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Mechanism-Based Design of a Photoactivatable Firefly Luciferase

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Supporting Information

ABSTRACT: We developed a photoactivatable firefly luciferase (pfLuc) whose activation can be controlled by light. A photocaged Lys analogue was site-specifically incorporated into fLuc to replace its key catalytic Lys residue, Lys529, rendering fLuc inactive until light-triggered removal of the caging group. This photoinduced gain of luminescence provides a facile approach for assessing the photolysis efficiency of this valuable photosensitive Lys analogue within the context of its carrier protein *in vitro* and in living cells. We further took advantage of the spatial and temporal activation feature of pfLuc for intracellular measurement of labile ATP levels without impairment of cellular physiology.

Photoactivation of intracellular proteins has recently emerged as a powerful strategy for spatial and temporal control of the activity of proteins, the most abundant biomolecules within a cell.[1] Various methods have been developed to employ light toward noninvasive modulation of protein functionality and/or localization in a native cellular context.[2] Among them, photocaged small molecules, typically bearing a light-cleavable blockage moiety attached to an effector molecule or an amino acid, have been utilized for *in situ* manipulation of protein activity with excellent efficiency and precision.[3] In particular, the repertoire of photocaged unnatural amino acids (UAAs) has been largely expanded lately, permitting the use of light to directly manipulate a specific amino acid residue on a given protein in diverse living species.[4] Also, besides modulating biologically important proteins such as p53 and kinases,[4d,e] photocaged UAAs have also been site-specifically incorporated into fluorescent proteins to generate photoactivatable marker proteins, commonly referred to as "molecular highlighters".[5]

The luciferase-based bioluminescent imaging/reporting technique features low background, high sensitivity, and quantitative capability as opposed to the fluorescent imaging methods.[6] The intracellular activation of bioluminescence probes in a spatial and temporal fashion may allow more precise tracking of the cellular events or gene expression within intact cells or animals.[7] This idea has been demonstrated by the development of "photocaged" bioluminescent substrates which, upon light activation, is converted into luciferin, the cognitive substrate of firefly luciferase (fLuc).[8] Yet, direct caging of fLuc, the corresponding bioluminescence enzyme, has not been achieved. Since the applications of current "caged" luciferin analogues are largely hampered by their low stability or poor membrane permeability, a genetically encoded "photocaged" luciferase may offer a better signal-to-noise ratio and higher specificity, which are essential for bioluminescence detection. Also, this approach may provide an independent, quantitative method for measuring the activation efficiency of the widely used photocaged Lys analogues within the context of their carrier proteins inside cells. Currently, the time scale for intracellular "decaging" of photocaged amino acids incorporated into the protein of interest (POI) can only be estimated by downstream biological events or by MS analysis, which can be inaccurate or time and labor consuming. A direct linkage between the photolysis efficiency and a bioluminescent readout would allow assessment of *in situ* free Lys generation from their photocaged precursors on the POI within living cells. Herein we report the development of such a photocaged fLuc, named pfLuc, whose masked catalytic activity can be restored upon exposure to light within intact cells.

Conversion of luciferin to the highly luminescent oxyluciferin is catalyzed by fLuc in a two-step process: the carboxylate group on luciferin is first adenylated with Mg-ATP by fLuc, which is then oxidized to yield the oxyluciferin product.[9] Lysine 529 on fLuc has been shown as a key catalytic residue for effective substrate orientation between luciferin and Mg-ATP (Scheme 1a), providing favorable polar interactions crucial for stabilizing the transition state that will ultimately yield the adenylated product.[10] Mutation of Lys529 to Arg caused a considerable reduction of fLuc activity (>600-fold), whereas the loss of the positively charged side chain from the K529Q and K529A mutants decreased its activity by over 1600-fold.[10] We envisioned that replacing this critical Lys residue with a genetically encoded photocaged Lys analogue may block the substrate binding in the active site and thus disrupt fLuc-catalyzed adenylation on luciferin (Scheme 1b,c). Photolysis with near-visible light to remove the caging group would regenerate a free Lys, leading to restored catalytic activity on fLuc.

We started by generating the photocaged version of fLuc using *o*-nitrobenzyloxycarbonyl-N^{ε}-lysine (ONBK, Scheme 1b), a photocaged Lys analogue carrying an *o*-nitrobenzyloxycarbonyl group that can be readily removed by 365-nm light.[4b] A mutant aminoacyl-tRNA synthetase (named "NBK-1") derived from the

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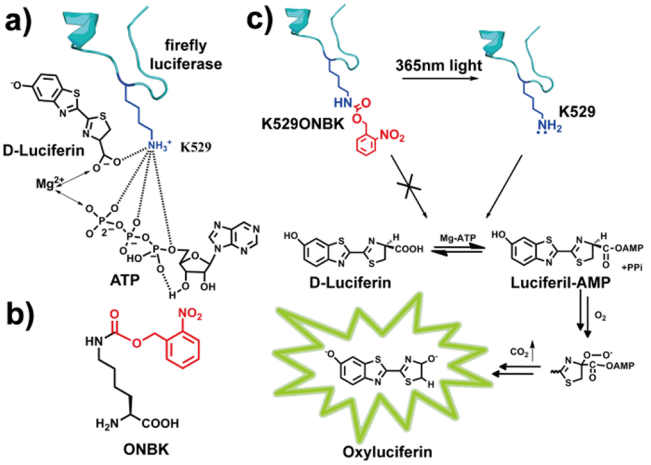
Scheme 1. Design of the pfLuc[a]



[a](a) Schematic representation of the "enzyme–substrate" complex of fLuc interacting with luciferin and Mg-ATP. The ε-ammonium ion of Lys529 forms H-bonds with luciferin and ATP. This interaction may help to stabilize the orientation of luciferin and Mg-ATP for adenylation reaction. (b) Structure of ONBK. (c)When Lys529 is replaced by ONBK, it no longer adenylates luciferine with Mg-ATP, resulting in abandoned enzymatic activity. Photolysis would regenerate free Lys and restore fLuc's activity in catalyzing the conversion of D-luciferin to the luminescent oxyluciferin in the presence of Mg-ATP.

pyrrolysyl-tRNA synthetase in *M. mazei* has been previously shown to work in conjunction with its cognitive pyrrolysyl-$tRNA^{Pyl}_{CUA}$ to site-specifically incorporate ONBK into proteins in both *E. coli* and mammalian cells. The codon corresponding to the crucial Lys529 residue on fLuc was mutated to the amber codon TAG followed by cotransfection with a plasmid containing both NBK-1 and $tRNA^{Pyl}_{CUA}$ into HEK293T cells. Expression of the full length fLuc carrying ONBK at residue 529 (fLuc-K529ONBK) in the presence of 1 mM ONBK was verified by immunoblotting analysis with an anti-His antibody to the C-terminal Histag on fLuc protein (Figure 1a, bottom). Expression of fLuc-K529ONBK was also successfully conducted in *E. coli* bacterial cells (Figure S1).

Lysate from cells expressing pfLuc was then subjected to luminescence analysis, which showed no measurable luciferase activity before photolysis. By contrast, a significant increase of bioluminescence signal was observed when the same batch of cell lysate was exposed to a low dose of UV irradiation (365 nm, 0.3 mW/cm^2; Figure 1a). Notably, the "Relative WT activity" of pfLuc (the luminescence intensity of photoactivated pfLuc deducted by the amount of protein and then normalized with that of wild-type fLuc; see Supporting Information for detailed calculation) after photolysis for 10 and 20 min reached 80% and 93%, respectively (Figure 1b). The time-dependent photolysis on aliquots of cell lysate bearing the same amount of pfLuc showed a photodeprotection half-life of 5.8 min (Figure 1c). In addition, we used the chemiluminescent channel from the ChemiDoc instrument (Bio-Rad) to directly monitor pfLuc activation. Lysate from HEK293T cells expressing pfLuc was transferred to a 96-well plate followed by photoactivation for different times (365 nm, 0.3 mW/cm^2, Figure 1d). Bioluminescence was clearly detectable with a photolysis time over 1 min. Therefore, pfLuc directly links the decaging efficiency of ONBK with a bioluminescent readout, offering a facile approach for measuring the activation efficiency of this widely used photocaged Lys analogue in the context of a POI.

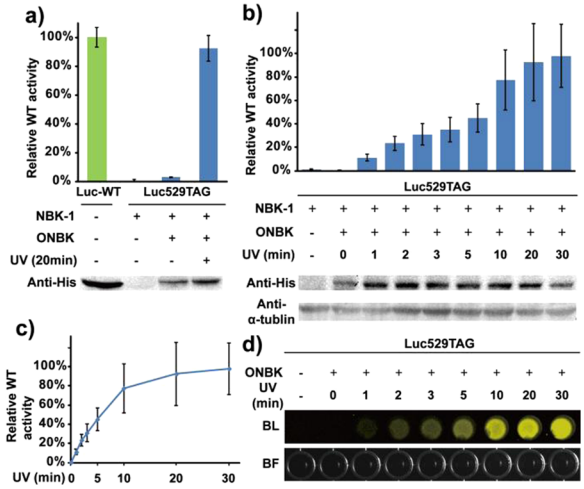


Figure 1. Generation and activation of pfLuc. (a) HEK293T cells expressing WT-fLuc or pfLuc supplemented with or without 1 mM ONBK were UV-irradiated (365 nm, 20 min, 0.3 mW/cm^2) and analyzed by luciferase assay. The relative luminescence of pfLuc was compared with that of WT-Luc and referred as "relative WT activity". Error bars represent ± s.d. from three independent experiments. Immunoblotting analysis was used to compare the amount of luciferase protein carrying a C-terminal Histag ($fLuc_{His6}$) being used. (b)Time-dependent activation of pfLuc in mammalian cells. Cells expressing pfLuc were irradiated for different times between 0 and 30 min before the luminescence signal was measured and normalized as "relative WT activity". Cells without ONBK supplementation were used as a control. Immunoblotting analysis was employed to show that the same amount of pfLuc protein was used for photolysis. Error bars represent ± s.d. from three independent experiments. (c) Photoactivation curve of pfLuc in HEK293T cells. (d)Visualization of time-dependent activation of pfLuc using the chemiluminescent channel in ChemiDoc with bright field (BF) images (bottom) taken as controls.

Next, we demonstrated photoactivation of pfLuc in living cells. HEK293T cells expressing pfLuc were treated with or without 365-nm light low-dose irradiation for 20 min (0.3 mW/cm^2) followed by imaging from the chemiluminescent channel on ChemiDoc (Figure 2a). Bright luminescence was observed for cells treated with 365-nm light, whereas the same batch of cells without UV treatment exhibited negligible background luminescence. Time-dependent photoactivation was also performed by irradiation of HEK293T cells expressing pfLuc in a 96-well optical bottom plate for varying times between 0 and 30 min (0.3 mW/cm^2). The bioluminescence images taken by ChemiDoc showed that a 3–5 min photolysis could yield visible luminescence, while a brighter level of luminescence could be observed from cells being photoactivated for 10 min or longer. To determine the effects of our UV-irradiation experiments on cell viability, HEK293T cells after UV-treatment (0.3 mW/cm^2, 20 min) were subjected to an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (Figure S3). No apparent cell death was observed, confirming that cells were viable throughout our experiments. Finally, two culture dishes containing HEK293T cells expressing pfLuc were covered by aluminum foils in patterns resembling a character "U" or a cross before photolysis. Only those cells that were not blocked by the aluminum foil exhibited bright luminescence, whereas the covered cells remained as the dark background, resulting in a clearly visible pattern (Figure 2c). Together, we showed that our pfLuc can be spatially and temporally activated by light in living cells.

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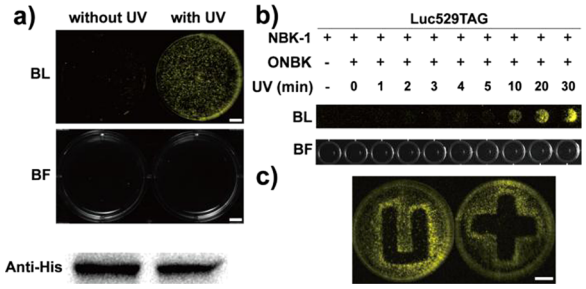

Figure 2. Monitoring the spatial-temporal activation of pfLuc in living cells. (a) HEK293T cells expressing pfLuc was treated with and without UV irradiation (365 nm, 20 min, 0.3 mW/cm^2) before bioluminescence (BL) images were directly taken on live cells (upper). Bright field (BF) images were taken (bottom) as a control. Immunoblotting analysis of the full-length pfLuc$_{His6}$ is shown below. Scale bars: 5 mm. (b) Time-dependent photoactivation of pfLuc in living cells. HEK293T cells expressing pfLuc were seeded in a 96-well optical bottom plate before being photoactivated for varying times. BL images (upper) and BF images (bottom) were taken on live cells. (c) Patterning of living cells expressing pfLuc via UV irradiation. HEK293T cells expressing pfLuc were irradiated for 20 min before visualization under ChemiDoc. Scale bar: 10 mm.

To further take advantage of its spatial and temporal activation feature, we employed pfLuc to measure labile ATP within living cells. As a central energy currency in all forms of living systems, ATP plays critical roles in diverse biological processes[11] and also serves as a signaling molecule with highly dynamic intracellular distribution.[12] Therefore, monitoring ATP generation and consumption, particularly those labile ATP molecules within living cells, is highly desirable.[13] Since fLuc catalyzes the conversion of luciferin to the highly luminescent oxyluciferin at the cost of ATP, fLuc has been routinely used as a luminescent reporter for ATP quantification in biological samples.[14] This fLuc-based detection method is advantageous over the fluorescent protein-based imaging technique in terms of quantitative ability, because a statistically significant number of cells can be analyzed with excellent sensitivity.[15] However, since fLuc-reporters typically measure the total ATP levels from cell extracts rather than intact cells, the free ATP concentration within cytoplasm or other compartments of a live cell cannot be obtained from this approach. Although attempts have been made for luminescence-based assessment of intracellular ATP levels by expressing fLuc in cells supplemented with the luciferin substrates, the utility of such methods is limited.[14a,16] A major issue is the consumption of intracellular ATP molecules by constitutively expressed fLuc in its active form, which may perturb cellular ATP homeostasis and thus cellular physiology. This cannot be circumvented by simply adding luciferin substrates to the fLuc-expressing cells right before the measurement, since heterogeneously distributed luciferin molecules in different subcellular compartments may affect the accuracy of such an analysis.[17] We reasoned that our pfLuc may help address these challenges as it mimics the inactive form of fLuc that is unable to consume ATP even when luciferin is present in cells. The subsequent photolysis would generate fully active pfLuc capable of hydrolyzing ATP, thus accurately detecting ATP levels, within a cell.

We first tested this hypothesis by intracellular expression and temporal activation of pfLuc for ATP measurement. As expected, the pfLuc-expressing cells without UV irradiation yielded no detectable signal when measured on cell extracts containing luciferin (Figure S12a). To activate pfLuc, we irradiated cells by 365-nm light for 10 min (0.3 mW/cm^2) followed by lysis of cells for luminescence measurement in the presence of luciferin. Indeed, a significant increase of luminescence was observed from the pfLuc-expressing cells under this photoactivation condition (Figure S12a). Further, the addition of sodium azide (NaN_3, an inhibitor of oxidative phosphorylation, OXPHOS) or 2-dexoy-D-glucose (2-DG, an inhibitor of glycolysis), two inhibitors known to impair intracellular ATP synthesis, were found to generate a lower luminescence signal than the nontreated cells (Figure S12b). Notably, concurrent treatment with 10 mM NaN_3 and 10 mM 2-DG led to a further decrease of luminescence signal, indicating the different mode-of-action for these ATP synthesis inhibitors. In addition, we replaced the primary energy source, glucose by fructose or galactose, which are metabolized at a slower rate than glucose and thus decrease ATP levels inside cells. As expected, cells grown in fructose- or galactose-supplemented medium both showed a significant drop in the cellular ATP level that was evident from our fLuc-based whole-cell measurement (Figure S4). Taken together, our pfLuc strategy allowed temporal activation of this powerful enzyme, enabling intracellular ATP measurement without perturbation of ATP homeostasis from a constitutively expressed fLuc.

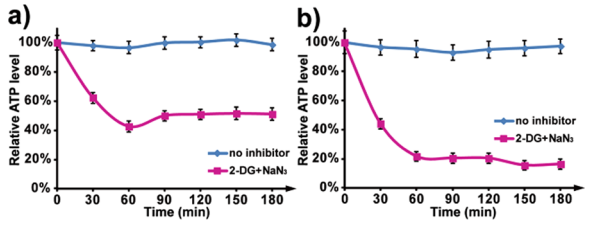

Figure 3. Monitoring intracellular ATP dynamics in live cells versus cell extracts. (a) Live HEK293T cells expressing pfLuc were preincubated with 1 mM luciferin for 180 min followed by the treatment with (magenta curve) and without (blue curve) inhibitors at time = 0 min. Bioluminescence from intact cells was taken at different elapsed time points with UV-activation (10 min, 0.3 mW/cm^2) conducted immediately before each measurement. Error bars represent ± s.d. of six independent experiments. (b) The pfLuc-expressing cells were treated with (magenta curve) and without (blue curve) the same inhibitors at time = 0 min, followed by UV-activation, cell lysis, and bioluminescence measurement at different elapsed time points in the presence of luciferin (1 mM). Error bars represent ± s.d. from six independent experiments.

Next, to further expand its utility, we employed pfLuc to monitor intracellular ATP dynamics according to an experimental procedure shown in Figure S6a. The intracellular ATP levels of live cells were found to be decreased by near 60% within 60 min, which was followed by a slight recovery (<10%) at ≥90 min. At time = 180 min, both inhibitors were washed away and cellular ATP recovery was monitored by pfLuc. As expected, the intracellular ATP level can be fully recovered with overnight incubation (~8 h, Figure S8).

For comparison, we also measured ATP on cell extracts according to a procedure shown in Figure S6b. Cellular ATP levels measured in this approach showed an over 80% decrease within 60 min after inhibitor treatment and remained stable afterward. ATP recovery was also observed after overnight incubation (~8 h) of cells with both inhibitors removed at time = 180 min (Figure S9). Taken together, in contrast to the cell-extract analysis that only measures the change in the total amount of cellular ATP, the temporal-activatable, cytoplasm-residing pfLuc is advantageous for noninvasive and specific monitoring of

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ATP fluctuation within the cytoplasmic space of living cells (Figure S11). Our pfLuc tool thus helped reveal that the whole-cell ATP level decreased to a larger extent than that in cytoplasm upon the treatment of ATP synthesis inhibitors.

Indeed, a higher degree of ATP depletion can be found in certain subcellular compartments such as mitochondria, which are major suppliers for cellular ATP that are sensitive to NaN_3-mediated inhibition of OXPHOS. This may explain the observed different degrees of ATP variation between the cytoplasm and entire cell. Furthermore, our live cell-based detection showed an evident ATP recovery process that was undetectable from measurements on cell extracts. This is in line with a previous report from a genetically encoded fluorescent ATP indicator.[18] The differences in terms of time scale and recovery extent between these two studies may be due to the different cell lines being used or the intrinsic variations between the luciferase- and GFP-based techniques. This recovery process suggested that cells are able to use alternative sources for ATP synthesis that are not inhibited by NaN_3 or 2-DG. Nevertheless, our study further underlined the importance of real-time monitoring of ATP dynamics with subcellular resolution. Targeting pfLuc to different organelles is currently underway in our laboratory, which may help resolve ATP distributions within different organelles.

In summary, we have developed a photoactivatable firefly luciferase by masking its catalytic Lys residue, Lys529, with a photocaged Lys analogue, ONBK. Light-triggered activation of pfLuc led to a gain-of-luminescence signal in cell extracts and within intact cells. Post-translational Lys modifications play critical roles in diverse cellular processes such as epigenetic histone regulations and p53 homeostasis. Genetically encoded photocaged Lys derivatives are highly valuable tools for studying these fundamental biological events with spatial and temporal precision. The pfLuc reported here may serve as a convenient and noninvasive bioluminescent reporter in assessing the photolysis efficiency of ONBK and potentially other photocaged Lys analogues[4d,e] in the context of their embedded proteins *in vitro* and in living cells. We further took advantage of the spatial and temporal activation feature of pfLuc for the measurement of labile ATP levels without interfering with cellular physiology, which presents as a formidable challenge for conventional luciferase-based ATP quantification methods. Finally, given that the pyrrolysine-based genetic-code expansion system used here for pfLuc creation has now been successfully extended to diverse living species including bacteria, yeast, mammalian cells, and, most recently, multicellular organisms,[19] our photocaging strategy on fLuc may find broad applications in a variety of these living systems.

■ ASSOCIATED CONTENT

Supporting Information

Experimental details, supplemental data, and references. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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