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Photocatalysis / Biocompatible Reaction

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

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Abstract: *The photomanipulation of bioactive molecules provides unique advantages with the high temporal and spatial precision of light. Here we report the first visible light uncaging reaction by photocatalytic deboronative hydroxylation in live cells. Using the 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. The effective visible light uncaging reaction enabled the light-inducible protein expression, the photomanipulation of membrane potentials, and the subcellular-specific photorelease of small molecules.*

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

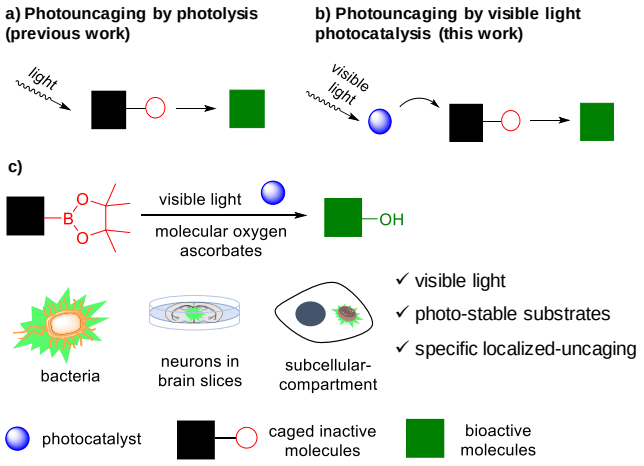

Figure 1. a) Photouncaging by photolysis. b) Photouncaging by photocatalysis. c) Visible light photocatalysis for boronate uncaging in live cells including bacteria, neurons and subcellular-compartment.

However, the suitable photocatalytic reactions for live cell applications are very limited currently due to the possible cytotoxicity and challenging compatibility with complex live cell environments.[4] To begin with, 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] In addition, the endogenous redox-active agents intracellularly or extracellularly such as molecular oxygen and anti-oxidants usually affect or inhibit photocatalytic reactions.[7] To this end, using molecular oxygen and endogenous anti-oxidants as reactants will be ideal for the designated photocatalytic reaction.[7a, 8] In this communication, we report the first visible light uncaging reaction by photocatalytic deboronative hydroxylation in live bacteria and mammalian cells including neurons (Figure 1c).

Molecular oxygen **1** has low triplet spin state (E_T = 94 kJ mol^{-1}), which can be photosensitized *via* energy transfer reaction to yield the highly cytotoxic singlet oxygen **2**.[9] The 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, 1 M versus normal hydrogen electrode) generates endogenous signaling molecules superoxides **3** and hydrogen peroxides **4**, which are inherently biocompatible.[8, 11] The key to enable the molecular oxygen for biocompatible photocatalytic reactions is to enhance the single-electron transfer pathway while reducing the energy transfer pathway (Figure 2a).

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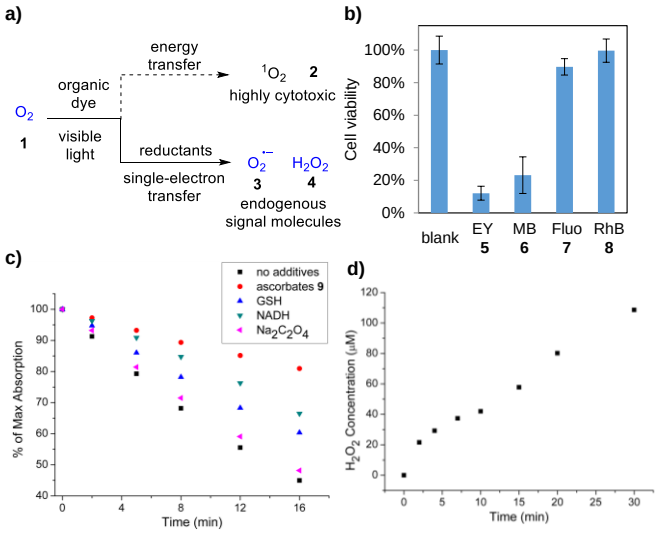

Figure 2. a) The use of suitable photocatalysts and reductants decreased generation of cytotoxic $^1O_2$ **2**. b) Phototoxicity of organic dyes in live human breast cancer (MCF-7) cells. The cell viability assay was performed with MTT method and error bars represent the standard deviation of measurements from four different samples. The sample of blank was used as 100% standard. c) The generation of $^1O_2$ **2** is 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), which impose potential phototoxicity to live cells with singlet oxygen **2**.[13] In contrast, the 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 a household compact fluorescence lamp (CFL) irradiation in the air atmosphere for 60 minutes, 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 anti-oxidants glutathione (GSH), nicotinamide adenine dinucleotide (NADH), or ascorbates **9** decreased the singlet oxygen **2** generation, and ascorbates **9** was the most effective (Figure 2c).[8, 16] We also measured the time-dependent generation of hydrogen peroxides **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 for conversion of superoxide anion **3** to hydrogen peroxides **4** (Figure S8). [8, 19]

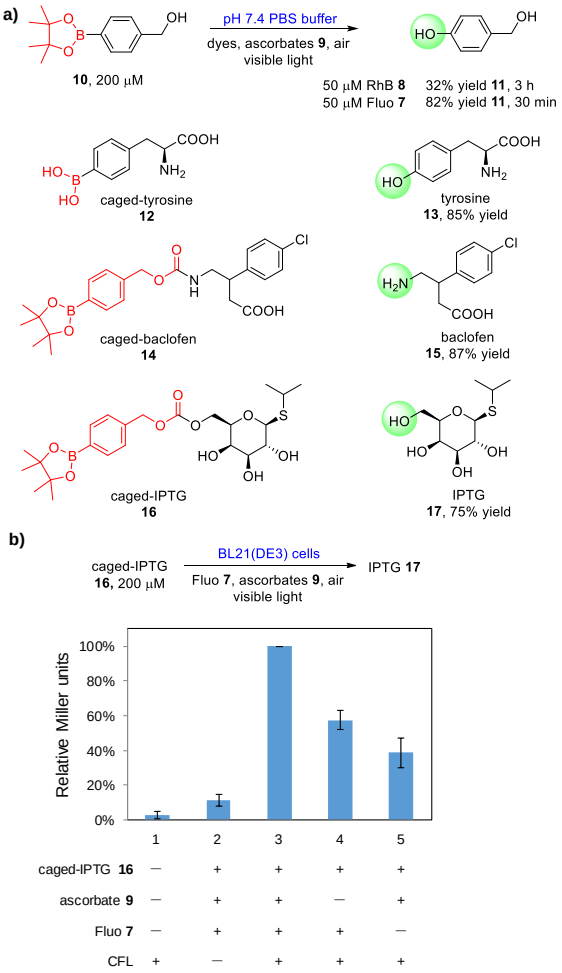

Figure 3. a) Photorelease of different functional groups with deboronative hydroxylation in pH 7.4 PBS buffer. b) Protein expression by photorelease of IPTG **17** in BL21 (DE3) cells. Protein expression level was determined by β-galactosidase (Miller) assay. Error bars represent standard deviations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

We next investigated the uncaging reactivity of Fluo **7** and RhB **8**. The organic boronates are readily available and stable molecular moiety for chemical biology applications, which 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** in 32% yield with RhB **7** and ascorbates **9** after 3 hours (Figure 3a).[21] When Fluo **7** was used, the optimal 82% yield of **11** was obtained within 30 minutes. The control experiments suggested the organic dyes, ascorbates, oxygen, and light were all critical for the reaction (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 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 receptor ($GABA_BRs$) **15** in 87% yield with LC-MS confirmation.[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 with LC-MS confirmation.[25]

The photouncaging of IPTG **17** was next 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 1-2 in Figure 3b). In contrast, 30 minutes of CFL irradiation increased the protein expression level by greater than 10 folds, suggesting the effective photouncaging of IPTG **17** in the live bacterial environment (lane 3). Notably, the endogenous anti-oxidants such as GSH, NADH, and ascorbates are capable for the visible light uncaging with 5 folds of increased protein expression levels, and the endogenous photosensitive flavin mononucleotide (FMN) can increase the protein expression by 3 folds, which provides a sub-optimal photouncaging method in live cells when exogenous additions of photocatalysts and reductants are impractical (lanes 4 and 5).[28]

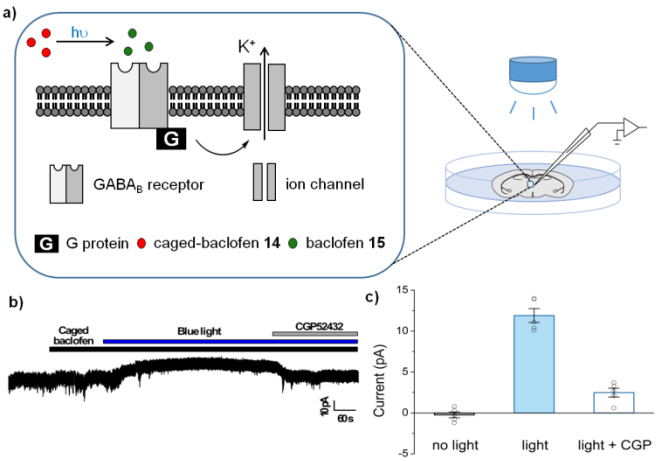

Figure 4. Photomanipulation of membrane currents in neurons from mice brain slices. a) Schematic illustration of the photouncaging experiments in neurons for neural circuitry studies. Photoreleased baclofen **15** stimulates $GABA_BRs$, and then G proteins are activated to open K^+ channels leading to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

After the bacterial cell applications and established biocompatibility with mammalian cells, we further corroborated its use in mice brain slices containing intact neural circuits. The 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 modulate synaptic transmission long-lastingly (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 baclofen due to 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.

Mouse brain slices containing lateral amygdala were then treated with caged-baclofen **14** to test the photorelease 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 4c, 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 minutes on the single patches, suggesting the activation of $GABA_BRs$ by photoreleased baclofen **15** (mean currents = 11.9 ± 0.9 pA, n = 5, p = 1.529E-04, *vs.* zero, paired Student's *t*-test).[30] We further applied CGP52432, the GABABR antagonist, to test if the outward current induced by GABABRs activation 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 as expected, which confirmed the selective photomanipulation of currents from $GABA_BRs$ by caged-baclofen **14** (mean currents = 2.5 ± 0.5 pA, n = 5, p = 5.540E-05, *vs.* no antagonist, paired Student's *t*-test).

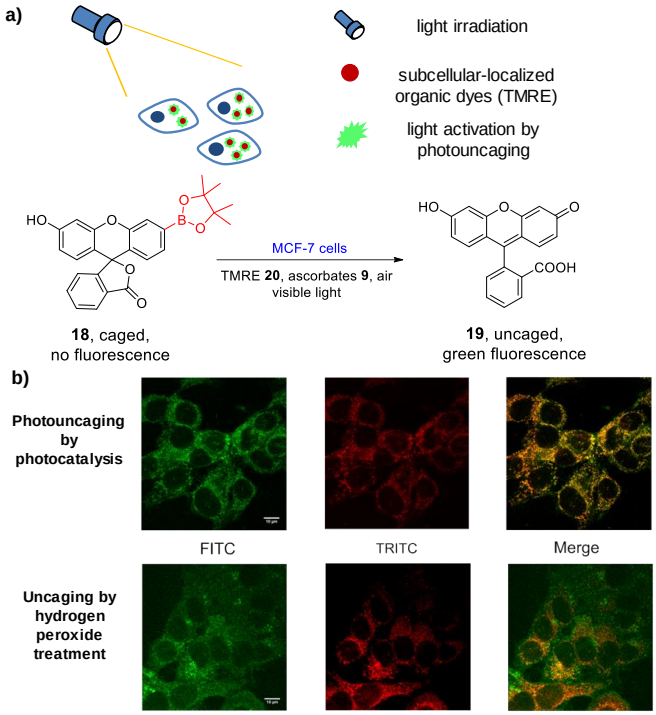

Figure 5. Subcellular-specific photorelease of small molecules in mammalian cells. a) Schematic illustration of subcellular-specific photouncaging with TMRE **20** in live cells. b) Confocal fluorescence imaging of MCF-7 cells after photouncaging with photocatalysis (top) and hydrogen peroxides **4**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

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, Figure S21) **20** as the photocatalyst, of which absorbance and fluorescence spectra are different from that of **19**.[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 minutes of CFL irradiation, the probe **18** is transformed to **19** with green fluorescence signals suggesting the generation of hydrogen peroxides in cells (Figure S22).

The TMRE **20** was previously used as a mitochondria-dye and we hypothesize it may photorelease hydrogen peroxides with mitochondrion-specificity.[32-33] After CFL irradiation for 30 min at 37 °C, the green fluorescence from **19** (FITC channel, Figure 5b) is observed to colocalize in the mitochondria with TMRE **20** (TRITC channel) under the confocal fluorescent microscope (top, Figure 5b). We speculate the photoreleased hydrogen peroxides are short-lived and undergo readily metabolic degradation with the highly abundant

peroxidase and catalase in live cell, which confine the boronate photouncaging reactions to the vicinity of photoexcited organic dyes.[8, 34] In sharp contrast, the treatment with hydrogen peroxide resulted in stochastic fluorescent signals without mitochondrion-specificity seen from the merged image (bottom, Figure 5b). These results confirmed the subcellular-specific photorelease of bioactive molecules in mammalian cells by visible light uncaging.

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 photouncaged *in vitro*, in bacteria, and in mammalian cells including neurons. The effective demonstration of light-inducible protein expression, photomanipulation of membrane potentials, and subcellular-specific photorelease highlight the powerfulness of this visible light uncaging method. In addition, 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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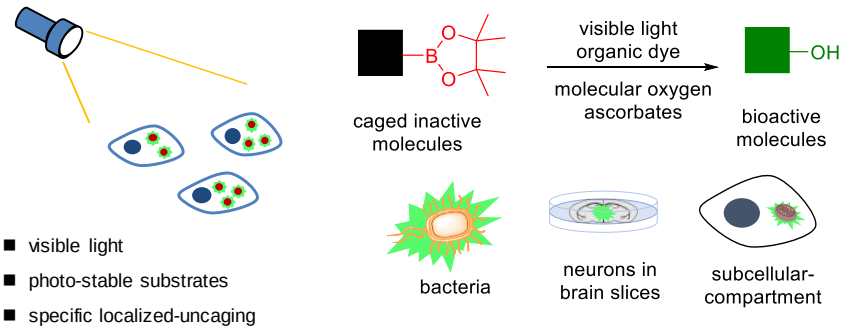
Photocatalysis Enables Visible Light Uncaging of Bioactive Molecules in Live Cells



The photomanipulation of bioactive molecules provides unique advantages with the high temporal and spatial precision of light. Here we report the first visible light uncaging reaction by photocatalytic deboronative hydroxylation in live cells. Using the 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. The effective visible light uncaging reaction enabled the light-inducible protein expression, the photomanipulation of membrane potentials, and the subcellular-specific photorelease of small molecules.