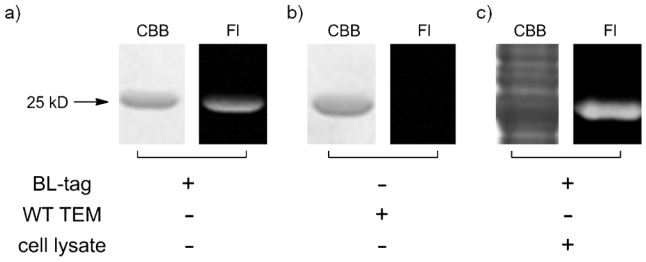

Figure 2. Specific labeling of BL-tag with TPA-Eu. a) BL-tag, b) WT TEM, and c) BL-tag mixed with HEK293T cell lysate were analyzed by electrophoresis. Fluorescent gel images were excited with a handheld UV lamp (λ_{ex} = 365 nm). CBB-stained gel image; CBB: Coomassie Brilliant Blue; Fl: fluorescent gel image.

TRL microscopy measurements of TPA-Eu were then performed. The TRL microscopy system (Figure S3 in the Supporting Information) was a slight modification of a reported system.[3b] Because this system excludes any short-lifetime luminescent signals, long-lifetime luminescent signals can be selectively detected. We confirmed the selective detection of long-lifetime PL of TPA-Eu over short-lifetime PL using silica gels (Figure S4d in the Supporting Information).

We next analyzed TPA-Eu-labeled cellular proteins using the TR microscopy system. BL-tag was fused to the extracellular region (N terminus) of epidermal growth factor receptor (EGFR) and expressed in HEK293T cells. The cells were treated with 10 μM TPA-Eu for 1 h at 37 °C, washed with buffer, and observed with a microscope (Figure 3a, left and center columns). Under UV excitation (λ = 340–390 nm), cellular autofluorescence severely interfered with the short-lifetime PL signals from TPA-Eu in the total PL images, which include no delay time, thereby providing almost identical results to steady-state measurements. On the other hand, the delayed PL images detected only TPA-Eu-labeled BL-EGFR. No long-lifetime PL was observed from the cells not expressing BL-EGFR.

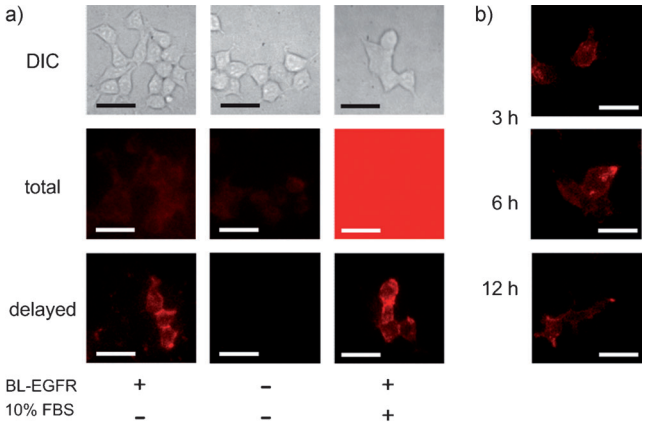

Figure 3. a) Microscopy images of HEK293T cells labeled with TPA-Eu. DIC: differential interference contrast images, total: total PL images, delayed: delayed PL images. b) Time-lapse imaging of HEK293T cells expressing BL-EGFR labeled with TPA-Eu. Incubation times are shown beside TR microscopy images. Scale bar: 50 μm.

The cells were also observed in the presence of 10% fetal bovine serum (FBS, Figure 3a, right column). In the total PL image, various fluorescent components in FBS interfered with the visualization of the TPA-Eu-labeled target proteins. However, the delayed PL image showed only long-lifetime components of the TPA-Eu signals on the cell surface. This property is useful for general bioimaging studies, because the inclusion of serum would enhance the robustness of living samples and would enable imaging over long periods. Long time-lapse imaging experiments, which can be performed in the presence of FBS, demonstrated no distinct dissociation of the labeled TPA-Eu from the target protein even after several hours (Figure 3b). This result demonstrated one of the advantages of our covalent-labeling-based method over previous technologies.[4]

Finally, we tested the application of TPA-Eu to lifetime-based multicolor imaging. As illustrated in Figure 4a, short- and long-lifetime PL signals, generated by organic dyes and lanthanide complexes, respectively, can be discriminately detected by the time-resolved gating technique, even if their emission spectra mostly overlap. After verifying the principle by using silica gels (Figure S3c–e in the Supporting Information), HEK293T cells expressing BL-EGFR were simultaneously labeled with TPA-Eu and MitoTracker Orange (MTO), which is a rhodamine-based fluorescent dye that stains mitochondria. The short-lifetime fluorescence of MTO was selectively visualized by accumulating the prompt PL component (delay time: 0 s, gate time: 10 μs; Figure 4c), while the long-lifetime PL of TPA-Eu on the cell-surface BL-EGFR was selectively detected as a delayed PL component

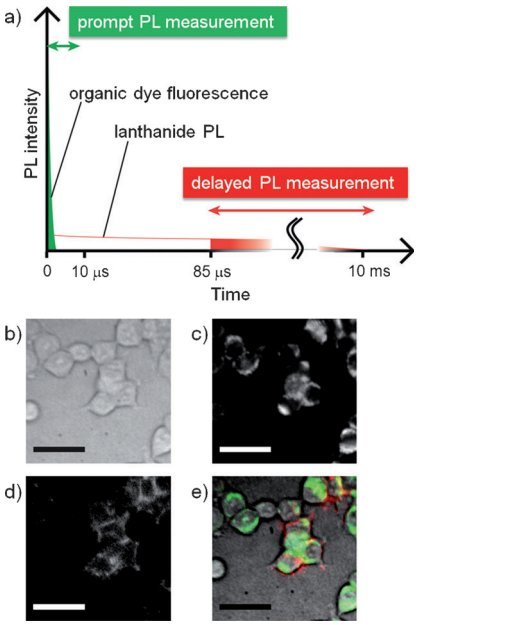

Figure 4. a) Illustration of time-gating discrimination of short- (organic fluorophores) and long-lifetime (lanthanides) luminescence components. b)–e) Lifetime-based multicolor imaging. Scale bar: 50 μm. b) DIC, c) prompt PL image, d) delayed PL image, and e) merged image. Short (MTO) and long (TPA-Eu) lifetime PL components were drawn with different pseudocolors (pseudo color: green for prompt PL and red for delayed PL).

(delay time: 85 μs, gate time: 10 ms; Figure 4d). These two signals can be overlaid with different pseudocolors (Figure 4e). Although frequency-domain fluorescence lifetime imaging microscopy (FLIM)[8] is an existing similar technique utilizing fluorescence lifetimes, this method requires a much longer accumulation time than our technique and also has the disadvantage of its high cost.

In conclusion, we have developed a novel protein imaging system based on covalent protein labeling with the lanthanide probe TPA-Eu combined with TRL microscopy. This technology separates live-cell imaging from background autofluorescence. The long-lifetime PL of TPA-Eu labels on cell-surface proteins can be selectively detected even in the presence of FBS. The covalent probe labeling enabled long time-lapse imaging lasting at least several hours. Both of these virtues are quite valuable especially for in vivo imaging experiments, because the autofluorescence of animal bodies severely hampers detection of faint PL signals, and in vivo studies usually take at least several hours. For further applications, intracellular protein labeling may be desired. As we expected from the anionic structure, TPA-Eu did not permeate into living cells. More hydrophobic or cationic lanthanide complexes should be chosen for intracellular protein labeling.

We also demonstrated a unique application—lifetime-based multicolor imaging—by exploiting pulse-gating technology. This multicolor imaging system would yield almost the same data as multicolor fluorescence imaging with different filter sets. Because this technique is orthogonal to the conventional wavelength-based multicolor imaging, simultaneous use of both wavelength-based and lifetime-based multicolor imaging techniques could increase the number of color channels. For example, three emission filter sets (blue, green, and red) and two lifetime settings (short and long) yield six channels. Considering that differently colored luminescent lanthanides such as terbium(III) or dysprosium(III) are also suitable for TRL measurements, simultaneous imaging of a larger number of proteins can be achieved in future.

Received: June 3, 2011
Published online: July 26, 2011

Keywords: imaging agents · labeling probes · proteins · time-resolved luminescence

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