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Chromophore-Assisted Light Inactivation of HaloTag Fusion Proteins Labeled with Eosin in Living Cells

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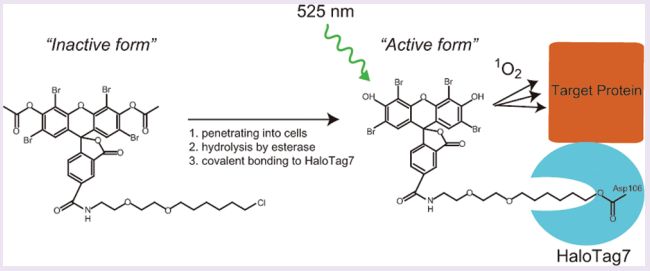
ABSTRACT: Chromophore-assisted light inactivation (CALI) is a potentially powerful tool for the acute disruption of a target protein inside living cells with high spatiotemporal resolution. This technology, however, has not been widely utilized, mainly because of the lack of an efficient chromophore as the photosensitizing agent for singlet oxygen ($^1O_2$) generation and the difficulty of covalently labeling the target protein with the chromophore. Here we choose eosin as the photosensitizing chromophore showing 11-fold more production of $^1O_2$ than fluorescein and about 5-fold efficiency in CALI of β-galactosidase by using an eosin-labeled anti-β-galactosidase antibody compared with the fluorescein-labeled one. To covalently label target protein with eosin, we synthesize a membrane-permeable eosin ligand for HaloTag technology, demonstrating easy labeling and efficient inactivation of HaloTag-fused PKC-γ and aurora B in living cells. These antibody- and HaloTag-based CALI techniques using eosin promise effective biomolecule inactivation that is applicable to many cell biological assays in living cells.



The evaluation of specific protein functions is a major challenge in cellular and molecular biology. Several technologies, such as genetic knockout, antisense RNA, and RNA interference are powerful tools for elucidating protein functions *in vivo*. However, these methods suffer from genetic compensation and low time resolution because the effect of loss depends on the lifetime of the protein.[1]

Chromophore-assisted light inactivation (CALI) allows the functional analysis of a target protein inside living cells with high spatiotemporal resolution.[2] CALI uses a chromophore molecule as a photosensitizer that, upon irradiation with intense light, yields short-lived reactive oxygen species (ROS), such as $^1O_2$, which have been shown to damage proteins in the close vicinity of the chromophore via the oxidation of methionine residues and cross-linking.[3] $^1O_2$ has a half-radius of photodamage of approximately 3−4 nm,[4] which is smaller than 8 nm, the average protein−protein interaction distance inside a cell,[5] enabling the inactivation of a specific protein with high spatial precision. Despite these attractive properties, CALI has not been widely used because of its limitations, including the low ROS production of the photosensitizer and the difficulty of specifically labeling the target protein with the photosensitizer.[6]

The original photosensitizer used for CALI was malachite green.[2] However, this chromophore required a high-energy laser because of its low ROS production efficiency. Currently, a xanthene-based chromophore, fluorescein, is often used because it is a better producer of ROS.[4,5,7] However, an even more potent photosensitizer is still desirable to eliminate the need for long-term illumination with an intense laser, which can cause unspecific photodamage.

Originally, to label a target protein with the CALI chromophore, the chromophore was first conjugated to a specific nonblocking antibody and then microinjected into the cell at a concentration high enough to saturate the target protein.[2] However, the laboriousness of this procedure greatly hampered the use of CALI. Therefore, several innovative strategies have been devised that use membrane-permeable chromophore ligands that bind to genetically encoded target sequences. The first such method used the biarsenical dyes FlAsH-EDT2 and ReAsH-EDT2, both of which are xanthene (fluorescein and rhodamine, respectively) derivatives, and bind to a short peptide motif containing four cysteine residues, which can be genetically fused to a protein of interest.[8−10] However, this method is limited by its cytotoxicity, which is caused by the nonspecific binding of the

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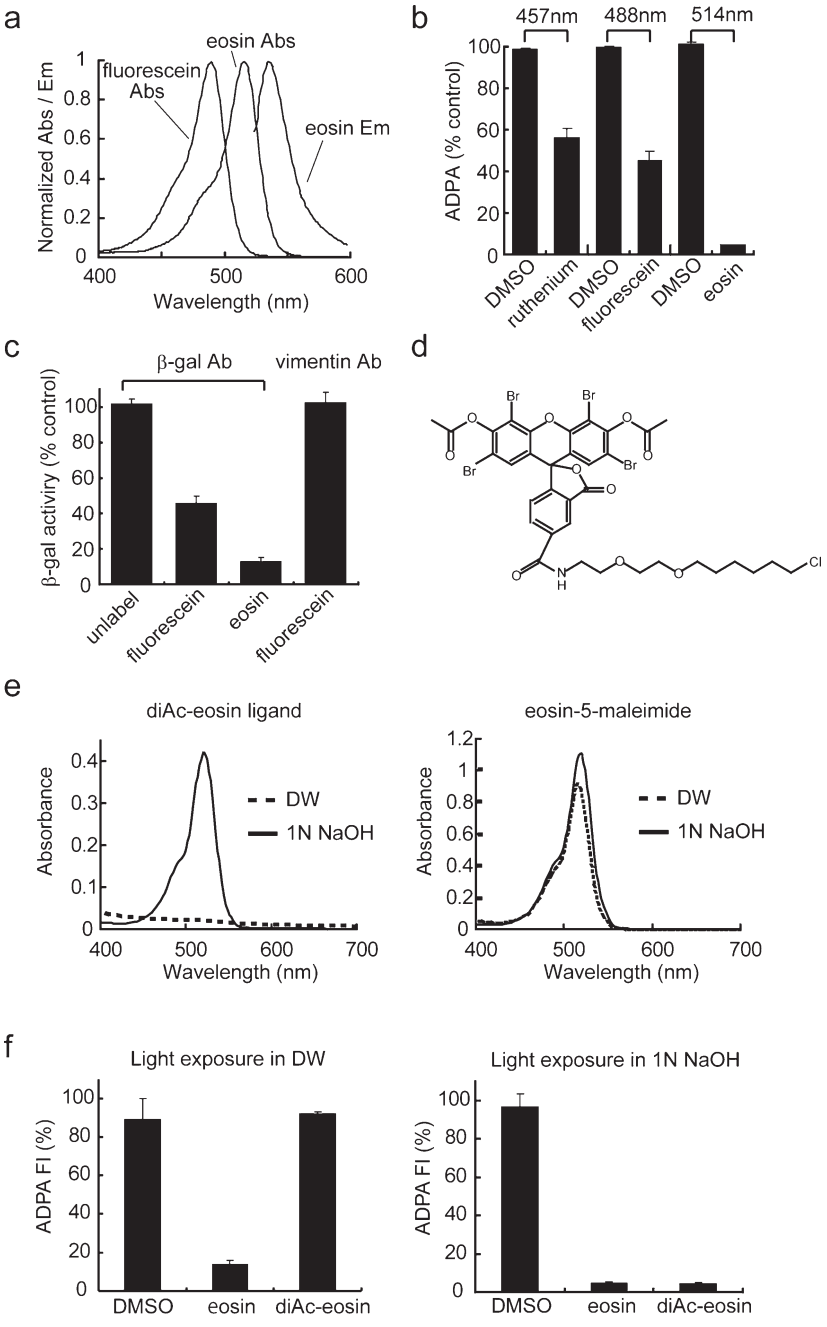

Figure 1. Comparison of photochemical properties of photosensitizing chromophores. (a) Absorption spectra of eosin and fluorescein and the emission spectrum of eosin. Both the absorption and fluorescence spectra were normalized by defining each peak absorption/fluorescence as 1.0. (b) Eosin generates $^1O_2$ more effectively than fluorescein and ruthenium. The amount of $^1O_2$ is shown by the quenching of ADPA fluorescence intensity after same power (0.28 W cm^{-2}) of the 457-, 488-, and 514-nm line of laser irradiation to ruthenium, fluorescein-5-maleimide (fluorescein), and eosin-5-maleimide (eosin), respectively. (c) Inactivation of β-galactosidase activity by CALI using a choromophore-labeled anti-β-galactosidase antibody or anti-vimentin antibody *in vitro*. (d) Molecular structure of diAc-eosin-AM (*N*-{2-[2-(6-chloro-hexyloxy)-ethoxy]-ethyl}-3′,6′-acetyleosin-(5,6)-amide). (e) Absorption spectra of diAc-eosin and eosin-5-maleimide (eosin) before and after their hydrolysis by NaOH. (f) $^1O_2$ generation by eosin-5-maleimide (eosin) and diAc-eosin in response to hydrolysis determined by the quenching of ADPA (anthracene-9,10-dipropionic acid) fluorescence intensity (FI). Abs, absorption; EM, emission. Error bars show SD.

dyes to cysteines in endogenous proteins.[10−12] As an alternative, a SNAP-tag-based labeling system for CALI has been reported.[13] This system utilized covalent labeling of SNAP-tag fusion proteins with fluorescein, and thereby fast and highly specific chromophore labeling has been achieved. The specificity of SNAP-tag-based CALI was demonstrated by acute inactivation of α-tubulin and γ-tubulin during cell division; nevertheless the intense illumination (over 67.5 kJ cm^{-2}) used in this report may raise the risk of nonspecific photodamage. As a similar technique, a method using HaloTag fusion protein labeled with Ru(II) tris-bipyridyl was established, which is an unusually efficient "warhead" for CALI, and markedly superior to the commonly used fluorescein.[14] Actually, Ru(II) tris-bipyridyl exhibited 2-fold stronger performance than fluorescein. However, the light

wavelength to expose Ru(II) tris-bipyridyl contains ultraviolet (>380 nm), which might induce photodamage/phototoxicity. Thus, there remains a need for more potent chromophore to generate $^1O_2$ by longer wavelength illumination for promising CALI.

Results and Discussion. *Characterization of Eosin as a CALI Dye.* To increase the efficacy of CALI, we selected another xanthene-based chromophore, eosin (2′,4′5′7′-tetrabromofluorescein), which has a high quantum yield of $^1O_2$ generation and has thus been used as an effective photooxidizer of diaminobenzidine in high-resolution electron microscopy studies.[15] The absorption peak of eosin is 517 nm (green light), which is 29 nm longer than that of fluorescein (Figure 1, panel a). This longer wavelength of excitation is an advantage in CALI, because cells' endogenous chromophores show less absorption in this range than in the violet/blue light range that is used for Ru(II) tris-bipyridyl and fluorescein excitation. In addition, eosin emits substantial detectable fluorescence, peaking at 538 nm (Figure 1, panel a), which permits the monitoring of intracellular eosin-conjugated proteins by fluorescence microscopy. Furthermore, the amount of the $^1O_2$ generated by eosin was 11 and 13 times as much as that generated by fluorescein and ruthenium, respectively (ADPA fluorescence 4.32 ± 0.22% with eosin, 45.1 ± 4.21% with fluorescein and 55.9 ± 4.43% with ruthenium; Figure 1, panel b). We also confirmed the superiority of eosin for the CALI of β-galactosidase by using an eosin-labeled anti-β-galactosidase antibody, which exhibited about a CALI efficiency 5-fold greater than that of the fluorescein-labeled antibody (Figure 1, panel c). These results motivated us to develop a method using eosin to label intracellular proteins in living cells.

Synthesis of Eosin HaloTag Ligand. The HaloTag labeling method is commonly used to label proteins in living cells with a fluorescent dye.[17,18] In the previous report, the early type of HaloTag protein (HT2) was used to induce protein inactivation with a photosensitizer, ruthenium.[14] In this study, we used HaloTag7, a second-generation haloalkane dehalogenase mutant, since the ability to form a covalent bond with the HaloTag ligand is stronger than that with HT2.[19] To create an eosin-linked ligand for HaloTag7, we synthesized a diAc-eosin-AM (Figure 1, panel d). Like commercially available diAc-FAM, diAc-eosin-AM has two acetyl groups that inhibit its photoabsorption activity. In the case of diAc-FAM, the diacetyl group is hydrolyzed by cellular esterase after its translocation into the cell by the AM group, which restores its absorption capacity. To examine whether the diAc-eosin-AM had the same property, we incubated it with 1 N NaOH *in vitro*. In response to hydrolysis, its absorption increased more than 20-fold over its prehydrolysis level (from 0.02 to 0.41; Figure 1, panel e), and the $^1O_2$ generation was markedly increased by hydrolysis (decrease in ADPA fluorescence intensity from 91.9 ± 1.13% to 4.21 ± 0.69%; Figure 1, panel f), suggesting that the diAc-eosin-AM could function as a photosensitizer only after incorporation into cells. These results inspired us to examine targeted protein labeling and inactivation in living cells using eosin.

Specific Labeling of Intracellular Target with Eosin HaloTag Ligand. To confirm whether diAc-eosin-AM could be applied to label specific intracellular protein, HaloTag7 fused with keratin and actin was expressed in living HeLa cells. After exposing the cells to diAc-eosin-AM and washing away the unbound dye, eosin fluorescence was clearly detected in the pattern of keratin and actin fibers, respectively (Figure 2, panel a). In addition, by expressing HaloTag7 with a mitochondria-targeting sequence,

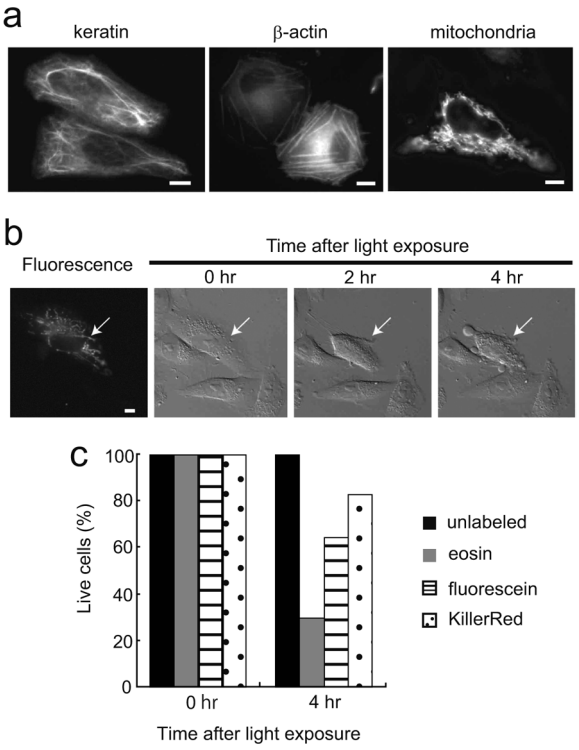

Figure 2. Protein and organelle labeling with eosin in living cells. (a) Localization pattern of HaloTag7-keratin, HaloTag7-actin, and mito-HaloTag7 labeled with eosin HaloTag ligand. (b) Induction of cell death (arrow) by destruction of mitochondrial function using eosin-based CALI. (c) Viability of cells containing unlabeled (n = 9 cells), 1 μM eosin-labeled (n = 10 cells), 1 μM fluorescein-labeled (n = 14 cells), and KillerRed expressing mitochondria (n = 21 cells) 4 h after light irradiation. Scale bars = 5 μm.

the specific labeling of mitochondria with eosin was also observed (Figure 2, panel a), indicating that diAc-eosin-AM with HaloTag7 can be used as an intracellular highlighter.

CALI of Specific Organelle and Protein Labeled with Eosin HaloTag. We next exposed mitochondria to intense light to destroy mitochondrial function in living cells. As expected, most of the cells emitting eosin fluorescence from mitochondria exhibited necrotic morphological change 4 h after light irradiation for 60 s (70%, n = 10, Figure 2, panels b and c). This efficiency was higher in eosin-labeled cells than with fluorescein (36%, n = 14, Figure 2, panel c) and unlabeled cells (0%, n = 9). Furthermore, the efficacy of the eosin-based CALI was also much greater than the KillerRed[20]-based mitochondrial dysfunction, which killed only 18% of the cells despite the longer exposure (90 s) to light (Figure 2, panel c). Taken together, these results demonstrate the powerfulness of eosin in CALI experiment *in vivo*.

Next, we examined the specific inactivation of Protein Kinase C-gamma (PKC-γ), by assessing the translocation ability by stimulation with TPA. For this purpose, PKC-γ was fused with HaloTag7 and mseGFP at the N- and C-termini, respectively (Figure 3, panel a). The HaloTag7-PKC-γ-mseGFP was successfully expressed in cytoplasm, labeled with eosin, and translocated to the plasma membrane within 5−15 min after TPA stimulation (Figure 3, panel b). In contrast, if the cells were irradiated with intense green light (520−560 nm), the HaloTag7-PKC-γ-mseGFP failed to show the translocation (Figure 3, panels c

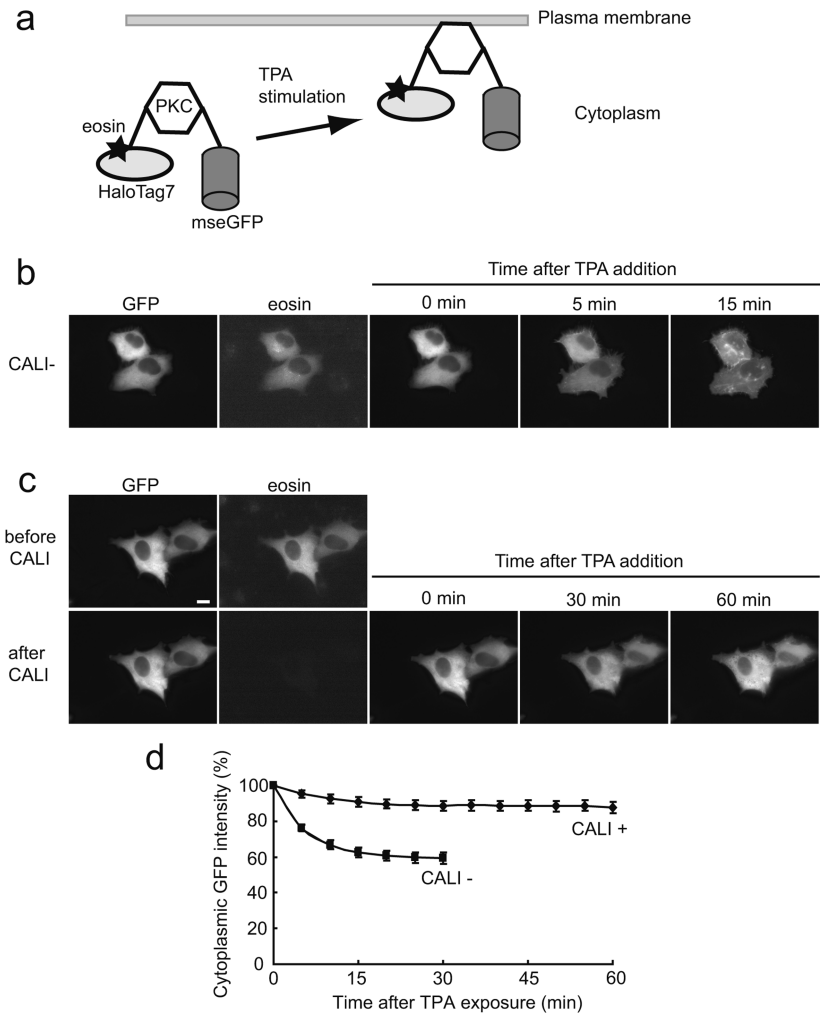

Figure 3. PKC-γ inactivation by eosin-based CALI in living cells. (a) Schematic representation of the HaloTag7-PKC-γ-mseGFP fusion protein and its translocation to the membrane after TPA(12-*O*-tetradecanoylphorbol 13-acetate) treatment. (b) Control experiment (CALI−). After TPA addition, PKC-γ successfully translocated to the plasma membrane. (c) CALI of PKC-γ. The eosin fluorescence disappeared after irradiation with intense light resulting in failure of PKC translocation to the plasma membrane. Cells were examined by time-lapse analysis for the CALI− (n = 21 cells) and CALI+ (n = 18 cells) conditions, respectively. Representative data are shown in panels b and c. (d) Time course analysis of PKC-γ translocation to the plasma membrane with (CALI+) or without (CALI−) CALI. The PKC-γ activation was evaluated by the decrease in fluorescence intensity in the cytoplasm due to the protein's translocation to the plasma membrane upon TPA stimulation. Error bars are SD. Scale bar = 5 μm.

and d), indicating the success of CALI. The input light energy totaled 0.792 kJ cm^{-2} (8.8 W cm^{-2} for 90 s), which was enough to bleach out the eosin fluorescence but 85 times lower than that (67.5 kJ cm^{-2}) used in a previous report in which SNAP-tagged α-tubulin was destroyed by fluorescein-based CALI,[14] indicating the power of the eosin-based CALI of target protein in living cells.

Regiospecific CALI of Eosin-Labeled HaloTag-Aurora B with a CW Laser. To validate whether our CALI method could be applied to acute inactivation of target protein in spatiotemporal manner, we chose aurora B as the target. Until now, inactivation of aurora B labeled with GFP was only conducted by multiphoton absorption-based CALI (MP-CALI) with intense irradiation of femtosecond pulsed laser.[21] To examine the possibility of eosin-based CALI of aurora B, we used a conventional cw laser as the light source. During the metaphase, eosin-labeled aurora B was observed at the spindle midzone (Figure 4, panel a). After laser irradiation in the central region of aurora B-eosin fluorescence, cell division was completely stopped or multinuclear structure was formed after cell division (100%, n = 11 cells, Figure 4, panels a and c). In contrast, unlabeled control cells seemed to be healthy, and normal cell division progressed within 2 h after laser irradiation (93%, n = 14 cells, Figure 4, panels b and c). These results indicate that eosin can be used for spatiotemporal inactivation of target proteins by single photon absorption using a cw laser equipped with commonly available microscope.

Perspective of CALI Technology in Bioscience. We have shown successful CALI for not only an enzyme with the eosin-labeled antibody but also organelle and intracellular proteins labeled with eosin-HaloTag without the risk of nonspecific photodamage caused by light illumination. The superior properties of this eosin-based CALI technique will be useful for the broad exploration of highly localized protein functions in living samples, particularly when it is used in combination with the "Flexi HaloTag" clone library. This library covers more than 4,000 (final clone number will reach to about 18,500) ORFs of the human genome fused to the HaloTag gene and inserted into a mammalian expression vector (http://www.kazusa.or.jp/kop/halotag/).

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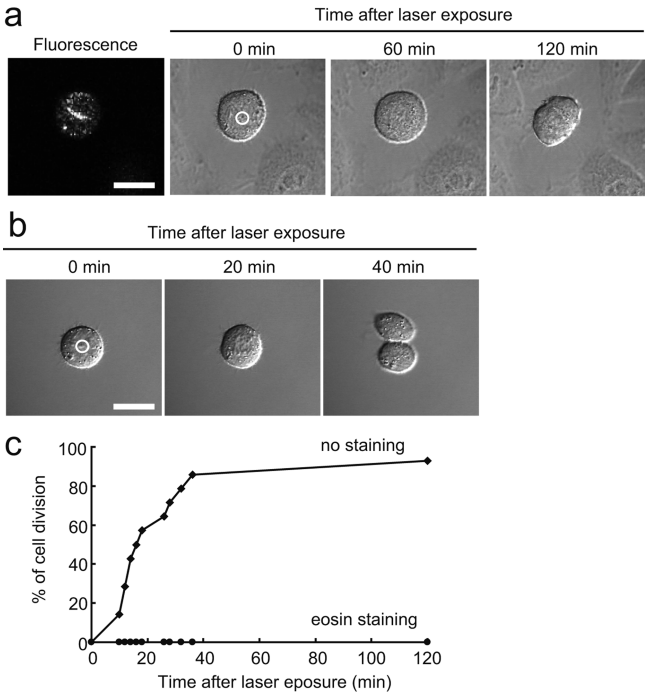

Figure 4. Interference of cell division by CALI on eosin-HaloTag-aurora B. (a) Failure of mitosis after 514 nm laser irradiation in the central region of the condensed chromosome where eosin-labeled aurora B was localized (leftmost panel) as revealed by time laps imaging (100%, $n = 11$). (b) Negative control experiment of aurora B-HaloTag without staining. Cell division normally occurred within 2 h after laser exposure (0%, $n = 14$). (c) Time course of cell division rate in eosin-stained or unstained cells after laser irradiation. Scale bar = 20 μm.

However, there still remains a potential drawback to HaloTag-based CALI, *i.e.*, the CALI phenotype could be obscured by the endogenous, unlabeled protein that is not susceptible to light inactivation. In this point of view, conventional antibody-based CALI would be applied in combination with eosin for spatio-temporal inactivation of target molecules. Alternatively, performing HaloTag-based experiments in deficient cells rescued with functional HaloTag-fusion proteins permits more complete loss of function to be achieved. As previously shown, a modified lentiviral system would be applicable for rapid and efficient generation of knockdown cell lines complemented with physiological levels of exogenous fusion proteins.[22]

METHODS

Gene Construction. mito-HaloTag7 expression vector was constructed by fusion of a duplicated mitochondrial targeting sequence derived from the subunit VIII precursor of human cytochrome C oxidase at the N-terminus of HaloTag7 and cloned into pcDNA3 vector (Invitrogen), yielding mito-Halotag7-pcDNA3. To construct a vector for keratin-HaloTag7 expression in mammalian cells, the HaloTag7 gene with an *Eco*RI and a *Not*I site at the 5′- and 3′-ends, respectively, was amplified by PCR and then substituted for mTFP1 in the mTFP1-keratin[23] vector, yielding HaloTag7-keratin-pcDNA3. To construct a vector for HaloTag7-actin expression in mammalian cells, the HaloTag7 gene with a *Nhe*I and *Xho*I site at the 5′- and 3′-ends, respectively, was amplified by PCR and then substituted for EGFP in pEGFP-Actin (Clontech), yielding pHaloTag7-Actin. To construct a vector for HaloTag7-PKCγ-mseGFP expression in mammalian cells, the HaloTag7 gene with a *Hind*III and a *Kpn*I site and a partial sequence encoding N-terminal portion of PKC-γ were amplified by PCR. The digested fragments were cloned into pcDNA3 simultaneously with a partial sequence encoding C-terminal portion of PKC-γ obtained from the pPKCγ-DsRed1 (Clontech) by digestion with *BamH*I/*Eco*RI, followed by insertion of mseGFP at the 3′-end of PKC-γ sequence using an *Eco*RI/*Xba*I site, yielding HaloTag7-PKCγ-mseGFP-pcDNA3. The mseGFP was made by introducing mutations of M153T, V163A, S175G,[24] and A206K,[25] using a method previously described.[26]

$^1O_2$ Measurement. $^1O_2$ was measured using anthracene-9,10-dipropionic acid (ADPA, Invitrogen) as described previously.[16] A laser with 3 mm diameter at 0.35 W cm^{-2} was used to illuminate a 10-μL sample on a Terasaki plate. Each chromophore concentration was 200 μM in methanol containing 100 μM ADPA. After light exposure, the quenching of ADPA fluorescence at 405-nm excited by 360 nm was measured using an F-2500 fluorescence spectrophotometer (Hitachi).

Labeling Antibody with Eosin. The anti-β-galactosidase and anti-vimentin antibodies were purchased from Cappel (no. 55976) and Sigma (no. V4630), respectively. The antibody (50 μg) was incubated with 40 μg mL^{-1} eosin-5-isothiocyanate (Invitrogen) in 0.5 M $NaHCO_3$ (pH 9.5) for 30 min at RT. The solution was then changed to 50 mM Tris-HCl (pH 7.4), 150 mM KCl using a NAP-5 column (GE Healthcare) to remove the free eosin-5-isothiocyanate dye.

CALI of β-Galactosidase. The dye-labeled antibody was diluted to 200 μg mL^{-1} and incubated with β-galactosidase (10 μg mL^{-1}) in PBS. A 10-μL reaction solution was irradiated with intense light (488 nm argon ion laser, 0.28 W cm^{-2}) for 30 s. After light exposure, the β-galactosidase activity was measured by the reduction in 420-nm absorption due to the cleavage of *o*-nitrophenyl-β-D-galactopyranoside (Invitrogen).

Synthesis of the HaloTag Ligand, diAc-Eosin. To synthesize carboxyeosin diacetate, succinimidyl ester (5 mg, 5.72×10^{-6} mol) and 2-[2-(6-chloro-hexyloxy)-ethoxy]-ethylammonium hydrochloride (6.6 mg, 2.86×10^{-5} mol) in 1 mL of CH_2Cl_2 was mixed with diisopropylethylamine (DIPEA) (5 μL, 3×10^{-5} mol), and stirred overnight. After evaporation of the solvent, 1 mL of acetic anhydride was stirred with the crude product for 0.5 h. The volatile reaction components were removed from the crude product, which was then purified by flash chromatography using a Teledyne Isco CombiFlash R_f on a 2 g prepacked silica column, with a 50% (v/v) to 75% (v/v) gradient of ethyl acetate in heptane. Carboxyeosin diacetate, succinimidyl ester was from Invitrogen. All other reagents and solvents for chemical synthesis were from Aldrich, Fisher, or VWR and were used without further purification.

CALI of Mitochondria and PKC-γ. HeLa cells cultured in Dulbecco's modified Eagle's medium containing 10% (v/v) heat-inactivated fetal calf serum on a glass-bottomed dish with a 35 mm diameter (Matsunami) were transfected with the expression vectors using Superfect (Qiagen) according to the manufacturer's protocol. diAc-eosin-AM ligand was added to the growth medium 24−30 h after transfection. After a 15-min incubation at 37 °C, the cells were washed with PBS several times and incubated for another 30 min in growth medium to completely remove free ligand. CALI was performed on an inverted microscope (TE2000, Nikon) using a 60x NA1.4 oil immersion objective (Apo-VC; Nikon). The samples were illuminated for 60 s with green light obtained through a 540AF30 interference filter (Omega). The light power was set to 8.8 W cm^{-2} by adjusting the aperture stop in the excitation optics. After irradiation with intense light, a DIC image or fluorescence image of the HeLa cells was taken by illumination with a lower-intensity light (<0.1 W cm^{-2}) using a cooled CCD camera, ORCA-AG (Hamamatsu Photonics) under the control of the AquaCosmos software (Hamamatsu Photonics). To induce PKCγ translocation, TPA (12-*O*-Tetradecanoylphorbol 13-acetate) was used.

CALI of Aurora B by CW Laser. For regiospecific CALI experiments, HeLa cells were arrested at G1/S transition using the double thymidine block method.[27] Ten hours after the second thymidine block,

CALI and DIC imaging of the cell division was started using the Olympus confocal inverted microscope FV1000 equipped with UPLSA-PO 60x 1.35 numerical aperture (NA) oil objective and multi-argon ion laser. 514 nm laser was locally exposed in the central region of aurora B-eosin fluorescence.

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REFERENCES

(1) Dorsett, Y., and Tuschl, T. (2004) siRNAs: applications in functional genomics and potential as therapeutics. *Nat. Rev. Drug Discovery 3*, 318–329.

(2) Jay, D.-G. (1988) Selective destruction of protein function by chromophore-assisted laser inactivation. *Proc. Natl. Acad. Sci. U.S.A. 85*, 5454–5458.

(3) Yan, P., Xiong, Y., Chen, B., Negash, S., Squier, T. C., and Mayer, M. U. (2006) Fluorophore-assisted light inactivation of calmodulin involves singlet-oxygen mediated cross-linking and methionine oxidation. *Biochemistry 45*, 4736–4748.

(4) Beck, S., Sakurai, T., Eustace, B. K., Beste, G., Schier, R., Rudert, F., and Jay, D. G. (2002) Fluorophore-assisted light inactivation: a high-throughput tool for direct target validation of proteins. *Proteomics 2*, 247–255.

(5) Linden, K.-G., Liao, J.-C, and Jay, D.-G. (1992) Spatial specificity of chromophore assisted laser inactivation of protein function. *Biophys. J. 62*, 956–962.

(6) Jacobson, K., Rajfur, Z., Vitriol, E., and Hahn, K. (2008) Chromophore-assisted laser inactivation in cell biology. *Trends Cell Biol. 18*, 443–450.

(7) Surrey, T., Elowitz, M. B., Wolf, P.-E., Yang, F., Nedelec, F., Shokat, K., and Leibler, S. (1998) Chromophore-assisted light inactivation and self-organization of microtubules and motors. *Proc. Natl. Acad. Sci. U.S.A. 95*, 4293–4298.

(8) Marek, K.-W., and Davis, G.-W. (2002) Transgenically encoded protein photoinactivation (FlAsH-FALI): acute inactivation of synaptotagmin I. *Neuron 36*, 805–813.

(9) Tour, O., Meijer, R.-M., Zacharias, D.-A., Adams, S.-R., and Tsien, R.-Y. (2003) Genetically targeted chromophore-assisted light inactivation. *Nat. Biotechnol. 21*, 1505–1508.

(10) Hearps, A., Pryor, M. J., Kuusisto, H. V., Rawlinson, S. M., Piller, S. C., and Jans, D. A. (2007) The biarsenical dye Lumio exhibits a reduced ability to specifically detect tetracysteine-containing proteins within life cells. *J. Fluoresc. 17*, 593–597.

(11) Martin, B.-R., Giepmans, B.-N., Adams, S.-R., and Tsien, R.-Y. (2005) Mammalian cell-based optimization of the biarsenical-binding tetracysteine motif for improved fluorescence and affinity. *Nat. Biotechnol. 23*, 1308–1314.

(12) Stroffekova, K., Proenza, C., and Beam, K.-G. (2001) The protein-labeling reagent FLASH-EDT2 binds not only to CCXXCC motifs but also non-specifically to endogenous cysteine-rich proteins. *Pflugers Arch. 442*, 859–886.

(13) Keppler, A., and Ellenberg, J. (2009) Chromophore-assisted laser inactivation of alpha- and gamma-tubulin SNAP-tag fusion proteins inside living cells. *ACS Chem Biol. 4*, 127–138.

(14) Lee, J., Yu, P., Xiao, X., and Kodadek, T. (2008) A general system for evaluating the efficiency of chromophore-assisted light inactivation (CALI) of proteins reveals Ru(II) tris-bipyridyl as an unusually efficient "warhead. *Mol. Biosyst. 4*, 59–65.

(15) Deerinck, T.-J. (1994) et al. Fluorescence photooxidation with eosin: a method for high resolution immunolocalization and in situ hybridization detection for light and electron microscopy. *J. Cell Biol. 126*, 901–910.

(16) Hoebeke, M., Piette, J., and van de Vorst, A.-J. (1991) Photosensitized production of singlet oxygen by marocyanine 540 bound to liposomes. *Photochem. Photobiol. B 9*, 281–294.

(17) Los, G.-V., Encell, L. P., McDougall, M. G., Hartzell, D. D., Karassina, N., Zimprich, C., Wood, M. G., Learish, R., Ohana, R. F., Urh, M., Simpson, D., Mendez, J., Zimmerman, K., Otto, P., Vidugiris, G., Zhu, J., Darzins, A., Klaubert, D. H., Bulleit, R. F., and Wood, K. V. (2008) HaloTag: a novel protein labeling technology for cell imaging and protein analysis. *ACS Chem Biol. 3*, 373–382.

(18) Los, G.-V., and Wood, K. (2007) The HaloTag: a novel technology for cell imaging and protein analysis. *Methods Mol. Biol. 356*, 195–208.

(19) Ohana, R.-F., Encell, L.-P., Zhao, K., Simpson, D., Slater, M.-R., Urh, M., and Wood, K.-V. (2009) HaloTag7: a genetically engineered tag that enhances bacterial expression of soluble proteins and improves protein purification. *Protein Expression Purif. 68*, 110–120.

(20) Bulina, M-E (2006) et al. A genetically encoded photosensitizer. *Nat. Biotechnol. 24*, 95–99.

(21) Tanabe, T., Oyamada, M., Fujita, K., Dai, P., Tanaka, H., and Takamatsu, T. (2005) Multiphoton excitation-evoked chromophore-assisted laser inactivation using green fluorescence protein. *Nat. Methods 2*, 503–505.

(22) Vitriol, E.-A., Uetrecht, A.-C., Shen, F., Jacobson, K., and Bear, J.-E. (2007) Enhanced EGFP-chromophore-assisted laser inactivation using deficient cells rescued with functional EGFP-fusion proteins. *Proc. Natl. Acad. Sci. U.S.A. 17*, 6702–6707.

(23) Ai, H.-W., Henderson, J.-N., Remington, S.-J., and Campbell, R.-E. (2006) Directed evolution of monomeric, bright and photostable version of Clavularia cyan fluorescent protein: structural characterization and applications in fluorescence imaging. *Biochem. J. 400*, 531–540.

(24) Crameri, A., Whitehorn, E.-A., Tate, E., and Stemmer, W.-P.-C. (1996) Improved green fluorescent protein by molecular evolution using DNA shuffling. *Nat. Biotechnol. 14*, 315–319.

(25) Zacharias, D.-A., Violin, J.-D., Newton, A.-C., and Tsien, R.-Y. (2002) Partitioning of lipid-modified monomeric GFPs into membrane microdomains of live cells. *Science 296*, 913–916.

(26) Sawano, A., and Miyawaki, A. (2000) Directed evolution of green fluorescent protein by a new versatile PCR strategy for site-directed and semi-random mutagenesis. *Nucleic Acids Res. 28*, E78.

(27) Bootsma, D., Budke, L., and Vos, O. (1964) Studies on synchronous division of tissue culture cells initiated by excess thymidine. *Exp. Cell Res. 33*, 301–309.