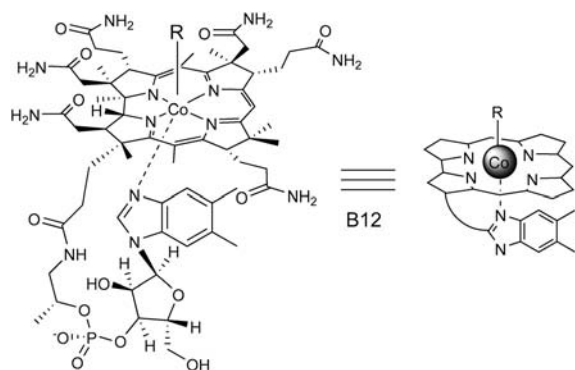


Merging of Confocal and Caging Technologies: Selective Three-Color Communication with Profluorescent Reporters**

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Light-cleavable (caged) agents^[1,2] are useful in addressing fundamental issues in cell biology, most notably drawing correlations between intracellular biochemical activity and cell behavior. However, currently used caged compounds are generally restricted to functional groups that respond to long UV/short visible wavelengths (approximately 360 nm). Recently, a few reports have appeared describing photolytic strategies that can discriminate between two caged bioactive species.^[3–7] These discriminating strategies are based, in large part, on studies of photolabile protecting groups by Brochet^[8] and Hagen and co-workers.^[9] These studies have laid the groundwork for a number of wavelength-distinguishable caging agents. We report the expansion of this concept to three wavelengths using the comparably weak (approximately 30 kcal mol^{−1}) cobalt–alkyl moiety in alkyl cobalamins^[10,11] as the light-cleavable bond.

Barker et al. described the photosensitivity of coenzyme B₁₂ (where R = 5'-deoxyadenosyl) over 50 years ago.^[12] Alkylated cobalamins absorb light at 340–380 nm, approximately 420 nm, and 500–570 nm. Illumination with light in



any of these regions induces cobalt–alkyl bond scission with quantum yields on the order of 0.1–0.4.^[13] To our knowledge,

the visible light sensitivity of the cobalt–alkyl bond has not been used to create caged bioactive compounds. This is not surprising since, unlike a caged hydroxy, carboxy, amido, or amino moiety, photolysis of a caged alkyl chain is not likely to generate a functional group essential for biological recognition or activity. However, we have shown that a photocleavable bond attached at a non-essential functionality can render a bioactive agent “caged” by a) controlling the cellular location of the agent^[14] or b) disrupting noncovalent interactions between the active agent and an appended inhibitor.^[15] We note that other examples of metal-based caging groups have been described, although they are cleaved at wavelengths less than 400 nm.^[16,17]

We,^[14,15] as well as others,^[18,19] have demonstrated that an independent measure of successful photolysis is feasible by linking bond cleavage to a fluorescent readout. We describe herein profluorescent reporters that respond to wavelength-distinct photolytic cleavage of nitrobenzyl, amino coumarin, and cobalamin moieties (Figure 1). The nitrobenzyl moiety is photolyzed at less than 410 nm with a quantum yield ($\Phi_{365\text{nm}}$) of approximately 0.1.^[6] We have reported the synthesis of quenched cassettes that display an increase in either TAMRA (NB-1) or FITC (NB-2) fluorescence upon photolysis of a nitrobenzyl linker, separating a QSY7 quencher from the fluorophore.^[14] NB-1 was designed with a cell-penetrating, mitochondrial-localization sequence (MLS)^[20] for cellular studies. We appended a 440 nm-sensitive diethylaminocoumarin^[3,5,7,9] to one of the aromatic amino moieties of rhodamine (Coum-3), which quenches the fluorescence of the Coum-3. The coumarin–rhodamine conjugate is linked to an MLS peptide using a polyethylene glycol (PEG) tether, which gives a cell-permeable profluorescent reporter of 440 nm photolysis. Finally, cobalamin has been shown to quench the fluorescence of appended fluorophores,^[1,21] which served as the basis for the design of Cob-4.

Cob-4 acts as a reporter of photolytic cleavage at 560 nm. In addition, Cob-4 is cell-permeable (see below) and consequently, attaching an MLS peptide is unnecessary. The unique spectral absorbances of these three groups allows selective cleavage of Cob-4 at 560 nm without interfering with the structural integrity of Coum-3, NB-1, or NB-2 and the selective cleavage of Coum-3, without interfering with the structural integrity of NB-1 or NB-2 (Supporting Information, Figure S1).

Photolysis of NB-1, Coum-3, and Cob-4 was monitored by an increase in rhodamine/TAMRA fluorescence. NB-1 and Coum-3 were photolyzed using a Hg-arc lamp with selective bandpass filters for 360 ± 25, 440 ± 10, and 560 ± 10 nm. NB-1 is only sensitive to photolysis with 360 nm light, resulting in a 24.2 ± 0.8 fold increase in fluorescence (Figure 2a and

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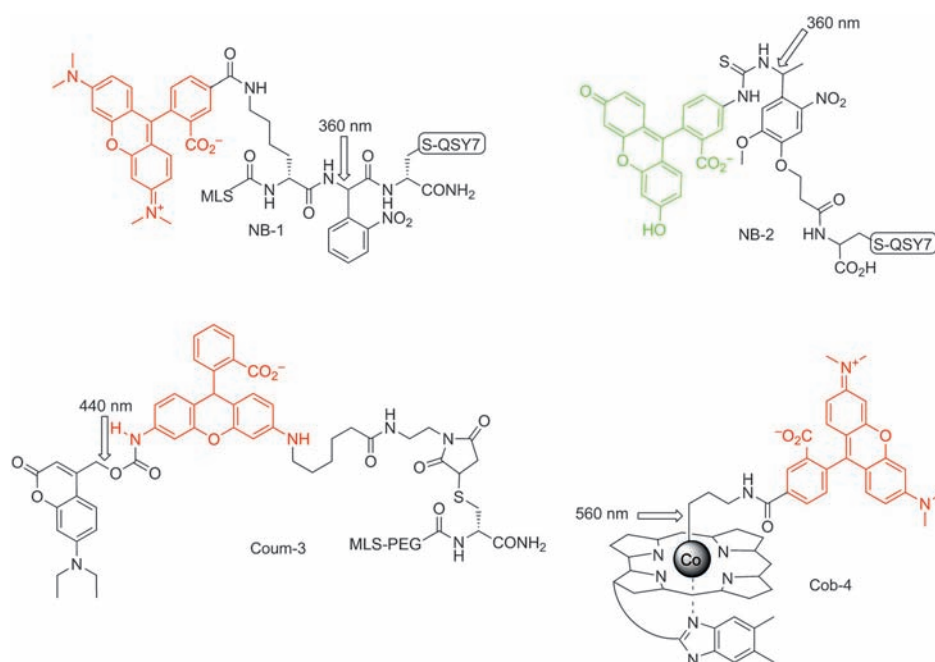


Figure 1. Chemical formulas of profluorescent reporters of photolysis, where TAMRA (red) in NB-1 and Cob-4 displays a $\lambda_{em}=580$ nm, Rhodamine 110 (red) in Coum-3 displays a $\lambda_{em}=540$ nm, and fluorescein (green) in NB-2 displays a $\lambda_{ex}=\lambda_{em}=520$ nm. The structure of the quencher QSY7 can be found in the Supporting Information.

Cob-4 (Figure 2c) is slower at 440 nm (◆) than at 360 nm (●) or 560 nm (▲), presumably because of the smaller absorbance at 440 nm. Finally, the overall yield for the photocleaved products is high. We have shown that nitrobenzyl derivatives very closely related to NB-1 and NB-2 undergo nearly quantitative photolytic conversion.^[14] In this study, both Coum-3 (Figure S3) and Cob-4 (Figure S4) were evaluated using LC-MS, where LC absorbance was used to determine photolysis yields and MS was used to characterize the products. Coum-3 undergoes photolysis in 92 % yield and Cob-4 in 97 % yield. Coum-3 gives a single product, whereas Cob-4 gives three (a predominant aldehyde, an intermediate hydroperoxide that is converted into the

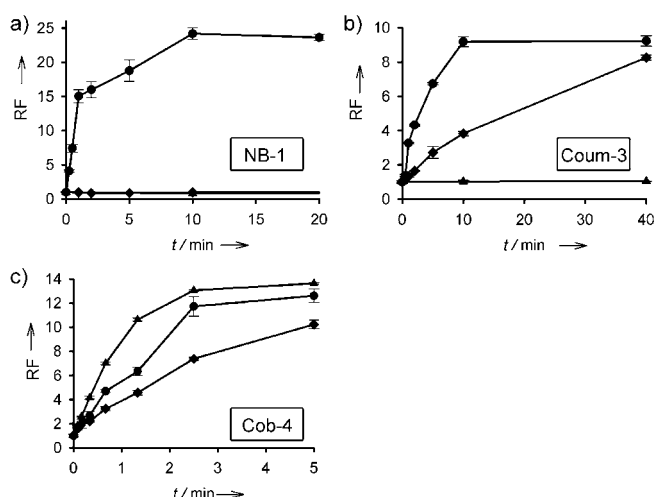


Figure 2. Fold increase of a) NB-1, b) Coum-3, or c) Cob-4 fluorescence upon photolysis with a Hg-arc lamp at 360 nm (●), 440 nm (◆) or 560 nm (▲). RF=relative fluorescence.

Table 1). Coum-3 is photocleaved at both 360 nm and 440 nm, giving a 9.2 ± 0.3 and 8.3 ± 0.2 fold fluorescent increase, respectively (Figure 2b). Like NB-1, Coum-3 fails to undergo photolysis at 560 nm. Finally, Cob-4 is exquisitely sensitive to photolysis at 360, 440, or 560 nm, resulting in a 12.6 ± 0.6 , 10.2 ± 0.3 , and 13.6 ± 0.1 fold fluorescent increase, respectively (Figure 2c). Unlike NB-1 or Coum-3, Cob-4 undergoes photolysis even under the comparatively low photon-flux conditions in a fluorimeter (versus that of a Hg-arc lamp). In addition, we note that photolysis of Coum-3 (Figure 2b) and

Table 1: Fold increase in fluorescence of NB-1, Coum-3, and Cob-4 upon photolysis.

Light source Sample	$\lambda_{\text{photolysis}}$	NB-1	Coum-3	Cob-4
Hg-arc lamp cuvette	360 nm	24.2 ± 0.8	9.2 ± 0.3	12.6 ± 0.6
	440 nm	1.0 ± 0.04	8.3 ± 0.2	10.2 ± 0.3
	560 nm	0.9 ± 0.04	1.0 ± 0.1	13.6 ± 0.1
laser/confocal microwells	355 nm	29.9 ± 2.6	5.5 ± 0.2	8.9 ± 0.5
	440 nm	0.9 ± 0.1	$1.5 \pm 0.1^{[a]}$	6.2 ± 0.1
	559 nm	1.1 ± 0.01	1.2 ± 0.01	8.1 ± 0.3
laser/confocal HeLa cells	355 nm	7.4 ± 0.5	3.9 ± 0.1	ND
	440 nm	1.4 ± 0.1	2.3 ± 0.1	ND
	559 nm	1.0 ± 0.1	1.0 ± 0.1	1.9 ± 0.1

[a] The 440 nm laser on the confocal microscope is not powerful enough to efficiently photolyze Coum-3 in microwells. ND=Not determined.

aldehyde, and a small amount of alkyl product; see Figure S4c).

We evaluated the wavelength-selective photolysis of Cob-4 in the presence of Coum-3 and NB-1 by taking advantage of the MLS sequences appended to Coum-3 and NB-1. In the presence of mitochondria, photolysis of Coum-3 and NB-1 generates an increase in fluorescence at 540 and 580 nm, respectively, which is associated with the mitochondria (Figure 3, Figure S8,S9). The mitochondrial fluorescence is observed after centrifuging the photolyzed mitochondria-containing solution and separating the pellet (mitochondrial fraction) from the supernatant (non-mitochondrial fraction). By contrast, photolysis of Cob-4, which lacks an MLS, only shows a fluorescence increase in the supernatant (Figure 3, Figure S8,S9). As predicted, illumination of an NB-1/Coum-

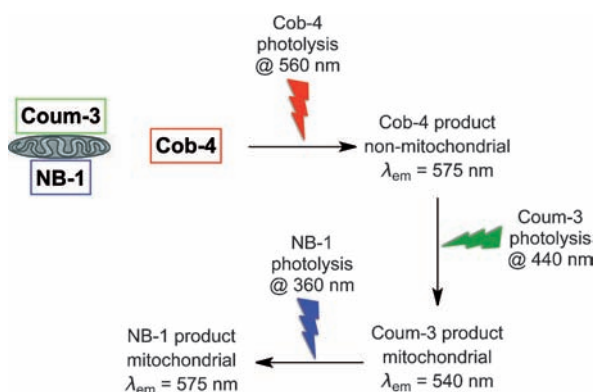


Figure 3. Strategy for demonstrating the wavelength-selective photolysis of Cob-4 in an NB-1/Coum-3/Cob-4 mixture. Cob-4 photolysis at 560 nm gives a mitochondria-free fluorescent response at 575 nm. Coum-3 photolysis at 440 nm gives a mitochondria-associated fluorescent response at 540 nm. Finally, NB-1 photolysis at 360 nm gives a mitochondria-associated fluorescent response at 575 nm.

3/Cob-4 mixture at 560 nm generates a fluorescence increase at 575 nm in the supernatant, characteristic of selective Cob-4 photolysis. Subsequent illumination at 440 nm provides a fluorescence increase at 540 nm in the pellet (mitochondria-associated), characteristic of Coum-3 photolysis. Finally, illumination at 360 nm, generates a fluorescence increase at 575 nm only in the pellet, characteristic of NB-1 photolysis (Figure 3, Figure S8,S9).

We integrated a 100 mW continuous-wave UV laser with a confocal microscope to photolyze cellular and other microscopic samples at 355 nm. Fluoview software was used to control the power and region of photolysis. Initial studies were performed using the photolabile profluorescent reporters in 10 μ L micro-wells. Photolysis of NB-1 and Coum-3 at 355 nm (100 mW) cleaves the photolabile bond as measured by increases in fluorescence of 29.9 ± 2.6 and 5.5 ± 0.2 fold, respectively (Figure S5 and Table 1). Photolysis of Cob-4 at 355, 440, and 559 nm results in 8.9 ± 0.5 , 6.2 ± 0.1 , and 8.1 ± 0.3 fold fluorescent increases, respectively (Figure S5 and Table 1). Selective photolysis of the cobalamin moiety in the presence of a nitrobenzyl group was monitored by imaging TAMRA

emission from Cob-4 and fluorescein emission from NB-2. As expected, 559 nm photolysis cleaves Cob-4 but not NB-2. NB-2 is cleaved upon subsequent exposure to 355 nm light (Figure S6). In addition, we have found that Cob-4 is much more sensitive to 355 nm illumination than NB-1, NB-2, or Coum-3, a result that is likely a consequence of a higher quantum yield for Cob-4 photolysis. Indeed, Cob-4 is selectively photolyzed in the presence of NB-2 at 355 nm by simply reducing the overall exposure (5 frames; where each frame represents exposure of all 512×512 pixels with a dwell time of 100 μ s per pixel). In contrast, 100 frames are required for complete NB-2 photolysis (Figure S7).

NB-1, Coum-3, and Cob-4 were shown to be cell permeable. As expected, only Cob-4-loaded HeLa cells display a response to photolysis at 559 nm. Specifically, Cob-4 cleavage is induced by imaging with the laser at 0.03 mW with a 2 μ s per pixel dwell time (Figure 4e,f and Figure S5c,d). In contrast, neither NB-1 (Figure 4a) nor Coum-3-loaded cells (Figure 4c) are affected by 559 nm light, even at ten times the power and five times the dwell time. Similarly, Coum-3-treated HeLa cells undergo photolysis at 440 nm (2.3 ± 0.1 fold fluorescent increase; Figure 4c,d) whereas NB-1-loaded cells are unaffected by 440 nm light (Figure 4a). Finally, both NB-1 and Coum-3 are susceptible to photolysis at 355 nm (Figure 4a,c and Figure S5a,b).

In summary, we established an order of photolytic selectivity that runs from long to short wavelengths: Cob-4

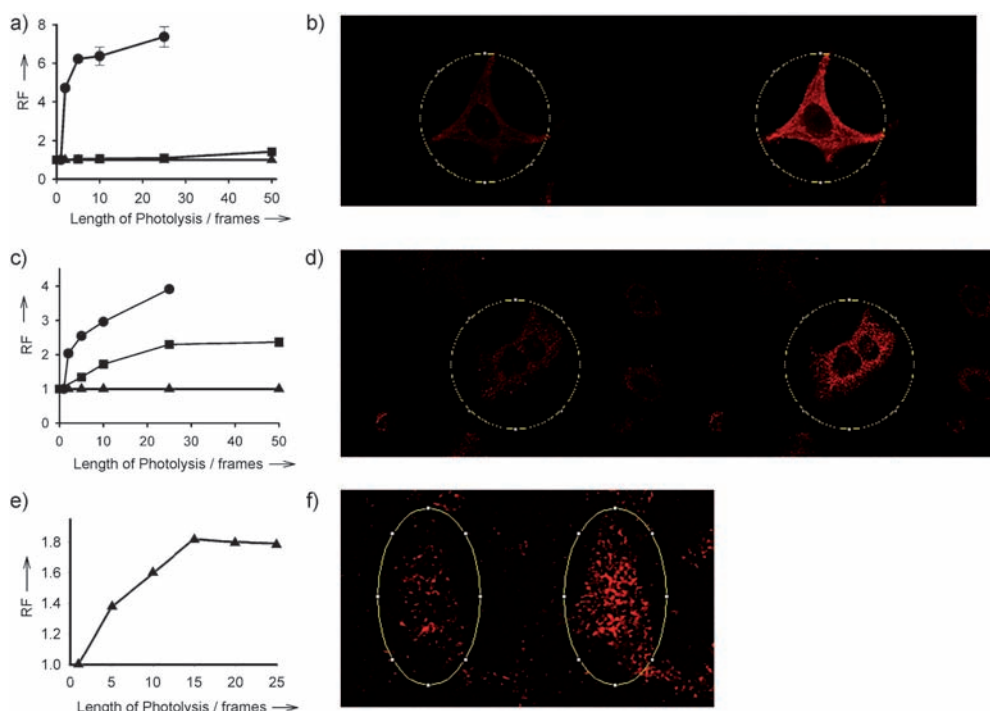


Figure 4. a) Quantification of the fluorescent increase of NB-1 in HeLa cells from photolysis at 355 (●), 440 (■), or 559 nm (▲). b) NB-1-loaded HeLa cells before and after photolysis at 355 nm. c) Quantification of the fluorescent increase of Coum-3 in HeLa cells from photolysis at 355 nm (●), 440 nm (■) or 559 nm (▲). d) Coum-3-loaded HeLa cells before and after photolysis at 440 nm. e) Quantification of the fluorescent increase of Cob-4 in HeLa cells after photolysis at 559 nm (▲). f) Cob-4-loaded HeLa cells before and after photolysis at 559 nm.

is selectively photolyzed at 559 nm in the presence of Coum-3 and NB-1/NB-2, whereas Coum-3 is selectively photolyzed at 440 nm in the presence of NB-1/NB-2. Cob-4 photolysis occurs in a fluorimeter (as do NB-1, NB-2, and Coum-3), as well as under a confocal microscope in microwells and living cells. This is the first time that selective photolysis has been reported for three different moieties at three different wavelengths. In addition, the difference in photolysis rates of NB-1/NB-2 and Cob-4 at 355 nm allows Cob-4 to be selectively cleaved in the presence of the NB-1/NB-2. The selective photolysis of three different moieties opens up possibilities of caging multiple bioactive molecules in a signaling network to probe their spatiotemporal influence in a single experiment.

Experimental Section

All experiments with NB-1, NB-2, Coum-3, and Cob-4 were performed in the dark to prevent inadvertent photolysis.

Photolysis of NB-1 and Coum-3 with Hg-arc lamp: Photolysis was performed using an Oriel 200 W Hg-arc lamp (model 68700) equipped with a beam-bending filter. A UV bandpass colored-glass 360 ± 50 nm filter (Newport, FSQ-UG1), 440 ± 10 nm bandpass filter (Newport, 10BPF10-440), and 560 ± 10 nm bandpass filter (Newport, 10BPF10-560) were used for wavelength-selective photolysis. Initial fluorescence of NB-1 (1 μM) or Coum-3 (1 μM) in PBS with DTT (1 mM) were read on a PTI Model 814 LPS-220B fluorimeter. Samples were photolyzed for specific time intervals up to 40 min on ice and then the fluorescence was re-read. Relative fluorescence (RF) changes were determined by the equation $RF = (F_t - F_0)/F_0$ where F_t is the fluorescence at time t and F_0 is the initial fluorescence. All experiments were performed in triplicate.

Photolysis of Cob-4 with a fluorimeter: Photolysis was performed using a PTI Model 814 LPS-220B fluorimeter. For photolysis at 560 nm, Cob-4 (1 μM) in PBS with DTT (1 mM) was excited at 560 nm while monitoring the emission at 580 nm. Photolysis at 360 and 440 nm was accomplished by excitation of Cob-4 (1 μM) in PBS with DTT (1 mM) to the appropriate wavelength for 0–5 min. Fluorescence of the photolyzed samples was then recorded with λ_{ex} = 560 nm and λ_{em} = 580 nm.

Photolysis of NB-1, Coum-3, and Cob-4 in HeLa cells with a confocal microscope: HeLa cells were loaded with a) NB-1 (5 μM) and MitoTracker Deep Red (20 nM), b) Coum-3 (5 μM) and MitoTracker Deep Red (20 nM), c) Tetramethylrhodamine ethyl ester (TMRE, 20 nM) and MitoTracker Deep Red (20 nM), or d) Cob-4 (80 μM) for 30 min at 37°C and then washed three times with L-15 media. Photolysis was carried out using the stimulation mode of Fluoview with images collected before and after stimulation for NB-1 and Coum-3. Photolysis was set at a dwell time of 100 μs per pixel for the defined region of interest using the following settings: a) 0–50 frames 355 nm (5 mW) or b) 0–100 frames 440 nm (30 mW). Imaging of NB-1 and Coum-3 was performed with a 60× oil immersion Plan S-Apo objective and a 559 nm (0.3 mW) laser. Photolysis of Cob-4 was carried out by exposing to 559 nm (0.03 mW) for 1–50 frames. TMRE-loaded cells were used for photobleaching curves under all photolysis experiments listed above. Image J software was employed for all image analysis. Fluorescent increases were determined as described above. All results are representative of 8–30 cells ± standard error (SE).

Photolysis of a mixture of NB-1, Coum-3, and Cob-4: Photolysis was performed at 560 nm using a PTI Model 814 LPS-220B fluorim-

eter and at 440 nm and 360 nm using the previously described filters with an Oriel 200 W Hg-arc lamp equipped with a beam-bending filter. A mixture of NB-1 (0.5 μM), Coum-3 (0.5 μM), and Cob-4 (0.5 μM) in PBS with DTT (1 mM) was photolyzed at 560 nm, 440 nm, or 360 nm and the emission spectra from 400–650 nm, excitation at 360 nm, was taken. For studies performed with mitochondria, bovine mitochondria (40 μL, 5.5 mg mL⁻¹ from Abcam) were incubated with 200 μL of the caged-fluorophore mixture in isotonic mitochondrial buffer for 5 min. The samples were centrifuged at 9500 × g for 10 min and the supernatant assayed. The pellet was washed three times, resuspended in phosphate-buffered saline (200 μL) and assayed.

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