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Photocatalysis Enables Visible-Light Uncaging of Bioactive Molecules in Live Cells

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Abstract: *The photo-manipulation of bioactive molecules provides unique advantages due to the high temporal and spatial precision of light. The first visible-light uncaging reaction by photocatalytic deboronative hydroxylation in live cells is now demonstrated. Using Fluorescein and Rhodamine derivatives as photocatalysts and ascorbates as reductants, transient hydrogen peroxides were generated from molecular oxygen to uncage phenol, alcohol, and amine functional groups on bioactive molecules in bacteria and mammalian cells, including neurons. This effective visible-light uncaging reaction enabled the light-inducible protein expression, the photo-manipulation of membrane potentials, and the subcellular-specific photo-release of small molecules.*

Biocompatible bond-cleavage reactions are able to restore biological functions by releasing endogenous biomolecules from associated carrier molecules.[1] The photo-uncaging reaction is a well-studied biocompatible bond-cleavage reaction induced by light irradiation, the fast reaction kinetics and spatial precision of which are advantageous for biological applications.[2] Recent development of photo-uncaging methods have called for 1) longer-wavelength light with a readily available light source for better biological penetration, 2) photo-stable substrates without need to be prepared on-site, and 3) location-specific uncaging.[2d–f] While many ingenious solutions have arisen from the existing photo-uncaging methods by photolysis (Figure 1 a), the emerging visible-light photocatalysis using photo-stable substrates may bring additional benefits and will be complementary to current photo-uncaging methods (Figure 1 b).[3]

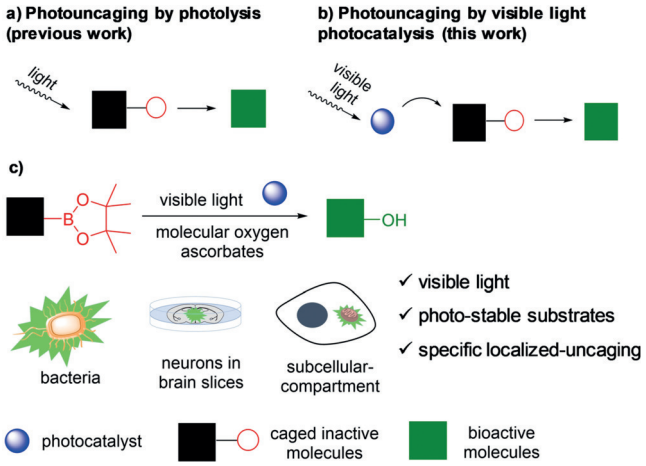

Figure 1. a) Photo-uncaging by photolysis. b) Photo-uncaging by photocatalysis. c) Visible-light photocatalysis enables boronate uncaging in live cells, including bacteria, neurons, and subcellular compartments.

The suitable photocatalytic reactions for live-cell applications are currently very limited due to possible cytotoxicity and the challenging compatibility issues associated with complex live-cell environments.[4] First, the undesirable metabolic toxicity of heavy metal photocatalysts should be avoided for live-cell application.[5] In this regard, some organic dyes have demonstrated well-accepted biocompatibility with versatile fluorescent applications in live cells under light irradiation.[6] Furthermore, endogenous redox-active agents, present intracellularly or extracellularly, such as molecular oxygen and antioxidants, usually affect or inhibit photocatalytic reactions.[7] To this end, using molecular oxygen and endogenous antioxidants as reactants would be ideal for the designated photocatalytic reaction.[7a,8] Herein, we report the visible-light uncaging reaction by photocatalytic deboronative hydroxylation in live bacteria and mammalian cells, including neurons (Figure 1 c).

Molecular oxygen **1** has a low triplet spin state (E_T = 94 kJ mol^{-1}), which can be photosensitized via the energy transfer reaction to yield the highly cytotoxic singlet oxygen **2**.[9] Singlet oxygen **2** is a contributing factor for cell death by the type II photodynamic process, which is not suitable for biocompatible reactions.[10] In contrast, the single-electron reduction of molecular oxygen **1** ($E_{1/2}^{red}$ = −0.16 V, 1M versus normal hydrogen electrode) generates endogenous signaling molecules superoxides **3** and hydrogen peroxides **4**, which are inherently biocompatible.[8,11] The key to enabling molecular oxygen **1** for biocompatible photocatalytic reactions is to

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enhance the single-electron transfer pathway while reducing the energy transfer pathway (Figure 2a).

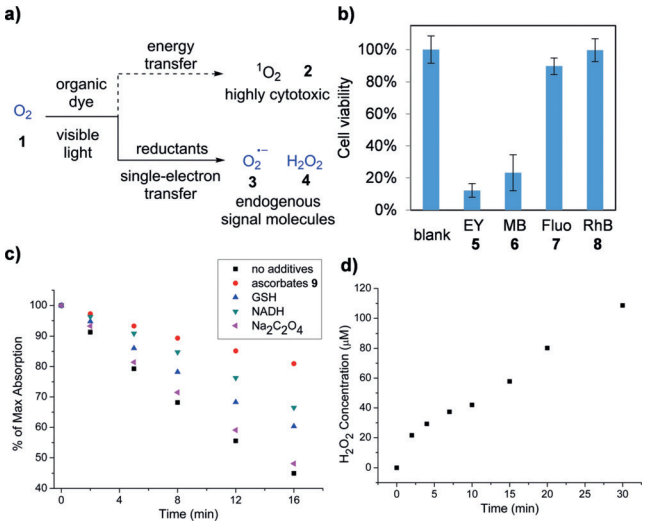

Figure 2. a) The use of suitable photocatalysts and reductants decreased the generation of cytotoxic $^1O_2$ **2**. b) Phototoxicity of organic dyes in live human breast cancer (MCF-7) cells. The cell viability assay was performed using the MTT method and error bars represent the standard deviation of measurements from four different samples. The sample of blank was used as a 100% standard. c) The generation of $^1O_2$ **2** decreased with the addition of reductants. d) The time-dependent curve of H_2O_2 **4** generation.

We first investigated the phototoxicity of different organic dyes **5**–**8** in the presence of molecular oxygen (Figure 2b). While Eosin Y (EY) **5** and Methylene Blue (MB) **6** are widely used photocatalysts in photoredox reactions,[12] their quantum yields of singlet oxygen are quite high ($\Phi_\Delta > 0.5$ in water), resulting in potential phototoxicity.[13] In contrast, Fluorescein (Fluo) **7** and Rhodamine B (RhB) **8** are rarely used in photocatalytic reactions;[5,12a] however, they are widely used fluorescent dyes for labeling and tracking in live cells, with low singlet oxygen quantum yields ($\Phi_\Delta < 0.1$ in water and methanol, respectively).[6,13] These different organic dyes were then tested for cell viability with human breast cancer (MCF-7) cells. Under compact fluorescence lamp (CFL) irradiation in the air atmosphere for 60 min, the MTT cell viability assay indicated only 10–20% survival rates for EY **5** and MB **6**, which was consistent with their high quantum yields of singlet oxygen.[14] In contrast, Fluo **7** and RhB **8** demonstrated >90% survival rate with live-cell compatibility.

We next measured the generation of singlet oxygen from Fluo **7** and probed the reductant effects on the photochemical pathways.[15] The addition of endogenous water-soluble antioxidants glutathione (GSH), nicotinamide adenine dinucleotide (NADH), or ascorbates **9** decreased the singlet oxygen **2** generation, and ascorbates **9** were the most effective (Figure 2c).[8,16] We also measured the time-dependent generation of hydrogen peroxide **4** from the reaction of Fluo **7** and observed its consistent formation (Figure 2d).[17,18] These results are consistent with the reductive quenching effect of ascorbates and their role in the conversion of superoxide anion **3** into hydrogen peroxides **4** (Supporting Information, Figure S8).[8,19]

We next investigated the uncaging reactivity of Fluo **7** and RhB **8**. The organic boronates are a readily available and stable molecular moiety for chemical biology applications and they are also known to undergo deboronative hydroxylation with hydrogen peroxides, however their reactivity with Fluo **7** and RhB **8** is unknown.[20] Under CFL irradiation in pH 7.4 PBS buffer in the air atmosphere, *p*-boronate-benzyl alcohol **10** formed the *p*-hydroxylbenzyl alcohol **11** with 32% yield after 3 h when using RhB **7** and ascorbates **9** (Figure 3a).[21] When Fluo **7** was used, the optimal 82% yield of **11** was obtained within 30 min. The control experiments suggested that the organic dyes, ascorbates, oxygen, and light were all critical to the reaction (Supporting Information, Tables S1 and S6).[22] Different endogenous functional groups present on bioactive molecules were then caged with boronates and tested for visible-light uncaging reactions. The phenyl boronic

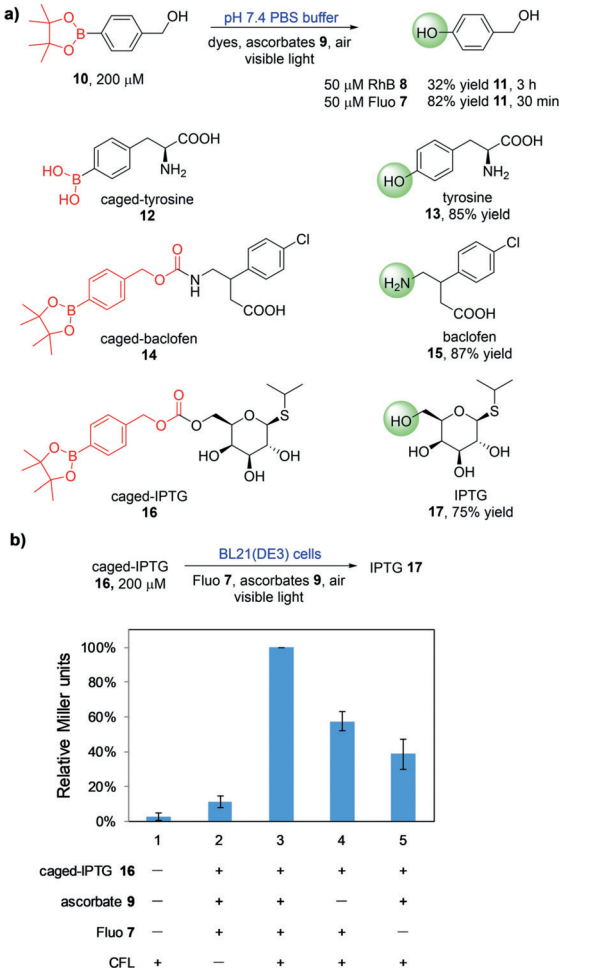

Figure 3. a) Photo-release of different functional groups with deboronative hydroxylation in pH 7.4 PBS buffer. b) Protein expression by photo-release of IPTG **17** in BL21 (DE3) cells. Protein expression level was determined using the β-galactosidase (Miller) assay. Error bars represent standard deviations from three independent experiments and lane 3 was used as a 100% standard.

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acid **12**, caged for the phenol group of tyrosine **13**, readily underwent deboronative hydroxylation and yielded the amino acid **13** in 85% yield (100 μM Fluo **7**).[23] The benzyl carbamate boronic ester **14**, caged for the amine group of baclofen **15**, underwent 1,4-elimination to restore the agonist of neuronal B type γ-aminobutyric acid receptors ($GABA_BRs$) **15** in 87% yield, confirmed by LC-MS.[24] The benzyl carbonate boronic ester **16**, caged for the alcohol group of isopropyl β-D-thiogalactoside (IPTG) **17**, underwent 1,4-elimination to release the protein expression inducer **17** in 75% yield, confirmed by LC-MS.[25]

The photo-uncaging of IPTG **17** was then tested for live bacterial applications (Figure 3b).[26,27] There was little activity in the absence of reagents with CFL irradiation or with reagents in the darkness (lanes 1 and 2 in Figure 3b). In contrast, 30 min of CFL irradiation increased level of β-galactosidase expression greater than 10-fold, suggesting the effective photo-uncaging of IPTG **17** in the live bacterial environment (lane 3 in Figure 3b). Notably, the endogenous antioxidants, such as GSH, NADH, and ascorbates, are compatible with visible-light uncaging of IPTG with an observed 5-fold increase in target protein expression levels. Furthermore, endogenous photosensitive flavin mononucleotide (FMN) can increase the level of target protein expression by 3-fold. These results suggest a sub-optimal photo-uncaging method in live cells when the exogenous addition of photocatalysts and reductants is impractical (lanes 4 and 5 in Figure 3b).[28]

We then corroborated the use of photocatalysis in mice brain slices containing intact neural circuits. γ-aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the central nervous system, which activates $GABA_BRs$ that are coupled to G-proteins and modulates synaptic transmission long-term (Figure 4a).[29] $GABA_BR$ agonists display strong therapeutic effects in a variety of neurological and psychiatric conditions.[29] However, the ubiquitous distribution of $GABA_BRs$ in neuronal and non-neuronal tissues limited the clinical use of the $GABA_BR$ agonist baclofen due to the unwanted, adverse effects of muscle relaxation.[24a] It is expected that the administration of baclofen **15** in a temporally and spatially specific way will help overcome this limitation.

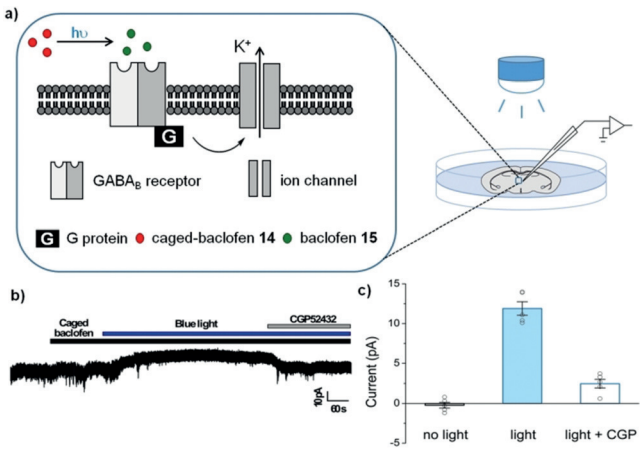

Figure 4. Photo-manipulation of membrane currents in neurons from mice brain slices. a) Illustration of the photo-uncaging experiments in neurons for neural circuitry studies. Photo-released baclofen **15** stimulates $GABA_BRs$, and then G proteins are activated to open K^+ channels leading to an outward current. b) Time-dependent changes of postsynaptic currents in the photo-uncaging process. c) Quantification of the changes of postsynaptic currents ($n=5$ each group). Data are shown as mean ± SEM.

Mouse brain slices containing lateral amygdala were then treated with caged-baclofen **14** to test the photo-release of baclofen **15** by photocatalysis.[24] The addition of combined caged-baclofen **14** (20 μM), Fluo **7** (10 μM), and sodium ascorbate **9** (40 μM) to artificial cerebrospinal fluid in the darkness did not induce any noticeable current (Figures 4b and c, mean currents = -0.2 ± 0.4 pA, $n=5$, $p=0.5429$, vs. zero, paired Student's *t*-test). Remarkably, after the exposure of blue LED light, an outward current occurred in about 2 min on the single patches, suggesting the activation of $GABA_BRs$ by photo-released baclofen **15** (mean currents = 11.9 ± 0.9 pA, $n=5$, $p=1.529E\text{-}04$, vs. zero, paired Student's *t*-test).[30] We further applied CGP52432, the $GABA_BR$ antagonist, to test if the outward current induced by the activation of $GABA_BR$ could be blocked.[24a,29] Under the voltage clamp mode of patch-clamp recording, the application of CGP52432 (10 μM) significantly abolished the $GABA_BR$-mediated current, which confirmed the selective photo-manipulation of currents from $GABA_BRs$ by caged-baclofen **14** (mean currents = 2.5 ± 0.5 pA, $n=5$, $p=5.540E\text{-}05$, vs. no antagonist, paired Student's *t*-test).

We lastly tested the visible-light uncaging method for intracellular mammalian cell applications. The Fluorescein-derived pinacol boronester **18** is a fluorogenic hydrogen peroxide probe, which undergoes deboronative hydroxylation to yield the fluorescent **19** in live cells (Figure 5a).[31] To circumvent the overlap of fluorescence signals between **19** and Fluo **7**, we chose the RhB derivative tetramethyl rhodamine ethyl ester (TMRE, Supporting Information, Figure S21) **20** as the photocatalyst.[32] The incubation of probe **18** in the darkness with 10 μM of TMRE **20** and 500 μM of ascorbates **9** resulted in no fluorescence from **19** in MCF-7 cells. After 30 min of CFL irradiation, the probe **18** was transformed to **19** with green fluorescence signals suggesting the generation of hydrogen peroxides in cells (Supporting Information, Figure S22).

TMRE **20** was previously used as a mitochondria-dye and we hypothesize it may enable the photo-release of hydrogen peroxide specifically in the mitochondria.[32,33] After CFL irradiation for 30 min at 37 °C, the green fluorescence from **19** (FITC channel, Figure 5b) is observed to colocalize with TMRE **20** in the mitochondria (TRITC channel; Figure 5b) under the confocal fluorescent microscope (top, Figure 5b). We speculate the photo-released hydrogen peroxide is short-lived and is readily degraded by endogenous peroxidase and catalase, which confines the boronate photo-uncaging reactions to the vicinity of photoexcited organic dyes.[8,34] In contrast, treatment with hydrogen peroxide resulted in stochastic fluorescent signals not specific to the mitochondria (merged image, bottom, Figure 5b). These results suggest the advantageous subcellular-specific photo-release of bioactive molecules in mammalian cells by visible-light photocatalysis.

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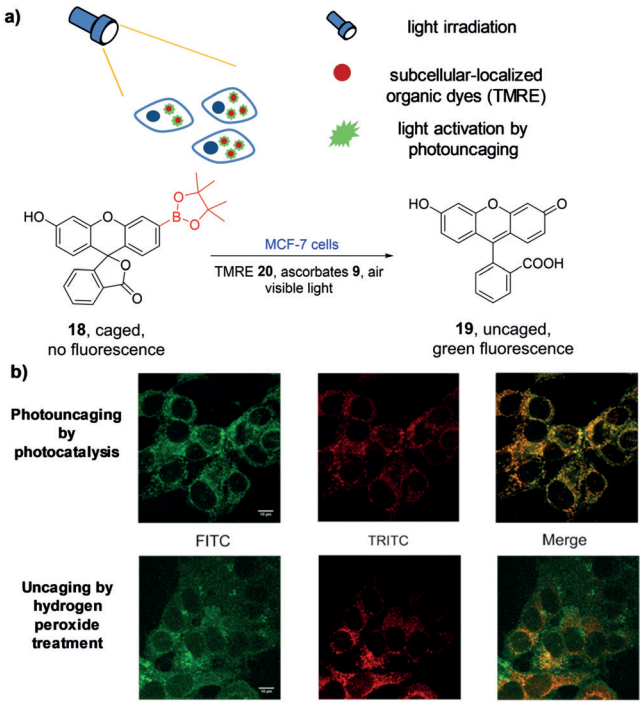

Figure 5. Subcellular-specific photo-release of small molecules in mammalian cells. a) Illustration of subcellular-specific photo-uncaging with TMRE **20** in live cells. b) Confocal fluorescence imaging of MCF-7 cells after photo-uncaging with photocatalysis (top) and hydrogen peroxide **4** treatment (bottom). FITC channel shows uncaged product **19** and TRITC channel shows TMRE **20**. Scale bar: 10 μm.

In conclusion, we have developed the first visible-light uncaging by photocatalysis in live cells via deboronative hydroxylation. The careful choice of organic dyes and reductants are critical for the photochemical pathways for hydrogen peroxide generation. The phenol, alcohol, and amine functional groups on bioactive molecules can be photo-uncaged in vitro, in bacteria, and in mammalian cells, including neurons. The effective demonstration of light-inducible protein expression, photo-manipulation of membrane potentials, and subcellular-specific photo-release highlight the power of this visible-light uncaging method. Furthermore, the dual roles of organic dyes (visualization and photocatalysis) in biological systems may bring many exciting new applications in related research areas.

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Conflict of interest

The authors declare no conflict of interest.

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