# SUPPLEMENTARY INFORMATION

**Methods, Schemes S1 – S11, Figures S1 - S26, and Tables S1 – S11**

## Methods

### General

Hydroxocobalamin hydrochloride ($B_{12a}$) was purchased from MP Biomedicals. TAMRA was purchased from AnaSpec. SulfoCy5 succinimidyl ester was purchased from Lumiprobe. Bodipy650 succinimidyl ester, MitoTracker Green, and Rhodamine B dextran (10,000 MW) were purchased from Invitrogen. Dylight800 succinimidyl ester and Dylight 488 phalloidin were purchased from Thermo Fisher Scientific. Doxorubicin was purchased from LC Laboratories. All other fluorophores and reagents were purchased from Sigma-Aldrich. All fluorophores and reagents were used without further purification. The 546 ± 10 nm bandpass filter was purchased from Newport. The 646 ± 10, 700 ± 10, 730 ± 10, and 780 ± 10 nm bandpass filters were purchased from Cheshire Optical. All imaging was performed on an Olympus IX81inverted fluorescence microscope with a Lambda LS3 xenon arc lamp and a Hamamatsu C8484 CCD camera.

### Cell Culture

HeLa cells (ATCC CCL-2) and REF52 cells (kindly provided by Professor Rudy Juliano) were grown in Dubelco's Modifified Eagle Medium (DMEM, high glucose), supplemented with 10% fetal bovine serum and 1% gentamycin/kanamycin, at 37°C in a 5% $CO_2$ atmosphere.

### Synthesis and characterization of cobalamin-TAMRA conjugate (Cbl-1)

Synthesis of β-(3-aminopropyl)cobalamin **1**: β-(3-aminopropyl)cobalamin **1** was prepared from hydroxocobalamin and 3-chloropropylamine hydrochloride according to a literature procedure.[15] Purification was achieved according to literature procedure[12] to afford an orange solid; ESI MS calcd. for $C_{65}H_{97}N_{14}O_{14}PCo$ ($M^{1+} + H^+$): *m/z* = 1388.7, found 1388.7; calcd. for ($M^{2+} + H^+$) *m/z* = 694.3, found 694.5; calcd. for ($M^{3+} + H^+$): *m/z* = 463.1, found 462.9.

Synthesis of Cobalamin-TAMRA Conjugate (**Cbl-1**): **Cbl-1** was prepared from β-(3-aminopropyl)cobalamin **1** and 5-carboxytetramethylrhodamine (TAMRA) according to a literature procedure.[12] Purification was achieved according to literature procedure[12] to afford a red solid, 82%; ESI MS calcd. for $C_{90}H_{118}N_{16}O_{18}PCo$ ($M^{2+}$): *m/z* = 900.4, found 900.5; calcd. for ($M^{3+}$): *m/z* = 600.3, found 600.3.

### Synthesis and characterization of β-(3-acetamidopropyl)cobalamin (Cbl-2)

Synthesis of β-(3-acetamidopropyl)cobalamin (**Cbl-2**): Acetic acid (0.0022 g, 38 μmol), *N*,*N*,*N*′,*N*′-tetramethyl-*O*-(*N*-succinimidyl)uranium tetrafluoroborate (TSTU, 0.0242 g, 80 μmol), and DIPEA (0.0234 g, 181 μmol), were mixed for 1 h in a 2:2:1 dimethylformamide:dioxane:water solution (250 μL). β-(3-aminopropyl)cobalamin **1** (0.0052 g, 3.7 μmol) was added and the reaction and the reaction was mixed for 18 h. The desired compound was purified by HPLC (semiprepative C-18 column) using a linear gradient binary solvent system (solvent A: 0.1% TFA/$H_2O$; solvent B: 0.1% TFA/$CH_3CN$)

with a ratio of A:B that varied from 97:3 (0 min) to 10:90 (40 min). Removal of solvent by lyophilization afforded an orange solid (0.0039 g, 73%); ESI MS calcd. For $C_{67}H_{99}N_{14}O_{15}PCo$ ($M^+ + H$): *m/z* = 1430.7, found 1431.7; calcd. for ($M^{2+} + H^+$): *m/z* = 715.3, found 715.5.

**Synthesis and characterization of cobalamin-fluorophore conjugates (Cbl-3, Cbl-4, Cbl-5, Cbl-6, and Cbl-7)**

General synthesis of cobalamin-fluorophore conjugates: The *N*-hydroxysuccinimide ester of a fluorophore (1 eq.), β-(3-aminopropyl)cobalamin **1** (1.5 eq.), and diisopropylethylamine (6 eq.) were mixed in dimethylformamide for 18 h. The desired compound was purified by HPLC (semiprepative C-18 column) using a linear gradient binary solvent system (solvent A: 0.1 % TFA/$H_2O$; solvent B: 0.1% TFA/$CH_3CN$) with a ratio of A:B that varied from 97:3 (0 min) to 10:90 (40 min). Removal of solvent by lyophilization afforded a solid.

Cobalamin-SulfoCy5 Conjugate (**Cbl-3**): blue solid, 89%; ESI MS calcd. for $C_{97}H_{134}N_{16}O_{21}PS_2Co$ ($M^{2+} + H_2O$): *m/z* = 1014.4, found 1013.6; calcd. for ($M^{3+} + H_2O$): *m/z* = 676.3, found 676.4.

Cobalamin-Atto725 Conjugate (**Cbl-4**): blue solid, 66%; ESI MS estimated[3] for $C_{90}H_{118}N_{16}O_{18}PCo$-Atto725 ($M^{2+}$): *m/z* = 892.9, found 893.2; estimated[3] for ($M^{3+}$): *m/z* = 595.3, found 595.9.

Cobalamin-Dylight800 Conjugate (**Cbl-5**): blue solid, 92%; ESI MS estimated[4] for $C_{90}H_{118}N_{16}O_{18}PCo$-Dylight800 ($M^{2+}$): *m/z* = 1141.1, found 1139.8; estimated[4] for ($M^{3+}$): *m/z* = 760.7, found 760.4.

Cobalamin-Alexa700 Conjugate (**Cbl-6**): blue solid, 72%, ESI MS found for $C_{90}H_{118}N_{16}O_{18}PCo$-Alexa700 ($M^{2+}$): *m/z* = 1179.9, found for ($M^{3+}$): *m/z* = 787.0.[5]

Cobalamin-BODIPY650 Conjugate (**Cbl-7**): blue solid, 88%, ESI MS calcd. for $C_{94}H_{124}N_{18}O_{17}PBF_2Co$ ($M^{2+}$): *m/z* = 957.9, found 958.7; calcd. for ($M^{3+}$): *m/z* = 638.3, found 638.6.

**Synthesis and characterization of coenzyme $B_{12}$-TAMRA conjugate AdoCbl-1**

Synthesis of coenzyme $B_{12}$-ethylenediamine conjugate **2**: Coenzyme $B_{12}$ (0.0209 g, 13 μmol) and 1,1′-carbonyldi-(1,2,4-triazole) (0.0142 g, 87 μmol) were added to an oven-dried round bottom flask. The vessel was purged with Ar. Dry dimethylformamide (0.2 mL) was added to the flask and the mixture was stirred at room temperature for 1 h. Ethylenediamine (0.0270 g, 450 μmol) was added to the reaction mixture and stirring continued for another 18 h. The desired compound was purified by HPLC (semiprepative C-18 column) using a linear gradient binary solvent system (solvent A: 0.1% TFA/$H_2O$; solvent B: 0.1% TFA/$CH_3CN$) with a ratio of A:B that varied from 97:3 (0 min) to 10:90 (40 min). Removal of solvent by lyophilization afforded an orange solid (0.0189 g, 86%); ESI MS calcd. for $C_{75}H_{107}N_{20}O_{18}PCo$ ($M^{2+}$): *m/z* = 832.9, found 833.4; calcd. for ($M^{3+}$): *m/z* = 555.2, found 556.2.

Synthesis of coenzyme $B_{12}$-TAMRA conjugate (**AdoCbl-1**): *N*,*N*,*N*′,*N*′-Tetramethyl-*O*-(*N*-succinimidyl)uranium tetrafluoroborate (TSTU, 0.0139 g, 46 μmol), TAMRA (0.0127 g, 30 μmol) and DIPEA (0.0230 g, 178 μmol), were mixed for 2 h in a 2:2:1 dimethylformamide:dioxane:water solution (250 μL). Coenzyme $B_{12}$-ethylenediamine conjugate **2** (0.0039 g, 2.3 μmol) was added and the reaction was mixed for 18 h. The desired compound was purified by HPLC (semiprepative C-18 column) using a linear gradient binary solvent system (solvent A: 0.1% TFA/$H_2O$; solvent B: 0.1% TFA/$CH_3CN$) with a ratio of A:B that varied from 97:3 (0 min) to 10:90 (40 min). Removal of solvent by lyophilization afforded a red solid (0.0026 g, 53%); ESI MS calcd. For $C_{100}H_{128}CoN_{22}O_{22}$ P ($M^{2+}$): *m/z* = 1039.4, found 1039.8; calcd. for ($M^{3+}$): *m/z* = 693.0, found 693.6; calcd. for ($M^{4+}$): *m/z* = 519.7, found 520.5.

**Synthesis and characterization of coenzyme $B_{12}$-fluorophore conjugates AdoCbl-2, AdoCbl-3, and AdoCbl-4**

General synthesis of cobalamin-fluorophore conjugates: The *N*-hydroxysuccinimide ester of a fluorophore (1 eq.), coenzyme $B_{12}$-ethylenediamine conjugate **2** (1.5 eq.), and diisopropylethylamine (6 eq.) were mixed in dimethylformamide for 18 hrs. The desired compound was purified by HPLC (semiprepative C-18 column) using a linear gradient binary solvent system (solvent A: 0.1% TFA/$H_2O$; solvent B: 0.1% TFA/$CH_3CN$) with a ratio of A:B that varied from 97:3 (0 min) to 10:90 (40 min). Removal of solvent by lyophilization afforded a solid.

Coenzyme $B_{12}$-SulfoCy5 Conjugate (**AdoCbl-2**): blue solid, 87%, ESI MS calcd. for $C_{107}H_{141}N_{22}O_{25}PS_2Co$ ($M^{2+}$ + $H_2O$): *m/z* = 1153.0, found 1152.7; calcd. for ($M^{3+}$ + $H_2O$): *m/z* = 768.6, found 768.8.

Coenzyme $B_{12}$-Atto725 Conjugate: blue solid (**AdoCbl-3**), 69%, ESI MS estimated for $C_{75}H_{106}N_{20}O_{18}PCo$-Atto725 ($M^{2+}$): *m/z* = 1031.4, found 1032.3; estimated for ($M^{3+}$): *m/z* = 687.6, found 688.5. Note: The formula and exact mass of Atto725 carboxylic acid have not been reported.

Coenzyme $B_{12}$-Dylight800 Conjugate (**AdoCbl-4**): blue solid, 90%, ESI MS estimated for $C_{75}H_{106}N_{20}O_{18}PCo$-Dylight800 ($M^{2+}$): *m/z* = 1279.6, found 1279.6; estimated for ($M^{3+}$): *m/z* = 853.1, found 853.2. Note: The formula and exact mass of Dylight800 have not been reported.

**Photolysis of MeCbl, Cbl-1 - Cbl-6, Cbl-Bod, AdoCbl, and AdoCbl-1 – AdoCbl-4**

General procedure: Photolysis of cobalamin-fluorophore conjugates was performed using an Oriel Xe flash lamp (800 mJ, 62 Hz) with selective bandpass filters for 546 ± 10, 646 ± 10, 730 ± 10, and 780 ± 10 nm. The conversion of alkylcobalamin to hydroxocoabalmin was determined by monitoring the absorption of the mixture at 350 nm[13] Perkin Elmer Lambda 2 UV/Vis spectrophotometer.

## Determination of Cbl-1, Cbl-3 – Cbl-6, Cbl-Bod, and AdoCbl-1 – AdoCbl-4 photolysis products

General procedure: Photolyzed samples (100 μL) were analyzed by LC/MS using a linear gradient binary solvent system (solvent A: 0.1% formic acid/$H_2O$; solvent B: 0.1% formic acid/$CH_3CN$) with a ratio of A:B that varied from 97:3 (0 - 5 min) to 3:97 (5 - 18 min).

## Determination of fluorescent increases resulting from photolysis of Cbl-3 – Cbl-6 and Cbl-Bod

General procedure: Fluorescence readings of cobalamin-fluorophore conjugate(s) solutions were observed using a PTI Model 710 LPS-220 before and after tuning the instrument to the appropriate photolytic wavelength.

## Synthesis and characterization of adenosine 3′,5′-cyclic monophosphate ethylenediamine derivatives Etd-cAMP and Ac-cAMP

**Etd-cAMP**: Ethylenediamine (0.1349 g, 2.2 mmol) and 8-bromoadenosine 3′,5′-cyclic monophosphate (0.0114 g, 28 μM) were mixed and microwave irradiated (Sharp domestic microwave, model number: R-1M50B) for 3 cycles (5 min). The addition of the reaction mixture to ether (3 mL) resulted in the formation of a precipitate, which was collected by centrifugation. The precipitate was washed two times with ether (3 mL). Further purification of the desired compound was achieved by HPLC (semiprepative C-18 column) using a linear gradient binary solvent system (solvent A: 0.1% TFA/$H_2O$; solvent B: 0.1% TFA/$CH_3CN$) with a ratio of A:B that varied from 97:3 (0 min) to 10:90 (40 min). Removal of solvent by lyophilization afforded a white solid (0.0098, 91%); $^1$H NMR (600 MHz, $D_2O$) δ = 3.28 (t, *J* = 5.59 Hz, 2H), 3.73 (t, *J* = 5.69 Hz, 2H), 4.14 – 4.20 (m, 2H), 4.35 – 4.44 (m, 1H), 5.03 (d, *J* = 5.5 Hz, 1H), 5.10 - 5.14 (m, 1H), 5.87 (s, 1H), 8.21 (s, 1H); $^{13}$C NMR (125 MHz, $D_2O$) δ = 42.8, 44.0, 71.2, 75.0, 75.7, 81.0, 95.1, 121.3, 145.8, 150.6, 152.8, 157.7; ESI HRMS calcd. for $C_{12}H_{18}N_7O_6P$ ($M + H^+$): m/z = 388.1134, found 388.1141; calcd. for ($M + Na^+$): m/z = 410.0954, found 410.0955.

**Ac-cAMP**: Acetic acid (0.0068 g, 113 μM), TSTU 0.0754 g, 250 μM), and DIPEA (0.0668 g, 517 μM) were mixed for 2 h in dimethylformamide (200 μL). **Etd-cAMP** (0.0101 g, 25 μmol) was added and the reaction mixed for 18 h. The desired compound was purified by HPLC (semiprepative C-18 column) using a linear gradient binary solvent system (solvent A: 0.1% TFA/$H_2O$; solvent B: 0.1% TFA/$CH_3CN$) with a ratio of A:B that varied from 97:3 (0 min) to 10:90 (40 min). Removal of solvent by lyophilization afforded a white solid (0.0089 g, 87%); $^1$H NMR (600 MHz, $D_2O$) δ = 1.82 (s, 3H), 3.40 (t, *J* = 5.87 Hz, 2H), 3.52 (t, *J* = 5.69 Hz 2H), 4.07 – 4.14 (m, 2H), 4.31 – 4.38 (m, 1H), 5.01 (d, *J* = 5.5 Hz, 1H), 5.10 - 5.14 (m, 1H), 5.81 (s, 1H), 8.18 (s, 1H); $^{13}$C NMR (125 MHz, $D_2O$) δ = 24.6, 42.5, 46.0, 71.4, 75.1, 76.1, 81.1, 95.3, 121.3, 145.9, 150.5, 152.9, 157.9, 178.8; ESI MS calcd. for $C_{14}H_{20}N_7O_7$ P ($M + H^+$): m/z = 430.1240; found 430.1240; calcd. for ($M + Na^+$): m/z = 452.1060, found 452.1061.

## Synthesis and characterization of Cbl-cAMP

Synthesis of **3**: **3** was prepared from hydroxocobalamin and 4-bromobutyric acid in the same manner as β-(3-aminopropyl)cobalamin (described above). Purification was achieved in the same manner as well to afford an orange solid (66%); ESI MS calcd. for $C_{66}H_{96}N_{13}O_{16}PCo$ ($M^+ + H^+$): *m/z* = 708.8, found 709.0.

Synthesis of **Cbl-cAMP**: **3** (0.0275 g, 19 μM) TSTU (0.0126 g, 42 μM) and DIPEA (0.0111 g, 86 μM) were mixed for 2 h in 2:2:1 dimethylformamide:dioxane:water solution (250 μL). **Edt-cAMP** (0.0057 g, 15 μmol) was added and the reaction mixed for 18 h. The desired compound was purified by HPLC (semiprepative C-18 column) using a linear gradient binary solvent system (solvent A: 0.1% TFA/$H_2O$; solvent B: 0.1% TFA/$CH_3CN$) with a ratio of A:B that varied from 97:3 (0 min) to 10:90 (40 min). Removal of solvent by lyophilization afforded a white solid (0.0089 g, 87%); ESI MS calcd. for $C_{78}H_{112}CoN_{20}O_{21}P_2$ ($M^+ + H^+$): *m/z* = 893.3, found 893.5; calcd. for ($M^{2+} + H^+$): *m/z* =595.6, found 596.2.

## Synthesis and characterization of cobalamin-doxorubicin conjugate Cbl-Dox

Doxorubicin (**Dox**, 0.0032 g, 5.5 μM), **3** (0.0074 g, 5.2 μM), *O*-(Benzotriazol-1-yl)-*N,N,N′,N′*-tetramethyluronium hexafluorophosphate (0.0064 g, 17 μM), and triethylamine (0.0017 g, 17 μM), were dissolved in DMSO (200 μL) and the reaction mixed for 18 hr. The desired compound was purified by HPLC (semipreparative C-18 column) using a linear gradient binary solvent system (solvent A: 0.1% TFA/$H_2O$; solvent B: 0.1% TFA/$CH_3CN$) with a ratio of A:B that varied from 97:3 (0 min) to 10:90 (40 min). Removal of solvent by lyophilization afforded an orange solid (0.0084 g, 83%); ESI MS calcd. for $C_{94}H_{126}CoN_{14}O_{25}P$ ($M + 2H^+$): *m/z* = 970.9, found 971.5.

## Treatment of REF52 cells with Ac-cAMP, CPT-cAMP, and Cbl-cAMP

General Procedure: REF52 cells were plated on 35 mm glass bottom dishes (MatTek Corp) and allowed to recover for 24 h. The normal cell culture medium was removed, the cells rinsed with PBS, and replaced with L15 media supplemented with 1% BSA only (negative control and illumination only control) or 1% BSA and 500 μM **Ac-cAMP**, **Cbl-cAMP** or **CPT-cAMP**. Cells were illuminated for 10 min with a Zeiss Colibri LED system at 530 nm followed by incubation at 37 °C for 30 min. After the incubation period, cells were fixed with 4% paraformaldehyde for 10 min at room temperature and labeled with Dylight 488-phalloidin via manufacturer's protocol. Imaging of stress fibers was accomplished utilizing the Olympus microscope specified above at 4-X magnification with a FITC filter ($\lambda_{ex}$ = 475 ± 25 nm, $\lambda_{em}$ = 540 ± 25 nm, 200 ms exposure time). Iamges were processed using ImageJ.

## Treatment of HeLa cells with Cbl-Dox

HeLa cells were seeded in 96-well plates (5000 cells/well) and allowed to recover for 36 h. The normal cell media was removed, the cells rinsed with PBS, and the media replaced with L15 supplemented with 1% BSA and 0.5% DMSO (negative control and irradiation only control), or L15 media supplemented with 1% BSA, 0.5 % DMSO and 10 μM **Dox** or **Cbl-Dox**.

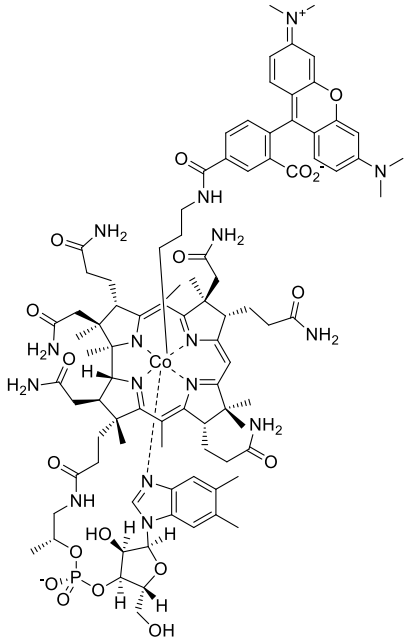
The cells were irradiated with a Zeiss Colibri LED system for 15 s (if necessary) 30 s, 45 s, and 1 min, 15 s at 530 nm followed by incubation at 37 °C for 24 h. Following incubation, the cells were subjected to a viability assay utilizing Cell Titer 96® Aqueous One Cell Proliferation Assay (Promega, absorbance read at 520 nm).

**Photolysis of Cbl-Bod on a widefield microscope**

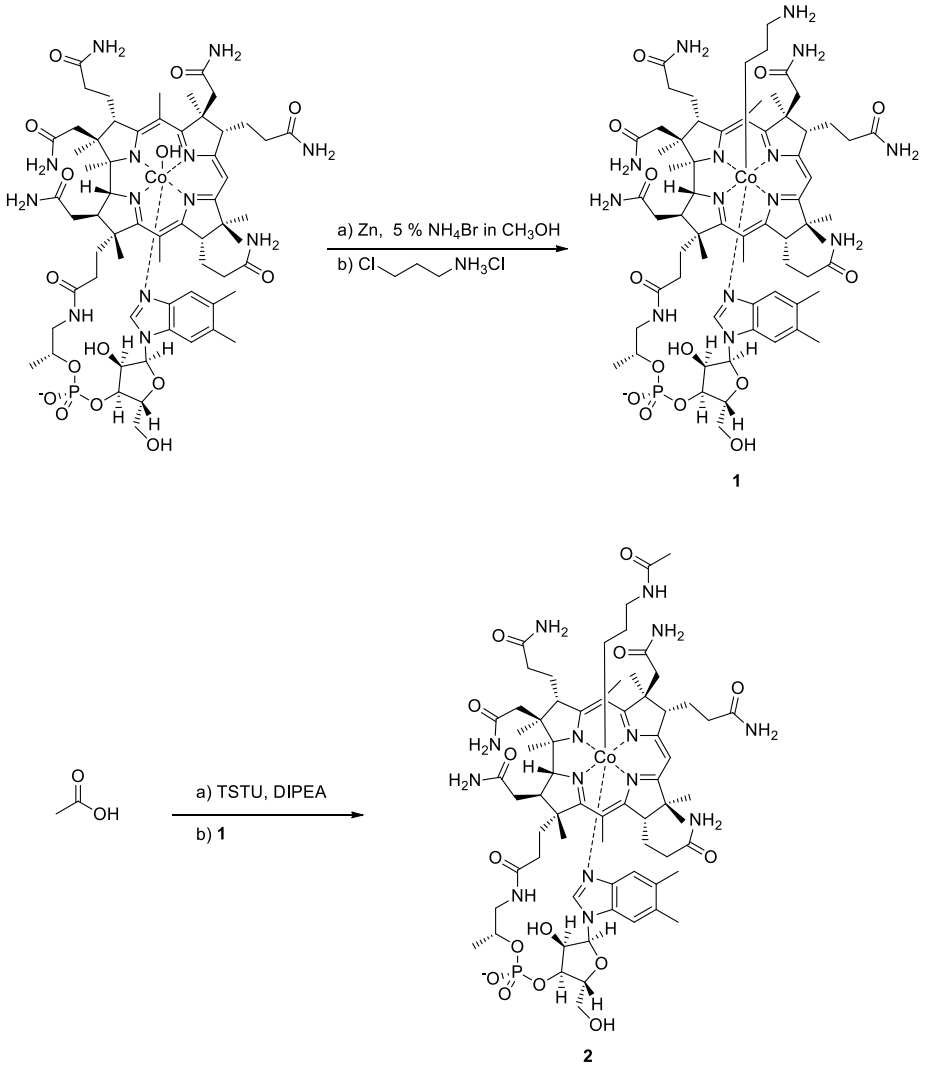
HeLa cells were plated on 35 mm glass bottom dishes (MatTek Corp) and allowed to recover for 24 h. The cells were loaded with **Cbl-Bod** in L15 supplemented with 1% BSA (500 nM, fluorescence increase and endosomal localization experiments, 125 nM for mitochondrial localization experiments, $\lambda_{ex}/\lambda_{em}$ = 650/665 nm) and MitoTracker Green (40 nM, $\lambda_{ex}/\lambda_{em}$ = 490/516 nm) or rhodamine B-dextran (1 mg/mL, $\lambda_{ex}/\lambda_{em}$ = 570/590) at 37 °C for 30 min followed by washing with PBS 3 times. Cells were illuminated using the Cy5 filter cube ($\lambda_{ex}$ = 650 ± 13 nm, $\lambda_{em}$ = 684 ± 25 nm) with a 500 ms exposure time (fluorescence increase experiments) or 1000 ms exposure time (mitochondrial localization experiments), every minute for 15 min. MitoTracker Green (20 ms exposure time) was visualized after photolysis was completed. All images were acquired with a 60x oil immersion UPlan S-Apo objective. Images were processed using a combination of MetaMorph and ImageJ. All results are representative of 5 - 10 cells.

## Schemes

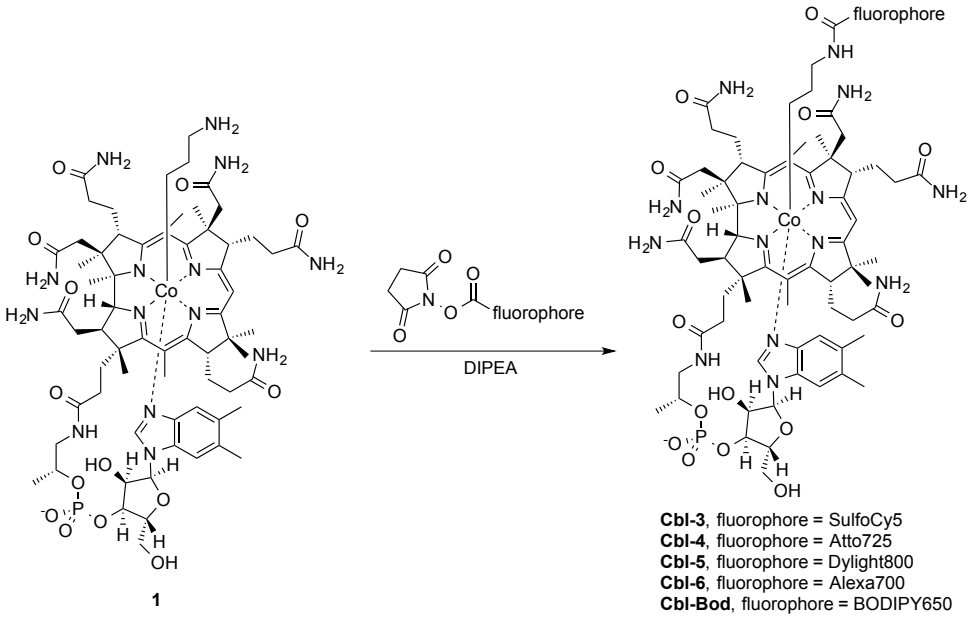
**Scheme S1.** Structure of cobalamin-TAMRA conjugate **Cbl-1**.



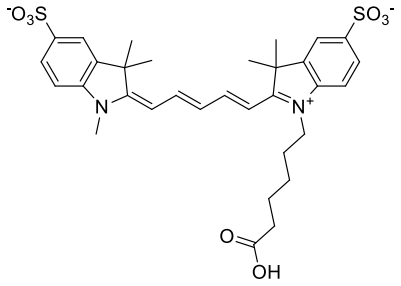
**Scheme S2.** Synthesis of β-(3-acetamidopropyl)cobalamin **Cbl-2**.



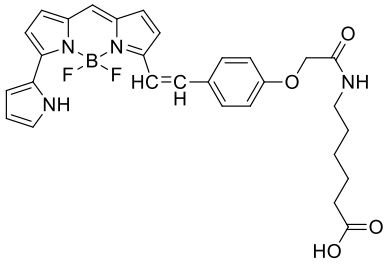
**Scheme S3.** General synthesis of cobalamin-fluorophore conjugates (**Cbl-3**, **Cbl-4**, **Cbl-5**, **Cbl-6**, and **Cbl-Bod**).



**Scheme S4.** Structures of SulfoCy5, carboxylic acid and BODIPY650, carboxylic acid. Note that the structures of Alexa700, Atto725 and Dylight800 have not been reported.

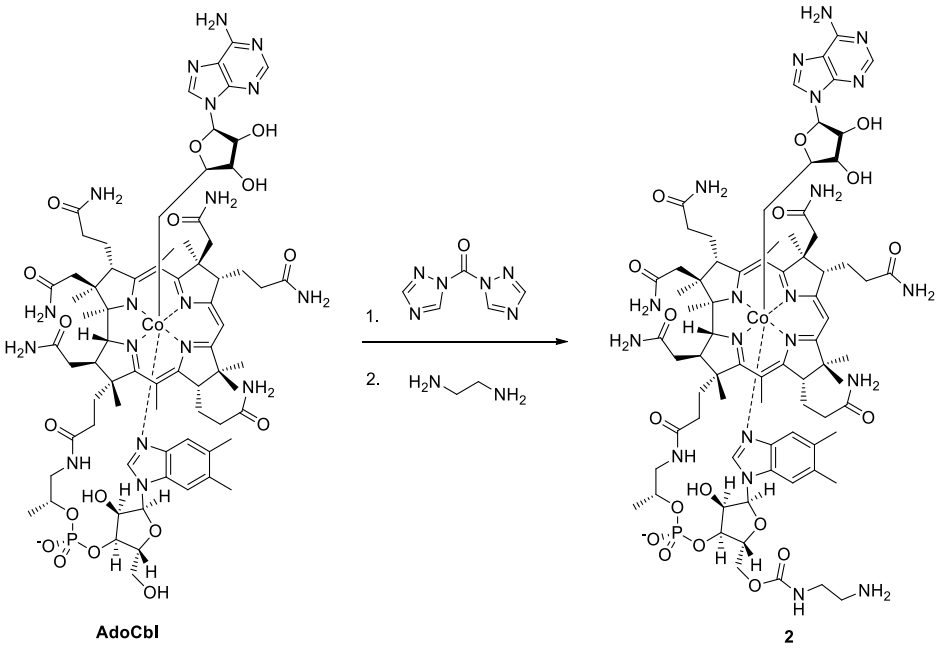


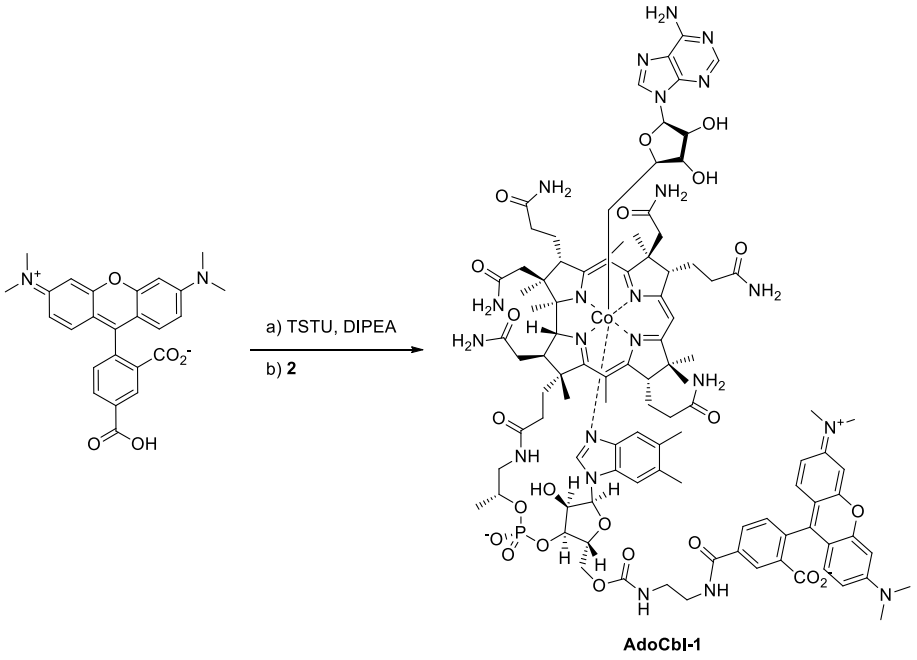

SulfoCy5, carboxylic acid



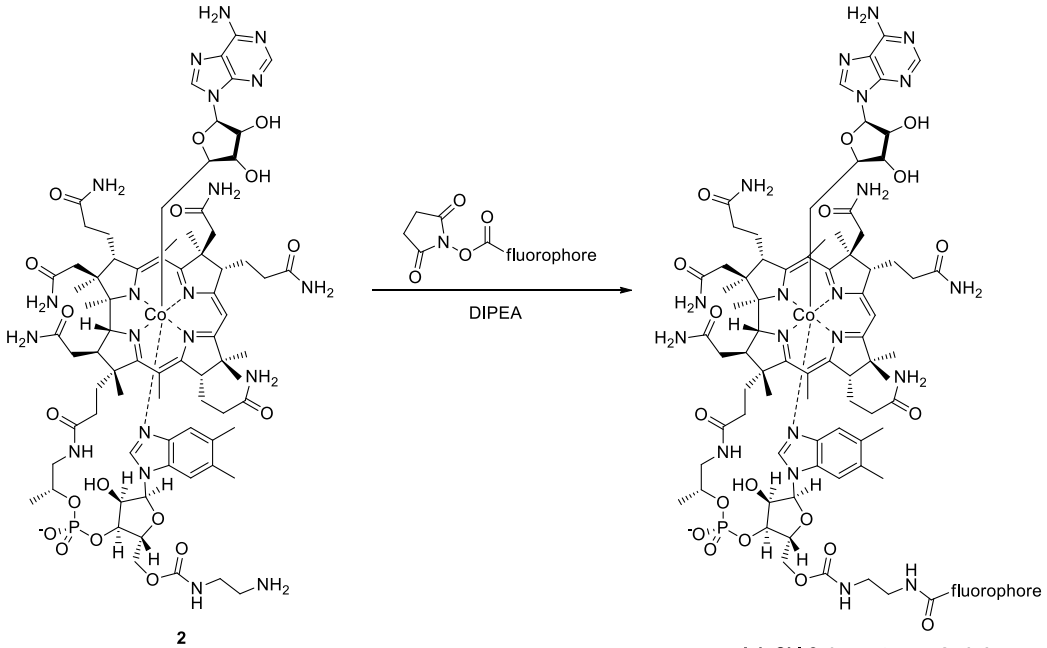

BODIPY650, carboxylic acid

**Scheme S5.** Synthesis of coenzyme $B_{12}$-TAMRA conjugate **AdoCbl-1**.



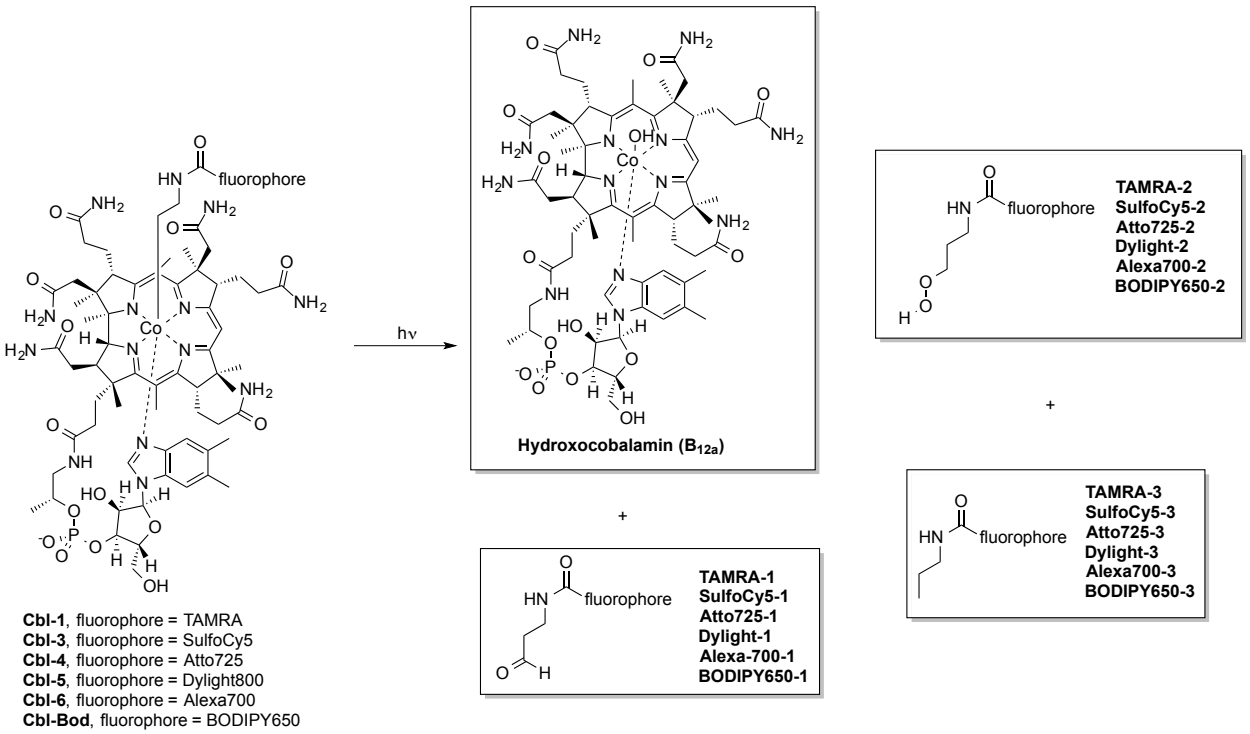
**Scheme S6.** General synthesis of coenzyme $B_{12}$-fluorophore conjugates **AdoCbl-2**, **AdoCbl-3**, and **AdoCbl-4**.




**2**

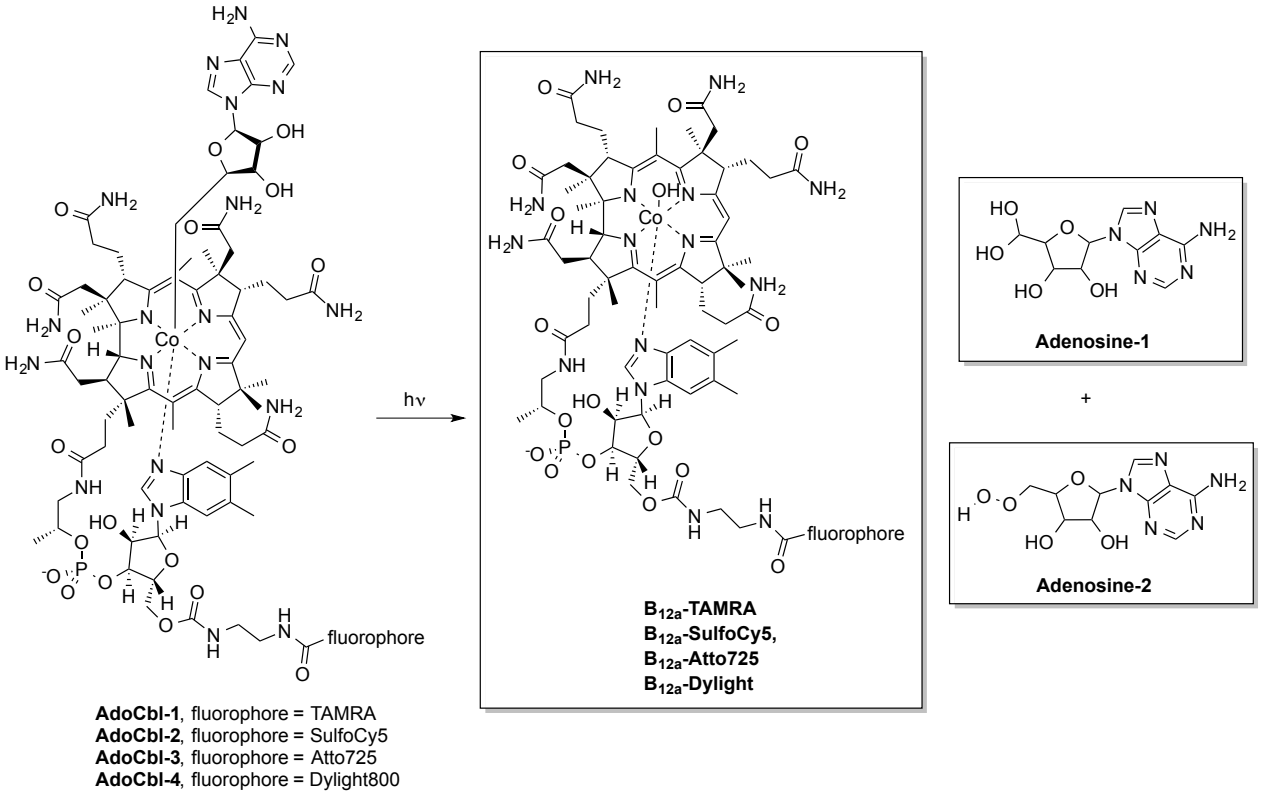
**AdoCbl-2**, fluorophore = SulfoCy5
**AdoCbl-3**, fluorophore = Atto725
**AdoCbl-4**, fluorophore = Dylight800

**Scheme S7.** General photolysis of cobalamin-fluorophore conjugates **Cbl-1**, **Cbl-3**, **Cbl-4**, **Cbl-5**, **Cbl-6**, and **Cbl-Bod**.




**Cbl-1**, fluorophore = TAMRA
**Cbl-3**, fluorophore = SulfoCy5
**Cbl-4**, fluorophore = Atto725
**Cbl-5**, fluorophore = Dylight800
**Cbl-6**, fluorophore = Alexa700
**Cbl-Bod**, fluorophore = BODIPY650

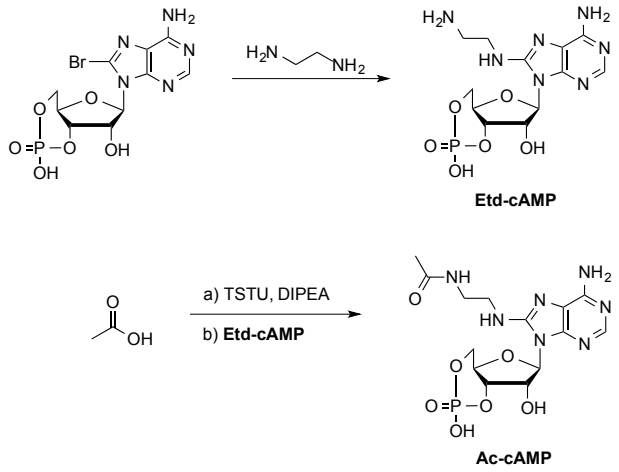
**Scheme S8.** Photolysis of AdoCbl-fluorophore conjugates **AdoCbl-1**, **AdoCbl-2**, **AdoCbl-3**, and **AdoCbl-4** furnishes hydroxocobalamin-fluorophore ($B_{12a}$-fluorophore) conjugates and **adenosine-1** and **adenosine-2**. Adenosine-1 and adenosine-2 were previously reported by Schwartz and Frey for adenosylcobalamin (**AdoCbl**), which we observe as well (Table S6) – see reference 10.



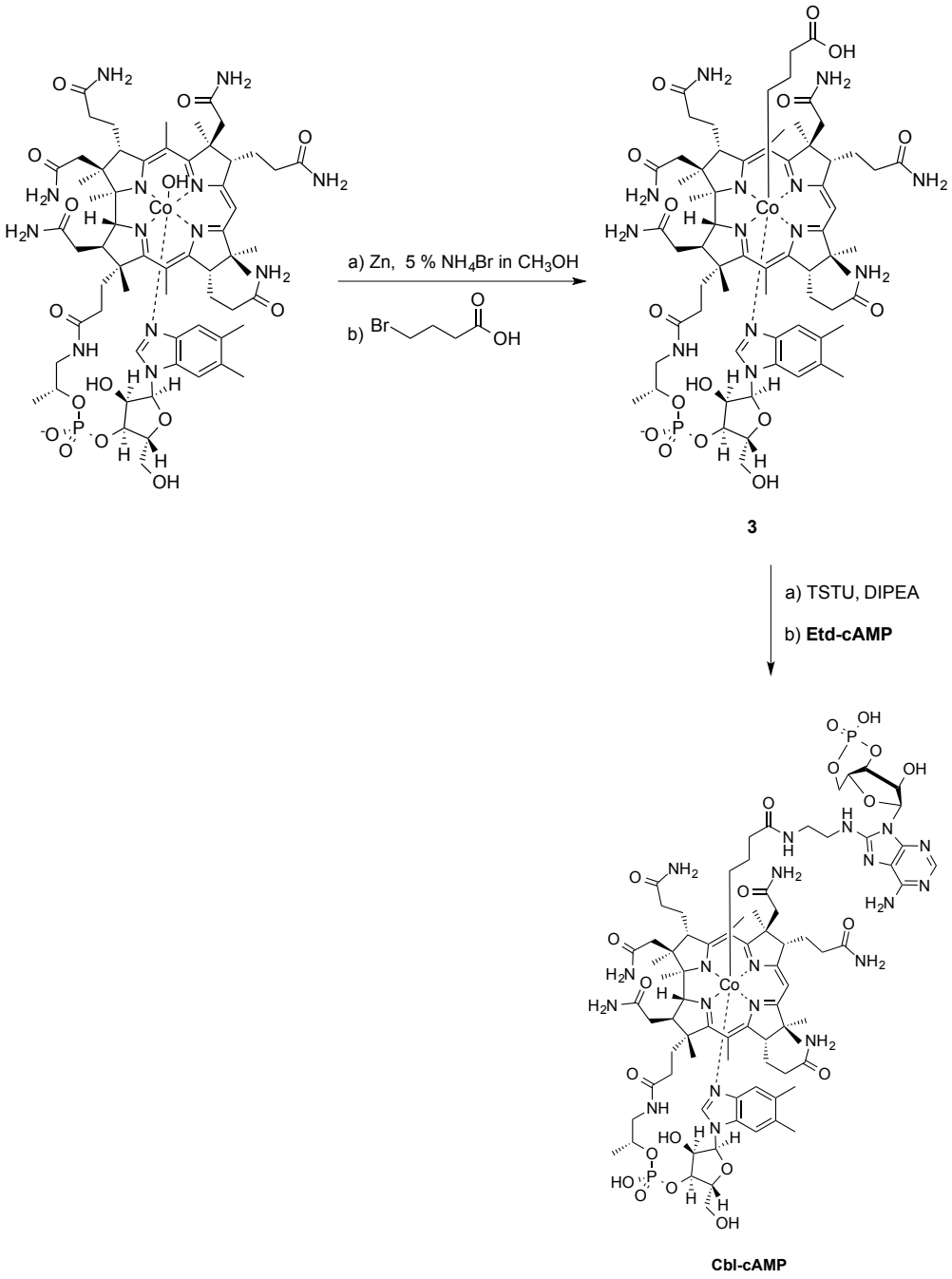

**AdoCbl-1**, fluorophore = TAMRA
**AdoCbl-2**, fluorophore = SulfoCy5
**AdoCbl-3**, fluorophore = Atto725
**AdoCbl-4**, fluorophore = Dylight800

**$B_{12a}$-TAMRA**
**$B_{12a}$-SulfoCy5,**
**$B_{12a}$-Atto725**
**$B_{12a}$-Dylight**

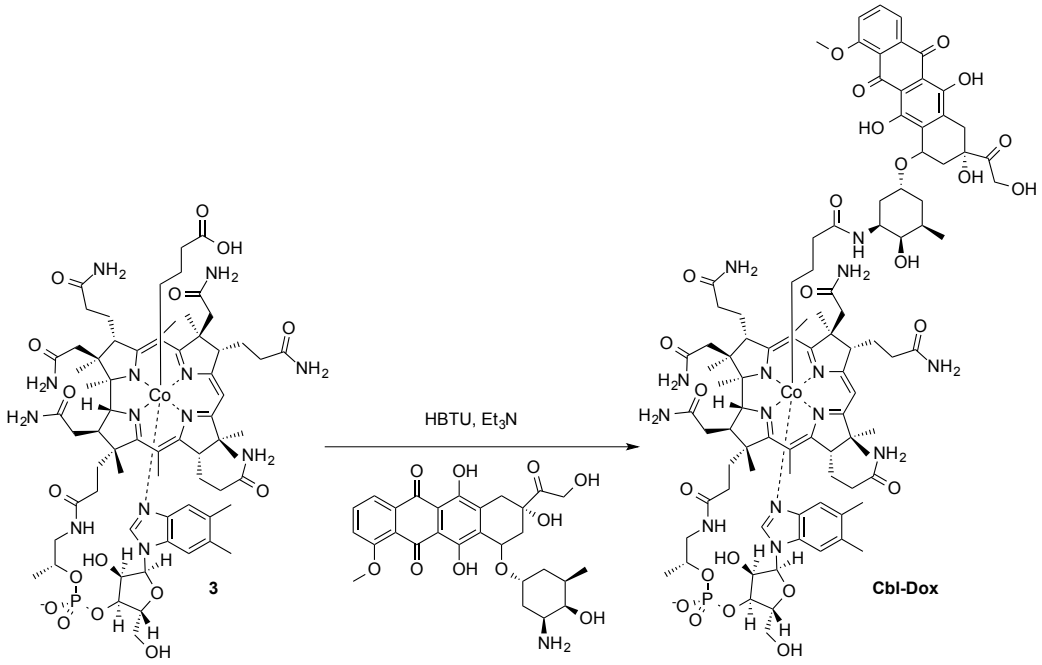
**Scheme S9.** Synthesis of adenosine 3′,5′-cyclic monophosphate derivatives **Etd-cAMP** and **Ac-cAMP**.



**Scheme S10. Synthesis of Cbl-cAMP.**



**Scheme S11. Synthesis of Cbl-Dox.**



**Figures**

**Figure S1.** Photoinduced conversion of **MeCbl** (10 μM, squares) to hydroxocobalamin (circles) using a Xe flash lamp at 546 ± 10 nm. Data are represented as averages with standard errors of three independent experiments.

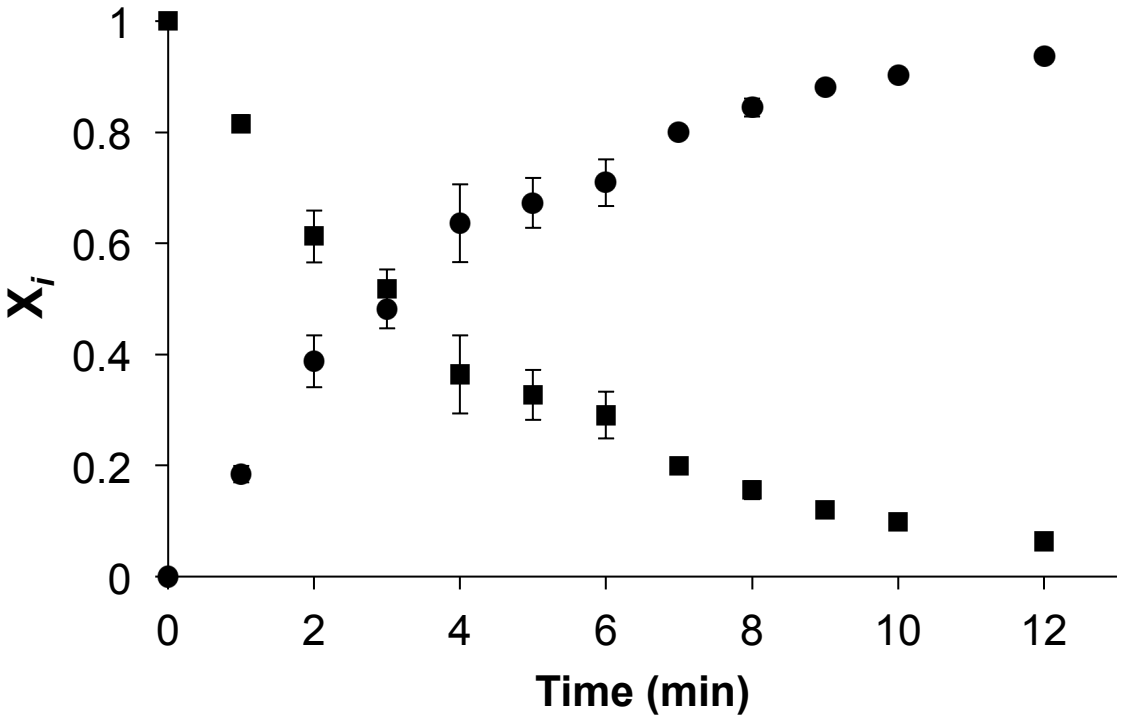


**Figure S2.** Photoinduced conversion of **Cbl-1** (10 μM, squares) to hydroxocobalamin (circles) using a Xe flash lamp at 546 ± 10 nm. Data are represented as averages with standard errors of three independent experiments.

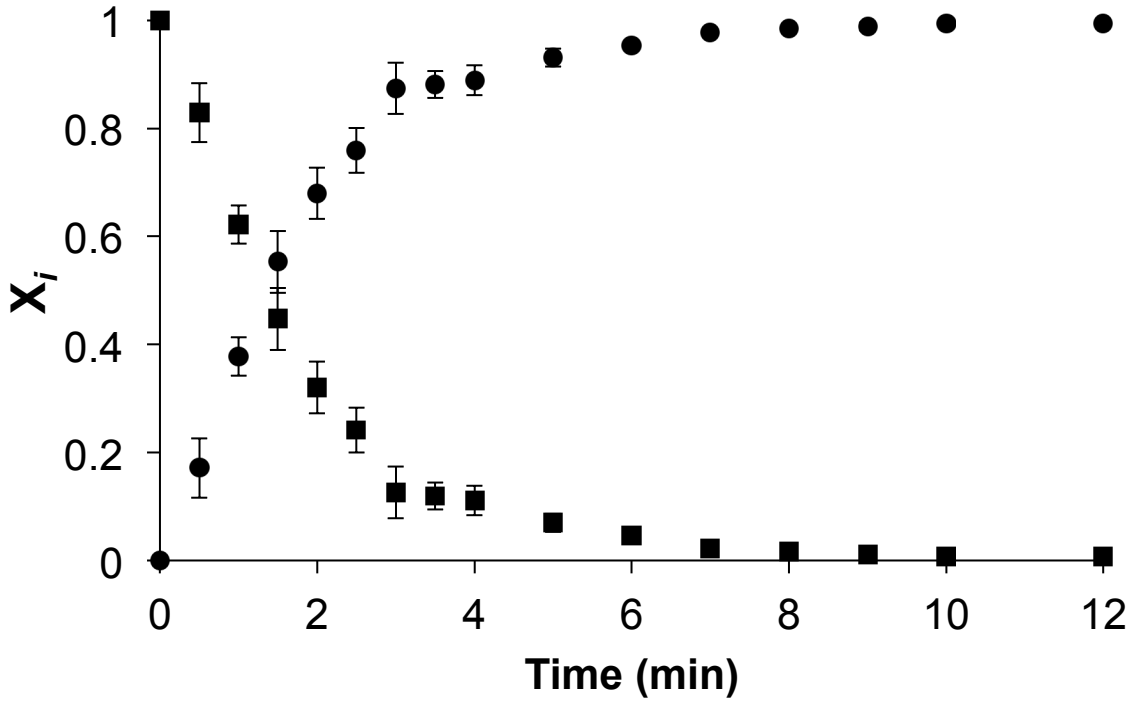


**Figure S3.** Photoinduced conversion of β-(3-acetamidepropyl)cobalamin (**Cbl-2**, 10 μM, squares) to hydroxocobalamin (circles) using a Xe flash lamp at 546 ± 10 nm. Data are represented as averages with standard errors of three independent experiments.

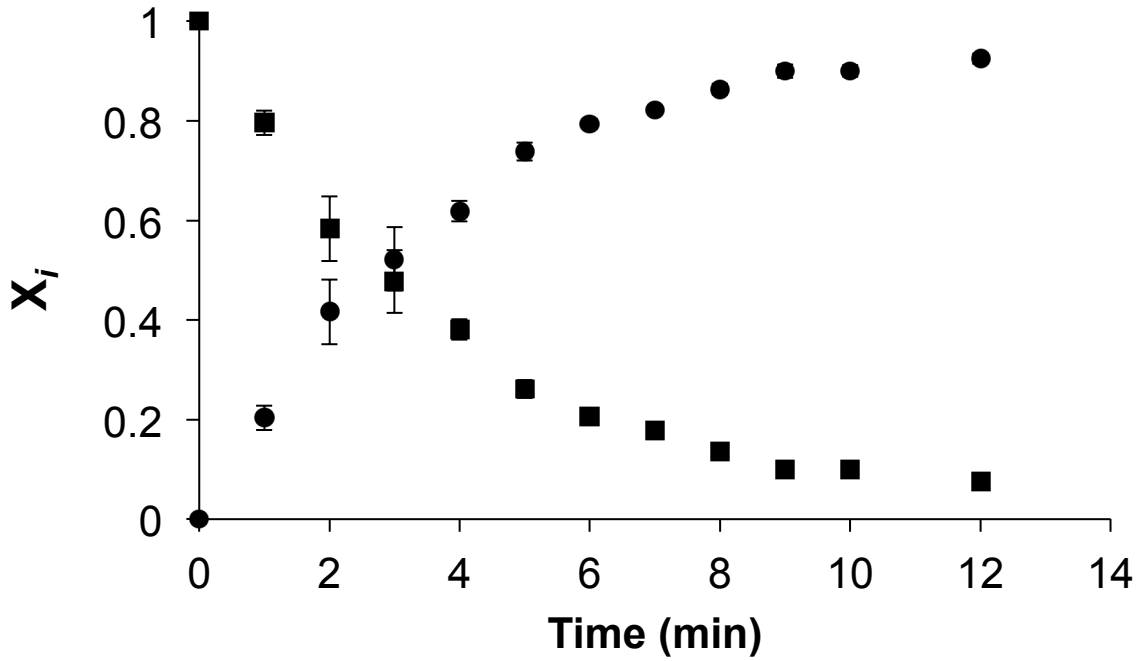


**Figure S4.** Photoinduced conversion **Cbl-3** (10 μM, squares) to hydroxocobalamin (circles) using a Xe flash lamp at 646 ± 10 nm. Data are represented as averages with standard errors of three independent experiments.

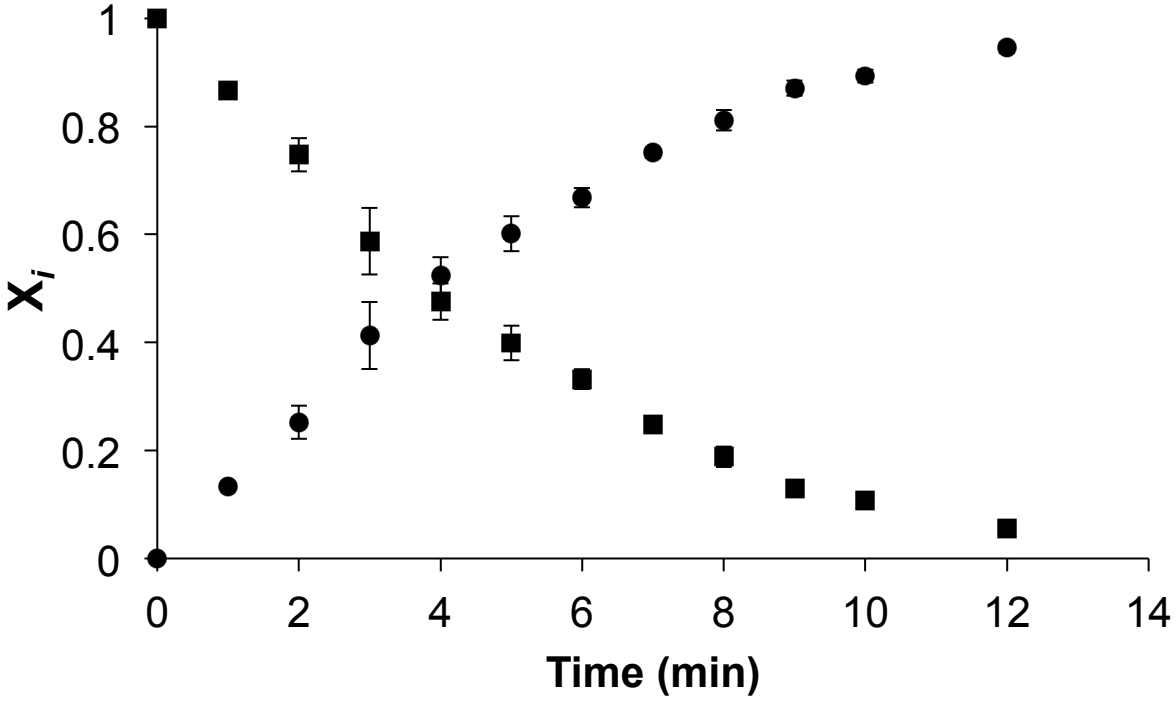


**Figure S5.** Photoinduced conversion of **Cbl-4** (10 μM, squares) to hydroxocobalamin (circles) using a Xe flash lamp at 730 ± 10 nm. Data are represented as averages with standard errors of three independent experiments.

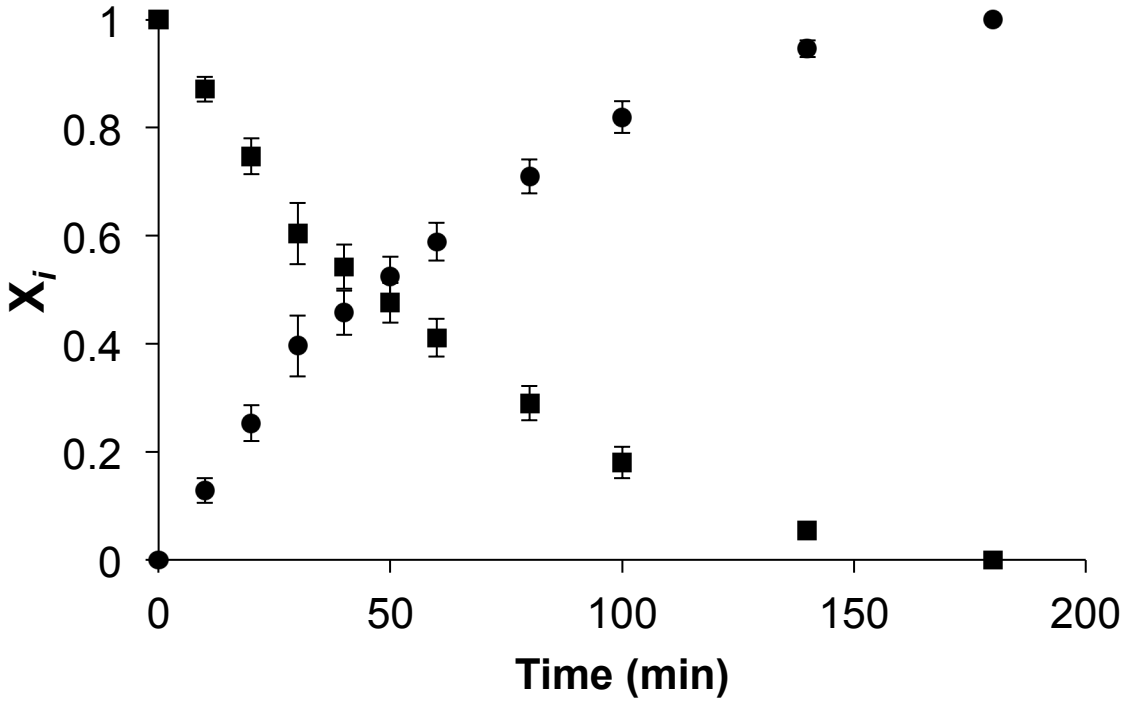


**Figure S6.** Photoinduced conversion of **Cbl-5** (10 μM, squares) to hydroxocobalamin (circles) using a Xe flash at 780 ± 10 nm. Data are represented as averages with standard errors of three independent experiments.

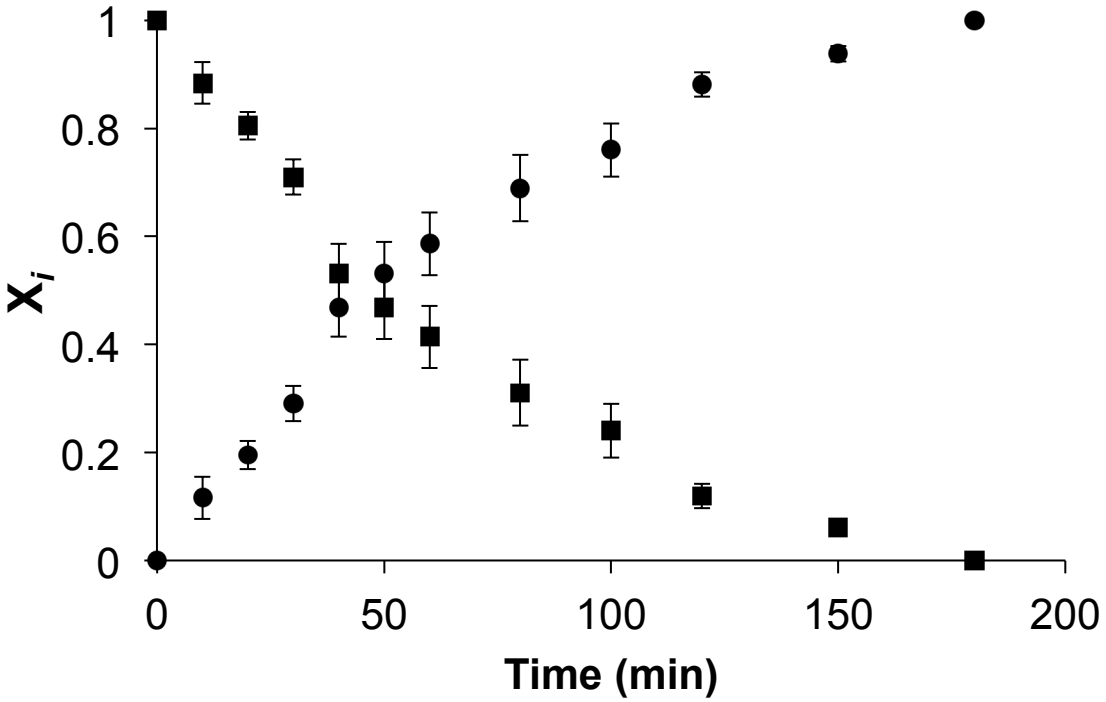


**Figure S7.** Fluorescent increase of **Cbl-1** (1 μM) photolyzed using a spectrofluorometer by excitation at 546 nm and monitoring the fluorescence emission at 580 nm. Data are represented as averages of three independent experiments.

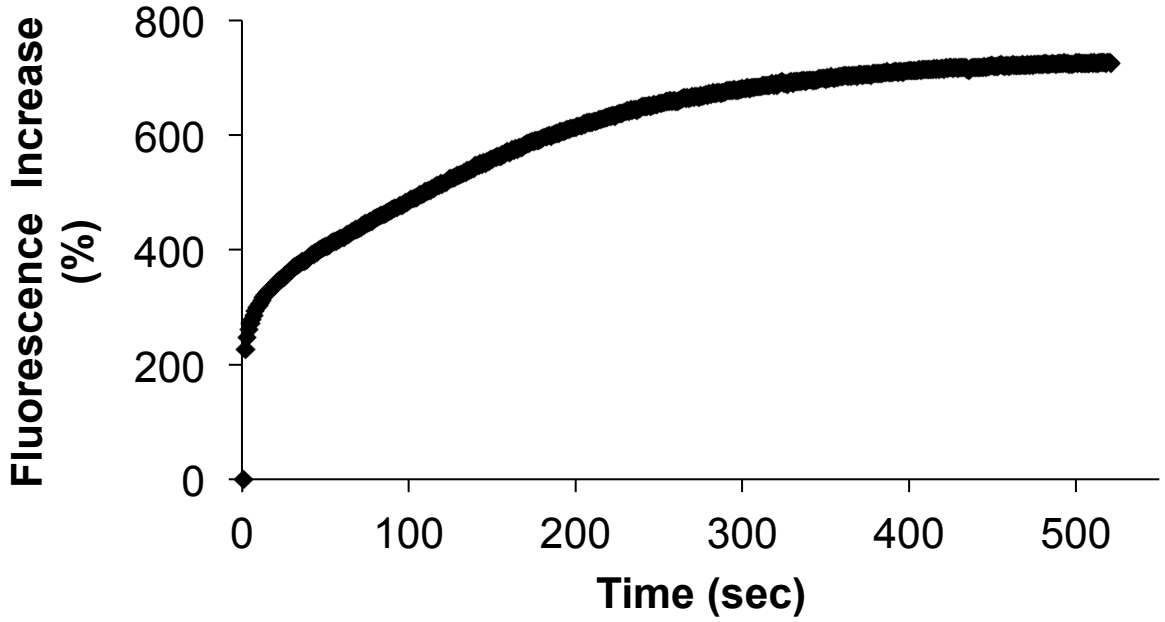


**Figure S8.** Fluorescence increase of **Cbl-1** (1 μM) photolyzed using a spectrofluorometer tuned to four different wavelengths (546 nm for 5 min, 646 nm for 5 min, 727 nm for 20 min, and 777 for 10 min). Data are represented as averages with standard errors for three independent experiments.

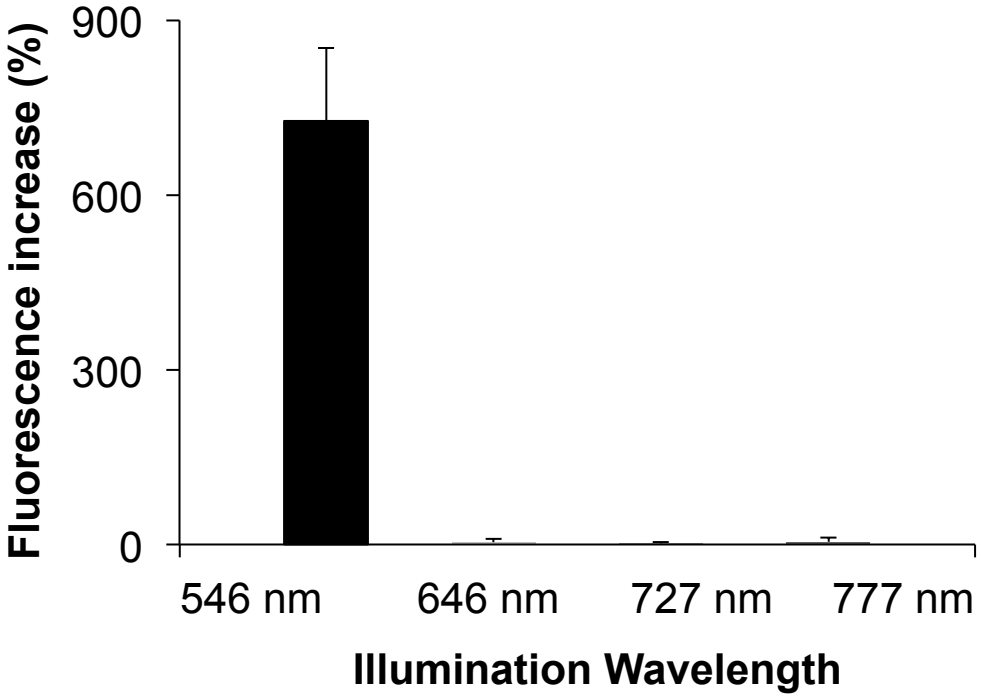


**Figure S9.** Fluorescent increase of **Cbl-3** (1 μM) photolyzed using a spectrofluorometer by excitation at 646 nm and monitoring the fluorescence emission at 662 nm. Data are represented as averages of three independent experiments.

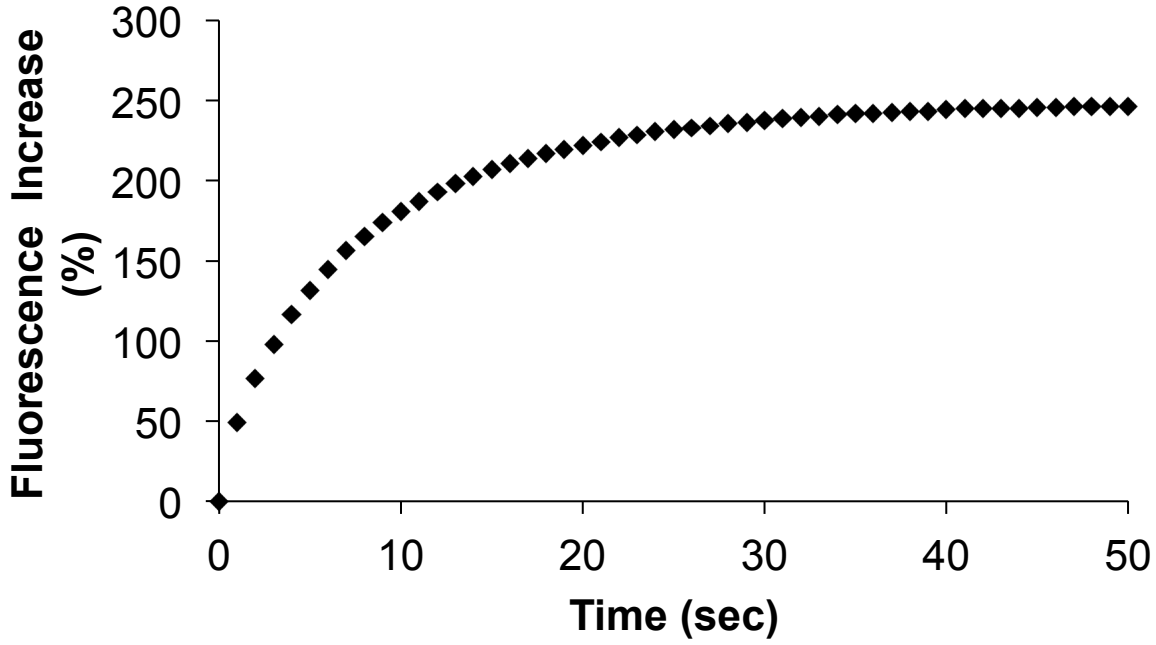


**Figure S10.** Fluorescence increase of **Cbl-3** (1 μM) photolyzed using a spectrofluorometer tuned to four different wavelengths (546 nm for 5 min, 646 nm for 5 min, 727 nm for 20 min, and 777 for 10 min). Data are represented as averages with standard errors for three independent experiments.

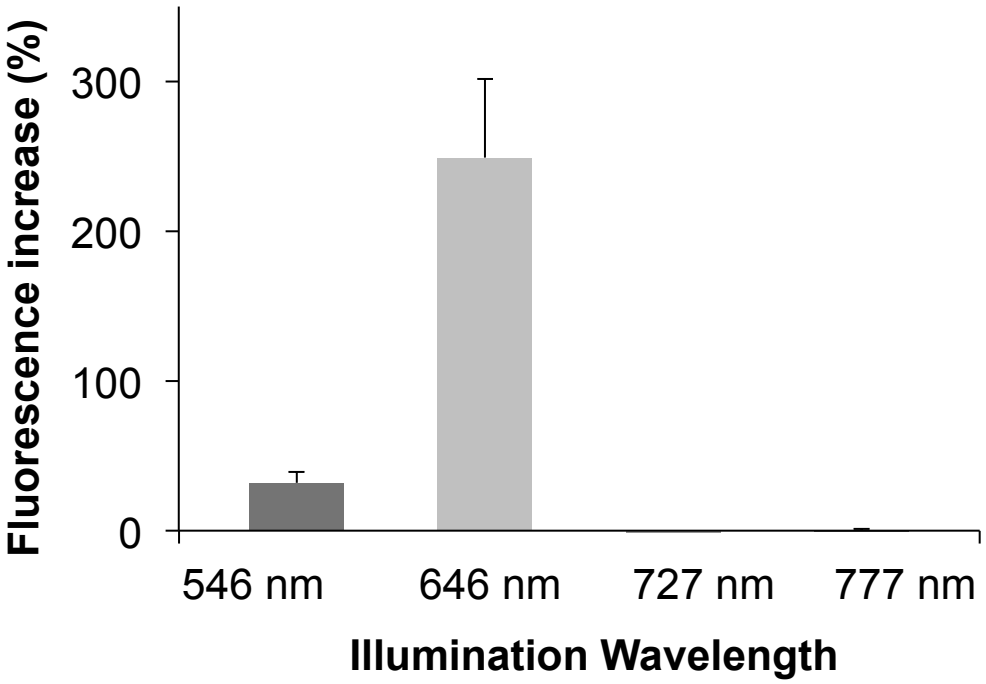


**Figure S11.** Fluorescent increase of **Cbl-4** (1 μM) photolyzed using a spectrofluorometer by excitation at 727 nm and monitoring the fluorescence emission at 752 nm. Data are represented as averages of three independent experiments.

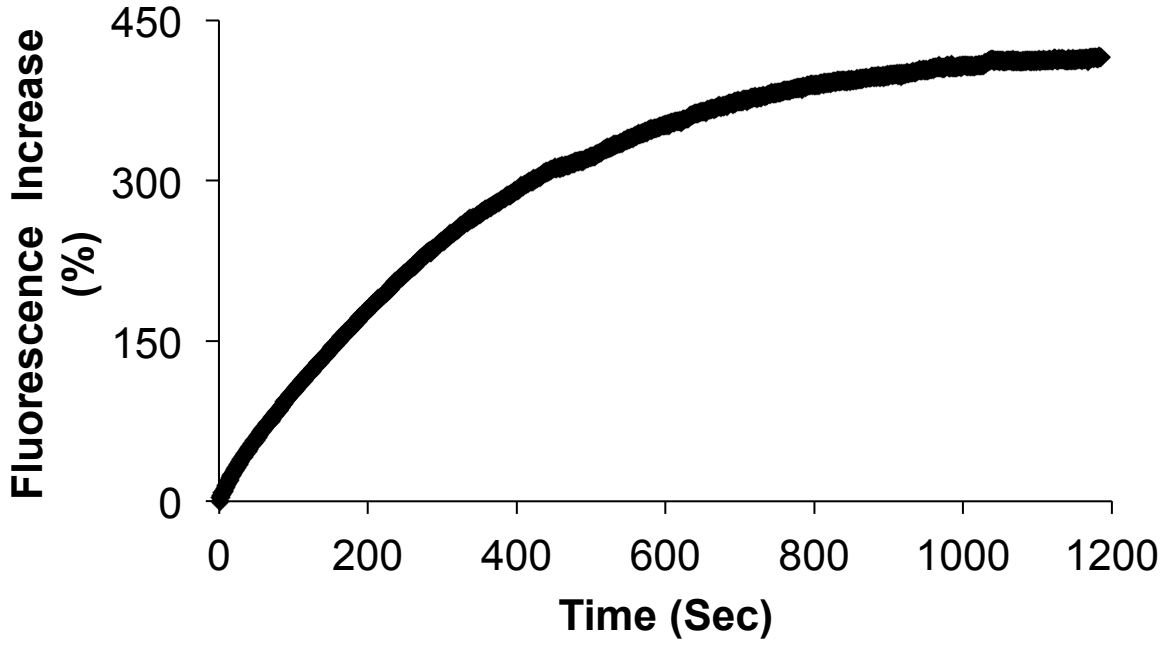


**Figure S12.** Fluorescence increase of **Cbl-4** (1 μM) photolyzed using a spectrofluorometer tuned to four different wavelengths (546 nm for 5 min, 646 nm for 5 min, 727 nm for 20 min, and 777 for 10 min). Data are represented as averages with standard errors for three independent experiments.

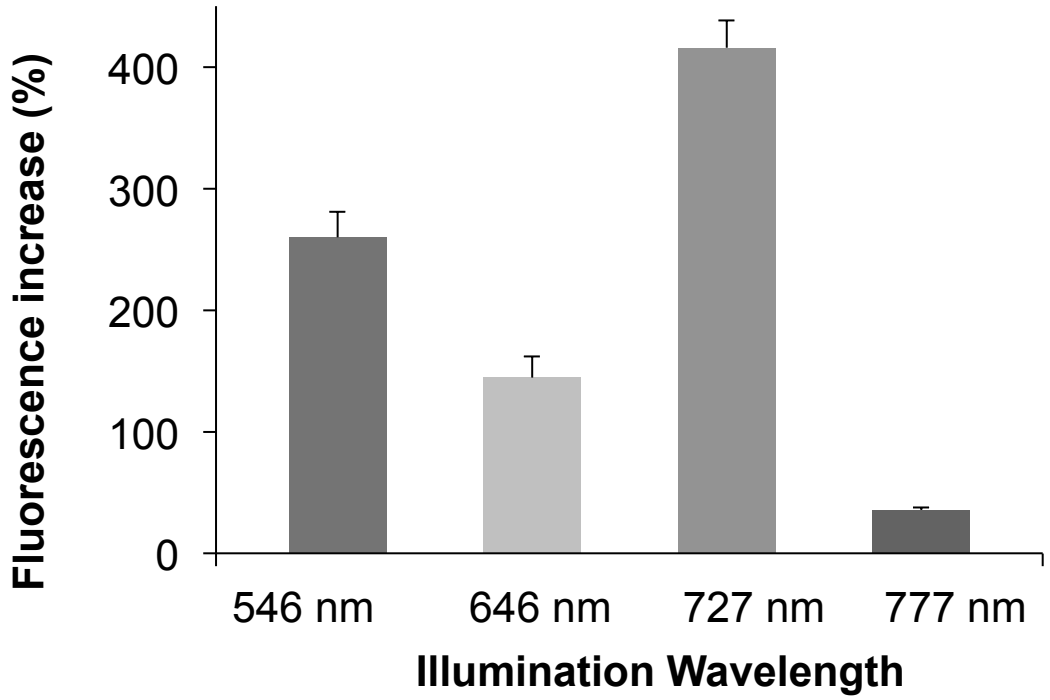


**Figure S13.** Fluorescent increase of **Cbl-5** (20 μM) photolyzed using a spectrofluorometer by excitation at 777 nm and monitoring the fluorescence emission at 794 nm. Data are represented as averages of three independent experiments.

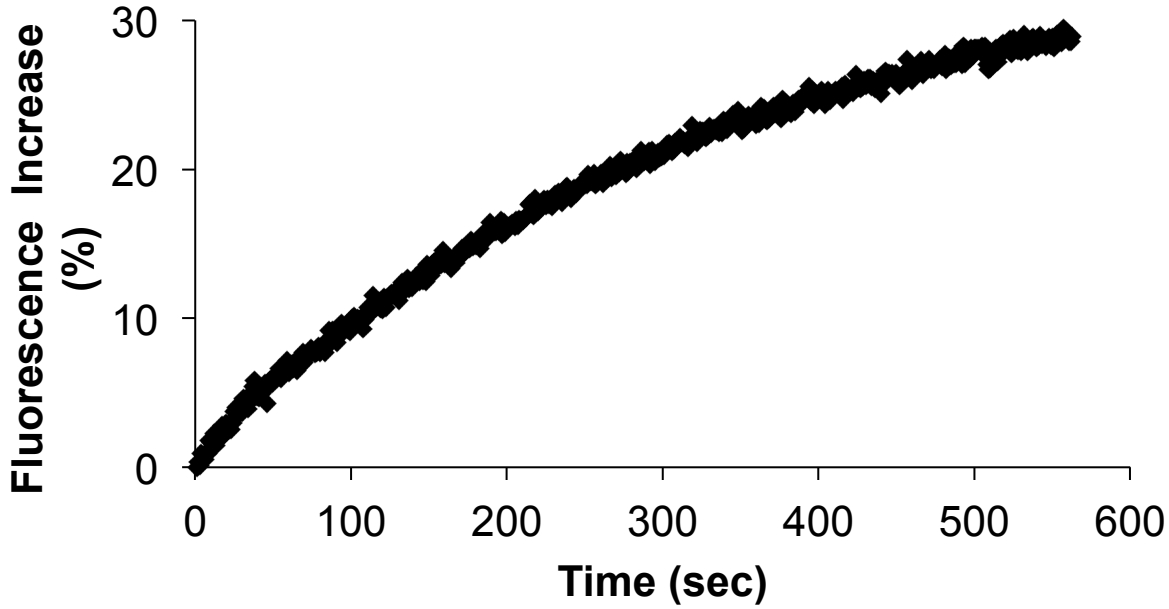


**Figure S14.** Fluorescence increase of **Cbl-5** (20 μM) photolyzed using a spectrofluorometer tuned to four different wavelengths (546 nm for 5 min, 646 nm for 5 min, 727 nm for 20 min, and 777 for 10 min). Data are represented as averages with standard errors for three independent experiments.

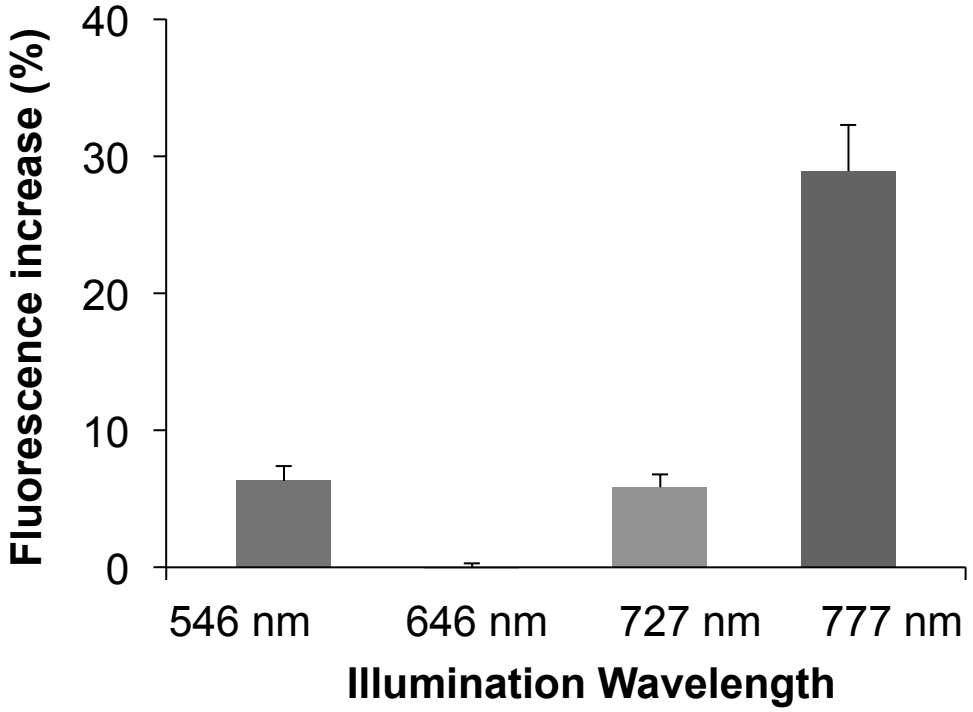


**Figure S15.** Overlay of absorption spectra of **Cbl-1 Cbl-3**, **Cbl-4**, **Cbl-5**, and **Cbl-6**.

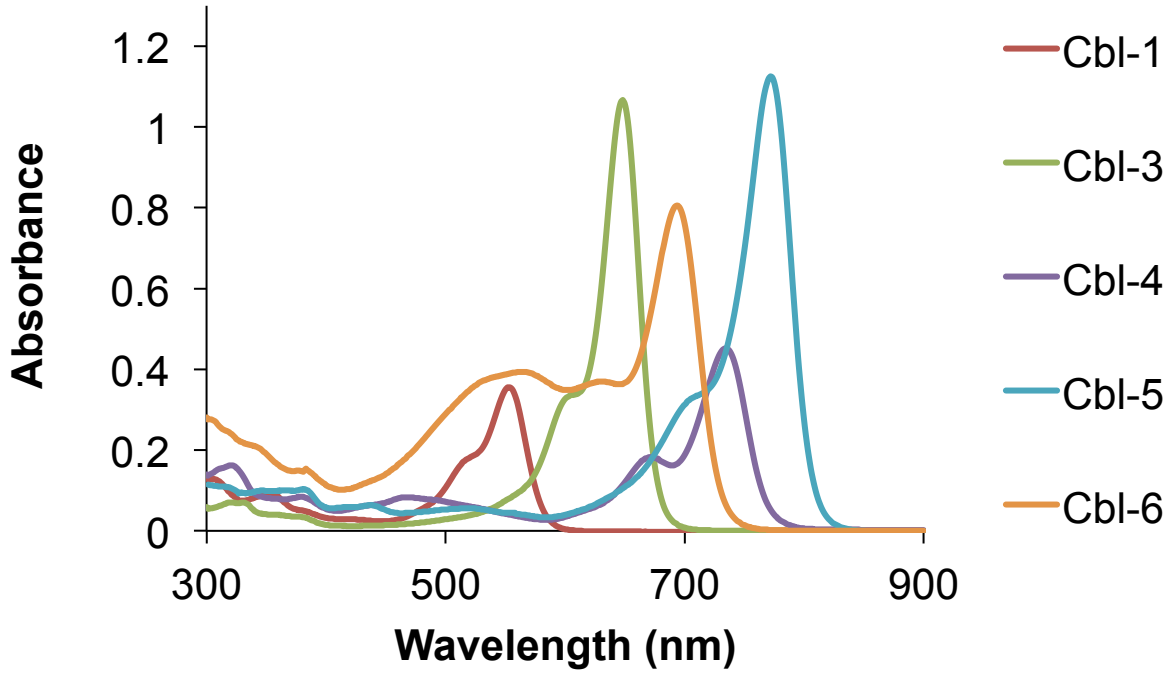


**Figure S16.** Fluorescent increase of **Cbl-6** (1 μM) photolyzed using a spectrofluorometer by excitation at 700 nm and monitoring the fluorescence emission at 715 nm. Data are represented as averages of three independent experiments.

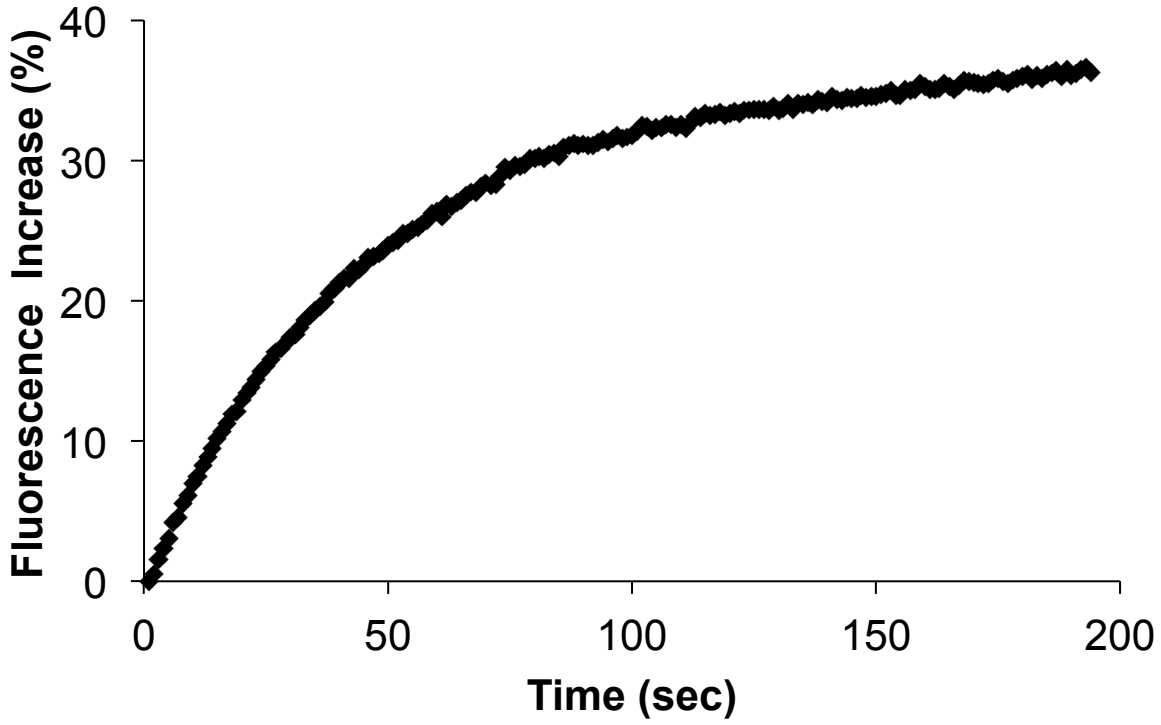


**Figure S17.** Fluorescence increase of **Cbl-6** (1 μM) photolyzed using a spectrofluorometer tuned to four different wavelengths (546 nm for 5 min, 646 nm for 5 min, 700 nm for 3 min, and 777 for 10 min). Data are represented as averages with standard errors for three independent experiments.

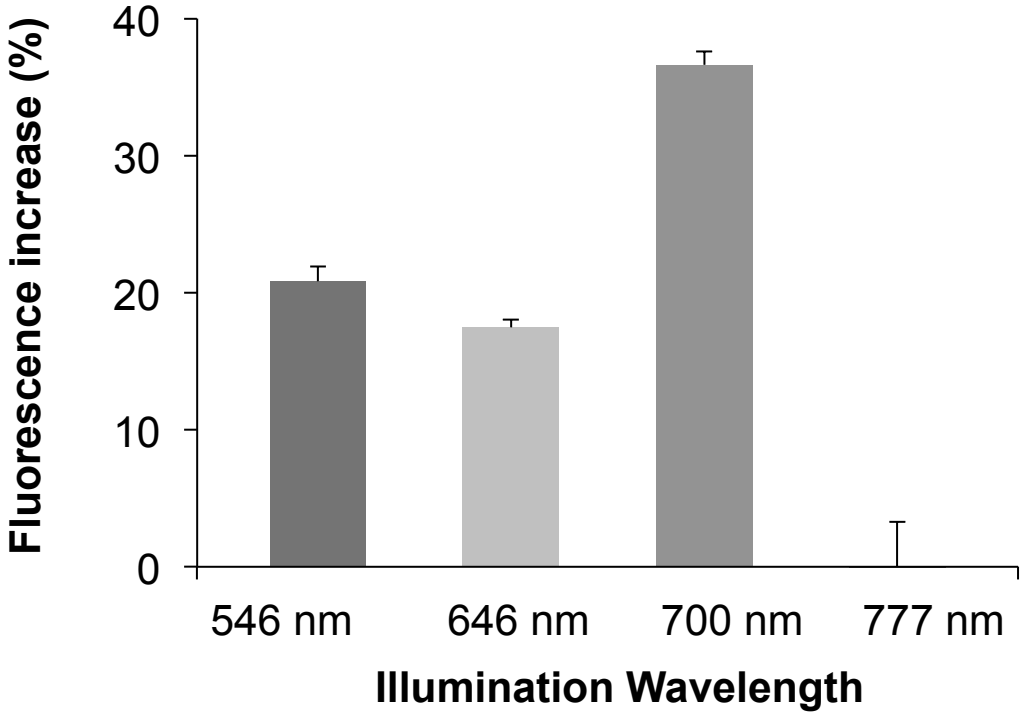


**Figure S18.** Overlay of absorption spectra of **Cbl-3** and **Cbl-5**.

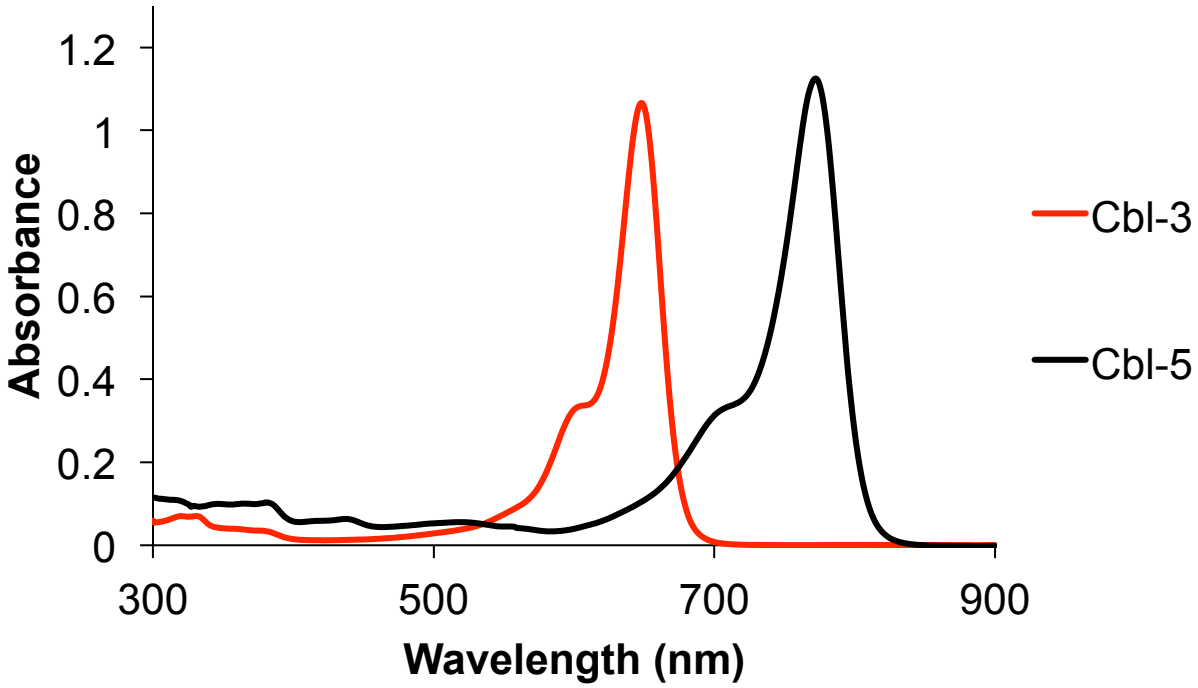


**Figure S19**. Selective photolysis of **Cbl-3** and **Cbl-5** via orthogonal illumination at 646 nm and 777 nm, respectively.

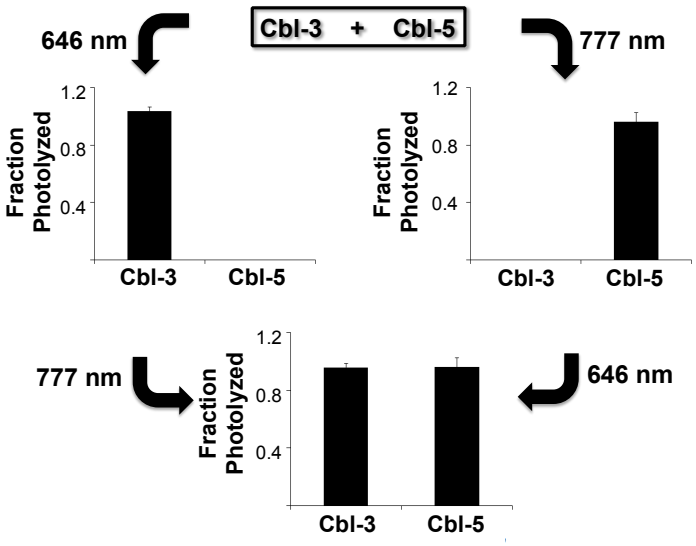


**Figure S20**. Photoinduced conversion of **AdoCbl** (10 μM, squares) to hydroxocobalamin (circles) using a Xe flash lamp at 546 ± 10 nm. Data are represented as averages with standard errors of three independent experiments.

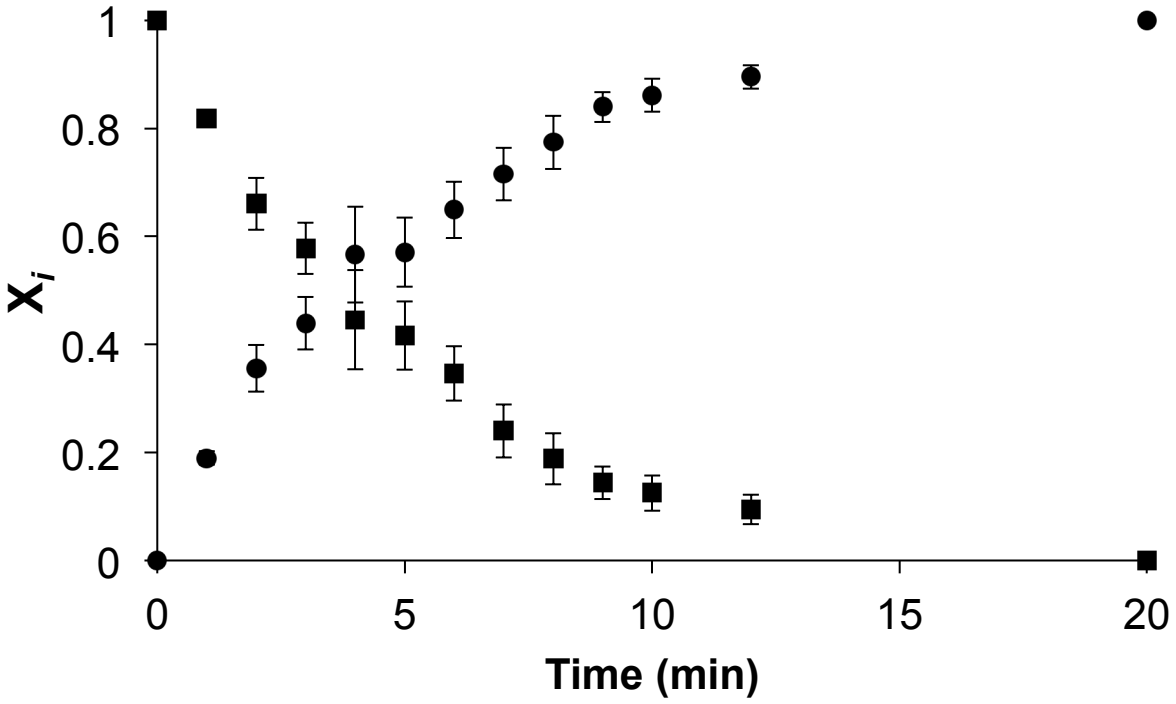


**Figure S21.** Photoinduced conversion **AdoCbl-1** (10 μM, squares) to hydroxocobalamin-TAMRA conjugate (circles) using a Xe flash lamp at 546 ± 10 nm. Data are represented as averages with standard errors of three independent experiments.

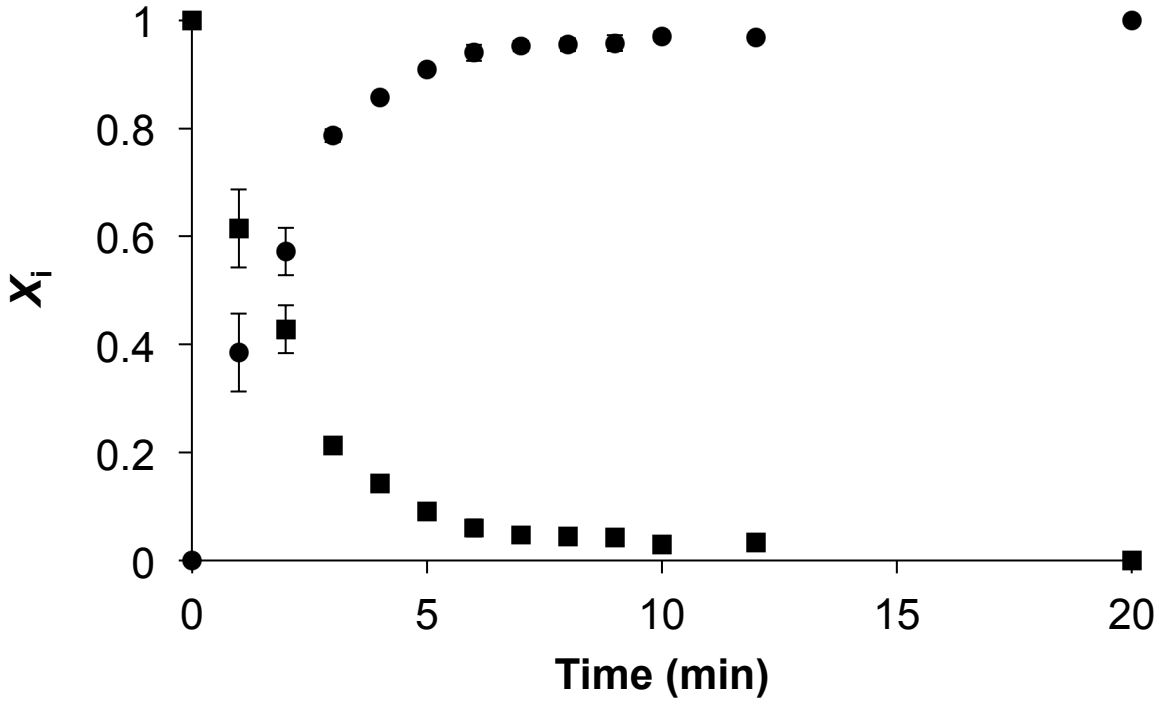


**Figure S22.** Photolysis of **AdoCbl** (10 μM, circles) and **AdoCbl-1** (10 μM, squares) using a Xe flash lamp at 546 ± 10 nm. Data are represented as averages with standard errors of three independent experiments.

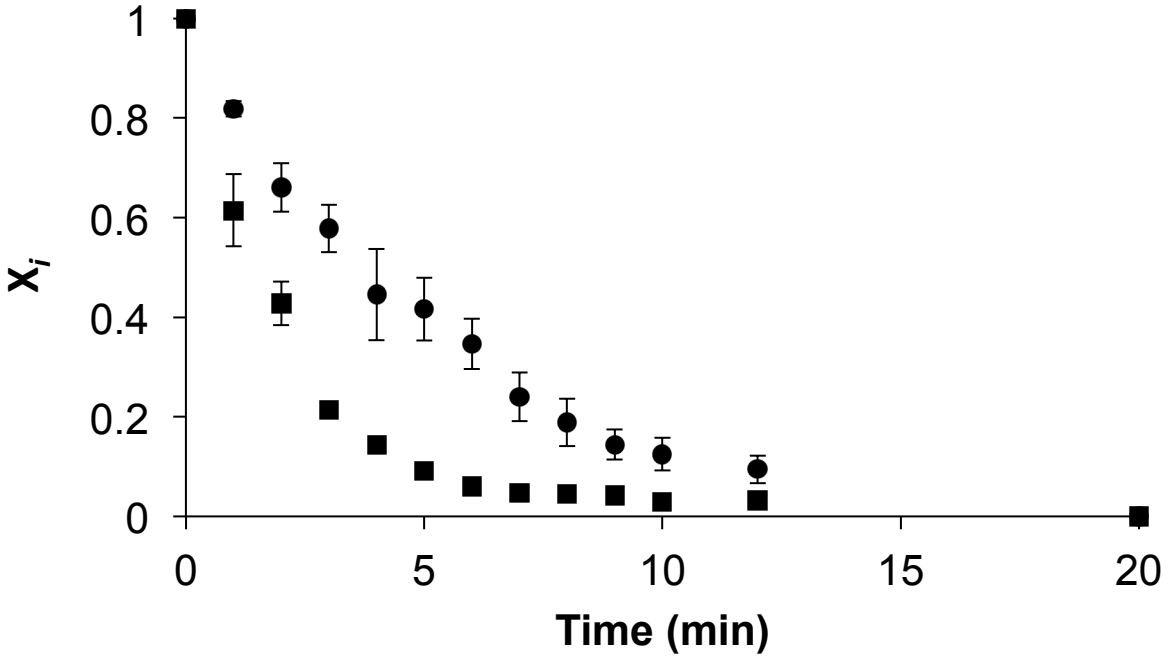


**Figure S23.** Fluorescent increase of **Cbl-Bod** solution (100 nM) photolyzed using a spectrofluorometer by excitation at 646 nm and monitoring the fluorescence emission at 660 nm. Data are represented as averages of three independent experiments.

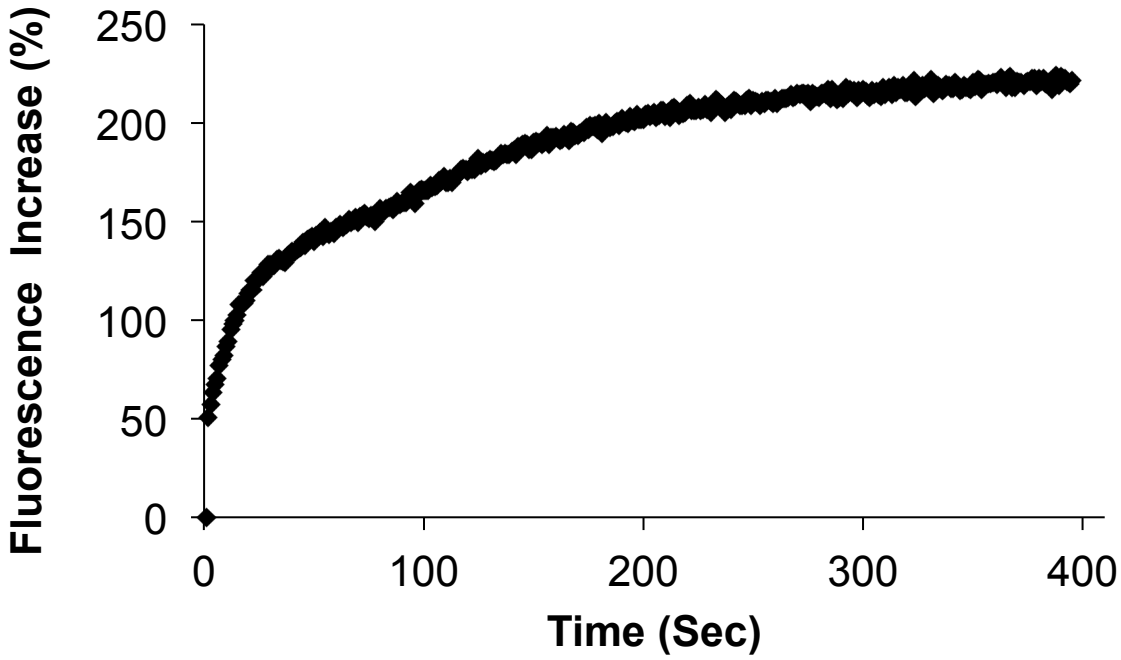


**Figure S24.** Fluorescence increase of a **Cbl-Bod** solution (100 nM) photolyzed using a spectrofluorometer tuned to four different wavelengths (546 nm for 5 min, 646 nm for 5 min, 727 nm for 20 min, and 777 for 10 min). Data are represented as averages with standard errors for three independent experiments.

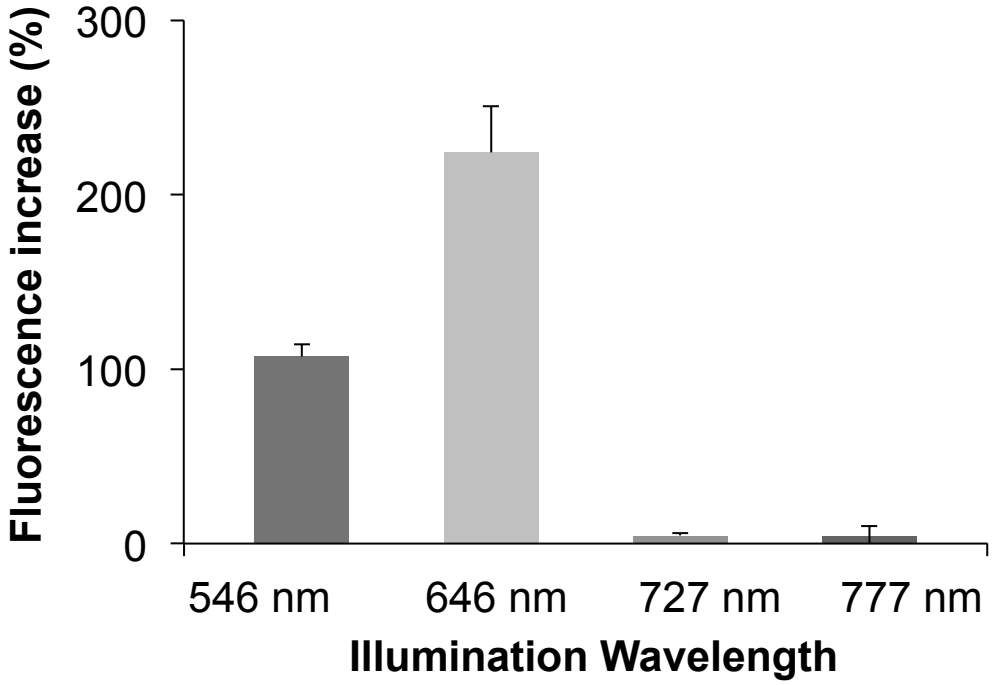


**Figure S25.** Fluorescence increase of **Cbl-Bod** in HeLa cells upon photolysis at 650 nm. (a) 0 min (b) 5 min (c) 10 min (d) 15 min. Imaging and photolysis (500 ms exposure time every minute) were accomplished utilizing an Olympus IX-81 widefield fluorescence microscope with a Cy5 filter cube.

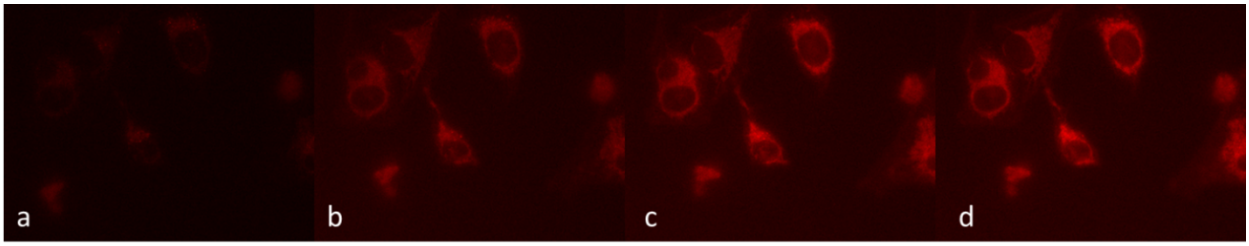


**Figure S26.** Fluorescence increase of HeLa cells loaded with **Cbl-Bod** as a function of time and imaged using a Cy5 filter cube.

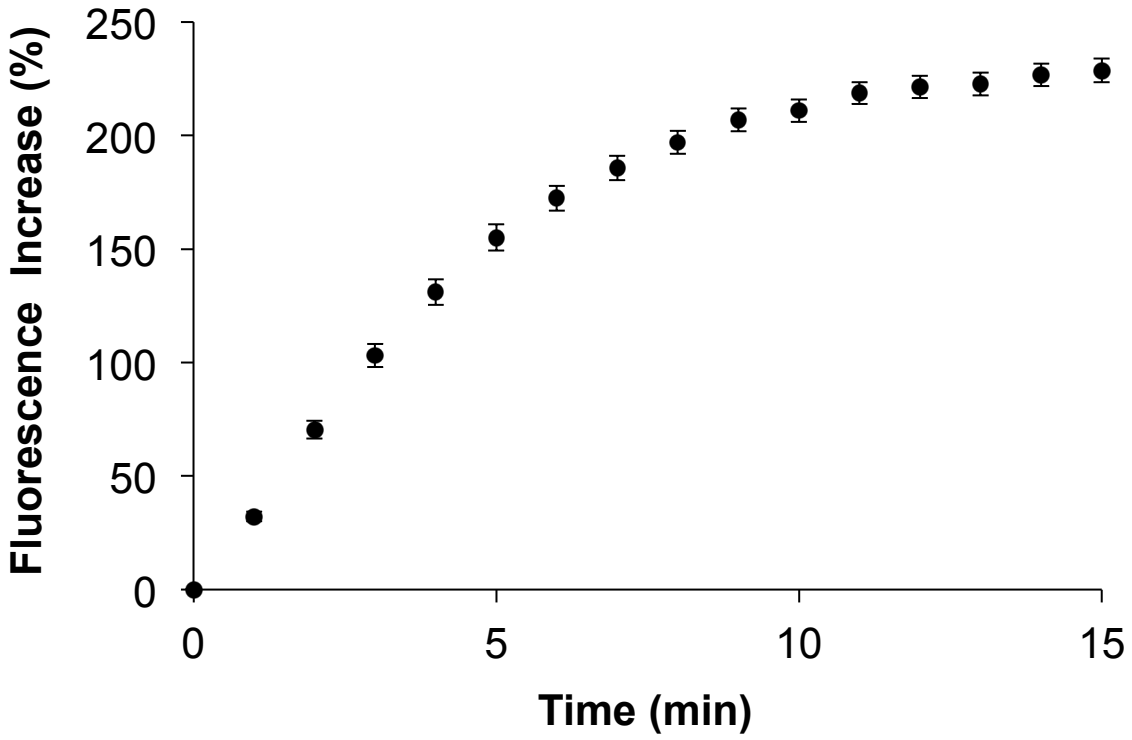
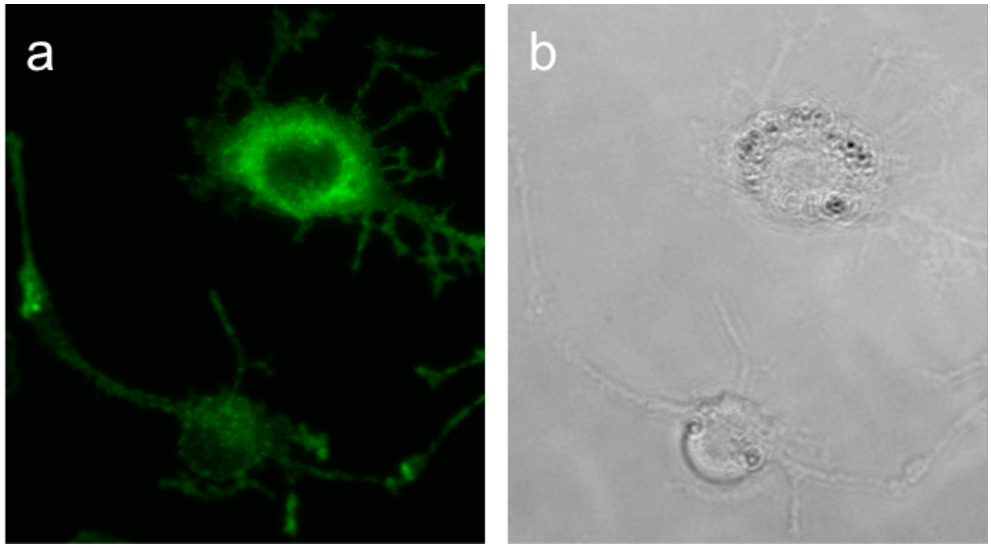


**Figure S27. Cellular response to Ac-cAMP**. REF52 cells were treated with **Ac-cAMP** (500 μM) for 30 min at 37 °C followed by labeling with Dylight 488-phalloidin. Cells show a loss in stress fiber formation similar to treatment with **Cbl-cAMP** (with illumination) as well as with the positive control **CPT-cAMP**. (a) Fluorescent image of Dylight 488-phalloidin staining upon treatment with **Ac-cAMP** (b) transmitted light image. Images are representative of 10 - 20 cells.



**Tables**

**Table S1. Cbl-1** conjugate (10 μM) stored in the dark and photolyzed (Xe flash lamp) at 546 ± 10 nm for 20 min.

| Photolysis Conditions | Retention Time (min) | Observed Mass | Designated Structure |
|---|---|---|---|
| Dark | 12.2 | 900.7 ($M^{2+}$), 600.8 ($M^{3+}$) | **Cbl-1** |
| | 10.0 | 665.1 ($M^{2+}$) | hydroxocobalamin |
| 546 nm | 12.8 | 486.2 ($M^{+}$) | TAMRA-1 |
| | 13.1 | 504.2 ($M^{+}$) | TAMRA-2 |
| | 13.7 | 470.2 ($M^{+}$) | TAMRA-3 |

**Table S2. Cbl-3** (10 μM) stored in the dark and photolyzed using a Xe flash at 646 ± 10 nm for 20 min.

| Photolysis Conditions | Retention Time (min) | Observed Mass | Designated Structure |
|---|---|---|---|
| Dark | 11.6 | 1013.6 ($M^{2+}$), 676.4 ($M^{3+}$) | **Cbl-3** |
| 646 nm | 9.7 | 665.0 ($M^{2+}$) | hydroxocobalamin |
| | 11.8 | 712.3. ($M^{+}$) | SulfoCy5-1 |
| | 12.0 | 730.2 ($M^{+}$) | SulfoCy5-2 |
| | 12.4 | 696.3 ($M^{+}$) | SulfoCy5-3 |

**Table S3. Cbl-4** (10 μM) stored in the dark and photolyzed using a Xe flash lamp at 730 ± 10 nm for 150 min.

| Photolysis Conditions | Retention Time (min) | Observed Mass | Designated Structure |
|---|---|---|---|
| Dark | 12.3 | 893.2 ($M^{2+}$), 595.9 ($M^{+}$) | **Cbl-4** |
| 646 nm | 9.7 | 665.0 ($M^{2+}$) | hydroxocobalamin |
| | 13.3 | 473.3 ($M^{+}$) | Atto725-1 |
| | 13.6 | 489.3 ($M^{+}$) | Atto725-2 |
| | 14.2 | 455.2 ($M^{+}$) | Atto725-3 |

**Table S4. Cbl-5** (10 μM) stored in the dark and photolyzed using a Xe flash lamp at 780 ± 10 nm for 3 h.

| Photolysis Conditions | Retention Time (min) | Observed Mass | Designated Structure |
|---|---|---|---|
| Dark | 12.9 | 1139.7 ($M^{2+}$), 760.4 ($M^{3+}$) | **Cbl-5** |
| 780 nm | 9.9 | 665.0 ($M^{2+}$) | hydroxocobalamin |

**Table S5. Cbl-6** (20 μM) after being stored in the dark and after being photolyzed using a Xe flash lamp at 700 ± 10 nm for 2 h.

| Photolysis Conditions | Retention Time (min) | Observed Mass | Designated Structure |
|---|---|---|---|
| Dark | 11.6 | 1179.9 ($M^{2+}$), 787.0 ($M^{3+}$) | **Cbl-6** |
| 700 nm | 9.794 | 665.0 ($M^{2+}$) | hydroxocobalamin |
| | 10.581 | 1061.0 ($M^{+}$), 531.3 ($M^{2+}$), 264.2 ($M^{4+}$) | Alexa700-2 |

**Table S6. AdoCbl** (10 μM) stored in the dark and photolyzed using a Xe flash lamp at 546 ± 10 nm (20 min), 646 nm (20 min), 730 nm (150 min), and 780 nm (3 h).

| Photolysis Conditions | Retention Time (min) | Observed Mass | Designated Structure |
|---|---|---|---|
| Dark | 10.4 | 790.4 ($M^{2+}$), 527.4 ($M^{3+}$) | **AdoCbl** |
| 546 nm | 2.6 | 284.1 ($M^{+}$) | Adenosine-1 |
| | 5.5 | 284.1 ($M^{+}$) | Adenosine-2 |
| | 9.6 | 664.9 ($M^{2+}$) | hydroxocobalamin |
| 646 nm | 10.4 | 790.4 ($M^{2+}$), 527.4 ($M^{3+}$) | **AdoCbl** |
| 730 nm | 10.5 | 790.4 ($M^{2+}$), 527.4 ($M^{3+}$) | **AdoCbl** |
| 780 nm | 10.4 | 790.4 ($M^{2+}$), 527.4 ($M^{3+}$) | **AdoCbl** |

**Table S7. AdoCbl-1** (10 μM) stored in the dark and photolyzed using a Xe flash lamp at 546 ± 10 nm (20 min), 646 nm (20 min), 730 nm (150 min), and 780 nm (3 h).

| Photolysis Conditions | Retention Time (min) | Observed Mass | Designated Structure |
|---|---|---|---|
| Dark | 11.7 | 1039.9 ($M^{2+}$), 520.4 ($M^{3+}$) | **AdoCbl-1** |
| 546 nm | 2.5 | 284.1 ($M^{+}$) | Adenosine-1 |
| | 5.4 | 284.1 ($M^{+}$) | Adenosine-2 |
| | 11.6 | 914.3 ($M^{2+}$), 609.8 ($M^{3+}$) | hydroxocobalamin |
| 646 nm | 11.7 | 1039.9 ($M^{2+}$), 520.4 ($M^{3+}$) | **AdoCbl-1** |
| 730 nm | 11.7 | 1039.9 ($M^{2+}$), 520.4 ($M^{3+}$) | **AdoCbl-1** |
| 780 nm | 11.7 | 914.3 ($M^{2+}$), 609.8 ($M^{3+}$) | **AdoCbl-1** |

**Table S8. AdoCbl-2** (10 μM) stored in the dark and photolyzed using a Xe flash lamp at 546 ± 10 nm (20 min), 646 nm (20 min), 730 nm (150 min), and 780 nm (3 h).

| Photolysis Conditions | Retention Time (min) | Observed Mass | Designated Structure |
|---|---|---|---|
| Dark | 11.6 | 1152.7 ($M^{2+}$), 768.8 ($M^{3+}$) | **AdoCbl-2** |
| 546 nm | 2.5 | 284.0 ($M^{+}$) | Adenosine-1 |
| | 4.9 | 284.1 ($M^{+}$) | Adenosine-2 |
| | 11.4 | 1027.4 ($M^{2+}$), 684.9 ($M^{3+}$) | $B_{12a}$-SulfoCy5 |
| 646 nm | 2.5 | 284.1 ($M^{+}$) | Adenosine-1 |
| | 5.3 | 284.1 ($M^{+}$) | Adenosine-2 |
| | 11.5 | 1027.0 ($M^{2+}$), 685.2 ($M^{3+}$) | $B_{12a}$-SulfoCy5 |
| 730 nm | 11.6 | 1152.5 ($M^{2+}$), 768.8 ($M^{3+}$) | **AdoCbl-2** |
| 780 nm | 11.6 | 1152.7 ($M^{2+}$), 768.8 ($M^{3+}$) | **AdoCbl-2** |

**Table S9. AdoCbl-3** (10 μM) stored in the dark and photolyzed using a Xe flash lamp at 546 ± 10 nm (20 min), 646 nm (20 min), 730 nm (150 min), and 780 nm (3 h).

| Photolysis Conditions | Retention Time (min) | Observed Mass | Designated Structure |
|---|---|---|---|
| Dark | 11.8 | 1032.3 ($M^{2+}$), 688.5 ($M^{3+}$) | **AdoCbl-3** |
| 546 nm | 2.4 | 284.1 ($M^{+}$) | Adenosine-1 |
| | 12.3 | 906.8 ($M^{2+}$), 604.8 ($M^{3+}$) | $B_{12a}$-Atto725 |
| 646 nm | 2.6 | 284.1 ($M^{+}$) | Adenosine-1 |
| | 12.0 | 1032.3 ($M^{2+}$), 688.5 ($M^{3+}$) | **AdoCbl-3** |
| | 12.2 | 907.4 ($M^{2+}$), 605.0 ($M^{3+}$) | $B_{12a}$-Atto725 |
| 730 nm | 2.4 | 284.1 ($M^{+}$) | Adenosine-1 |
| | 4.4 | 284.1 ($M^{+}$) | Adenosine-2 |
| | 12.3 | 906.7 ($M^{2+}$), 604.8 ($M^{3+}$) | $B_{12a}$-Atto725 |
| 780 nm | 11.9 | 1032.4 ($M^{2+}$), 688.5 ($M^{3+}$) | **AdoCbl-3** |

**Table S10. AdoCbl-4** (10 μM) stored in the dark and photolyzed using a Xe flash lamp at 546 ± 10 nm (20 min), 646 nm (20 min), 730 nm (150 min), and 780 nm (3 h).

| Photolysis Conditions | Retention Time (min) | Observed Mass | Designated Structure |
|---|---|---|---|
| Dark | 12.8 | 1279.6 ($M^{2+}$), 853.3 ($M^{3+}$) | **AdoCbl-4** |
| 546 nm | 2.4 | 284.0 ($M^{+}$) | Adenosine-1 |
| | 4.9 | 284.0 ($M^{+}$) | Adenosine-2 |
| | 12.5 | 1153.5 ($M^{2+}$), 769.0 ($M^{+}$) | $B_{12a}$-Dylight800 |
| 646 nm | 12.8 | 1279.0 ($M^{2+}$), 853.0 ($M^{3+}$) | **AdoCbl-4** |
| 730 nm | 2.4 | 284.0 ($M^{+}$) | Adenosine-1 |
| | 4.7 | 284.1 ($M^{+}$) | Adenosine-2 |
| | 12.6 | 1154.2 ($M^{2+}$), 769.2 ($M^{3+}$) | $B_{12a}$-Dylight800 |
| 780 nm | 2.1 | 284.0 ($M^{+}$) | Adenosine-1 |
| | 12.4 | 1154.2 ($M^{2+}$), 769.2 ($M^{3+}$) | $B_{12a}$-Dylight800 |

**Table S11. Cbl-Bod** (20 μM) stored in the dark and photolyzed using a Xe flash lamp at 646 ± 10 nm for 2 h.

| Photolysis Conditions | Retention Time (min) | Observed Mass | Designated Structure |
|---|---|---|---|
| Dark | 14.0 | 958.7 ($M^{2+}$), 639.5 ($M^{3+}$) | **Cbl-Bod** |
| 646 nm | 9.6 | 665.0 ($M^{2+}$) | hydroxocobalamin |
| | 16.9 | 586.3 ($M^{+}$) | BODIPY650-3 |