

Supporting Information

Multiphoton Activation of Photo-SPAAC 'Click' Reagents Enables In-situ Labelling with Submicron Resolution

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General Methods. Flash chromatography was performed using 40-63 μm silica gel. All NMR spectra were recorded in CDCl_3 using a 400 MHz instrument. Electronic spectra were taken using a Carry-300 Bio UV-Vis spectrometer. Spectral changes in photo-ODIBO-linked hydrogels were monitored by absorbance spectroscopy using a Thermo Scientific Nanodrop 2000 spectrophotometer. The gels were aligned in the 1mm gap between the quartz pedestals of the instrument, which were first wetted with buffer.

Two-photon excitation experiments in solution were performed using an amplified femtosecond laser system (Mira Optima 900 F and Legend Elite, Coherent Inc.) coupled with a Topaz-C optical paramagnetic amplifier operating at 1 kHz and producing 140 fs duration pulses. The laser beam was attenuated by a diaphragm with a 6.15 mm (800 nm) or 5.5 mm (660 and 690 nm) opening. 1.2 mL of sample solution was placed in a 1X1 cm quartz cell. The cell was placed inside a massive blackened brass cell holder attached to a metal bench within a temperature controlled optical enclosure. The consumption of starting materials was followed using a UV spectrophotometer. The power output of the laser was measured after the diaphragm and cuvette holder with and without sample. At the concentration of the substrate used in these experiments the loss of energy after the sample is mostly due to the losses at the phase boundaries, which allows us to evaluate the laser power within the sample as an average of the above two values. Using these parameters, we have calculated the distribution of light intensity and squared light intensity within the pulse assuming an ideal Gaussian pulse shape. The limits of integration for the calculation of the squared light intensity were set to ± 100 fs from the center of the pulse, at which points the value I drops to less than 0.3% of the maximum. Due to the dimensions of the beam, the irradiated volume was smaller than the total volume of the sample. The fraction of irradiated volume, f_V was equal to 0.25 in the 800 nm experiment, and 0.19 in the 660 and 690 nm experiment. The lifetime of **1a,c** in the course of the 2PE experiment ($\tau > 2.5 \times 10^4$ s) was much longer than required for diffusive mixing of the sample. We therefore can re-write equation 1 in a differential form:

$$\frac{dC}{dt} = -C \cdot f_V \cdot k_{obs} \quad (\text{S1})$$

Where k_{obs} is described by eq. 2. From eq. S1, we can express the changes of concentration in the sample as:

$$C(t) = C_0 \cdot e^{-k_{obs} \cdot f_V \cdot t} \quad (S2).$$

The duration of irradiation experiments required to achieve certain conversion were therefore scaled down by the factor of 0.25 (800 nm) or 0.175 (660 and 690 nm).

2PE product analysis. Methanol solutions of photo-ODIBO (**1a**, 200 μ M) alone (Figure S1A) or in the presence of 2-fold excess of butyl azide (Figure S1B) were irradiated for 90 min* using 800 nm pulses of Ti:S laser. The resulting photolysates were analyzed by HPLC, following absorbance at 275 nm and using methanol-water mixtures as an eluent (Figure S1). The instrument response was calibrated using authentic samples of cyclopropenones **1a,c** and isomeric triazoles **3a**. 800 nm irradiation of **1a** resulted in 13% conversion of photo-ODIBO producing ODIBO (**2a**) as a single product (Fig. S1A). Similar irradiation of a methanol solution containing photo-ODIBO (**1a**) and butyl azide results in the clean formation of two isomers (head-to-head and head-to-tail) of triazole **3a** (Scheme 1, R' = Bu), confirming decarbonylation into functional ODIBO (**2a**). The products were identified by HPLC using authentic triazoles as a reference (Fig. S2B).

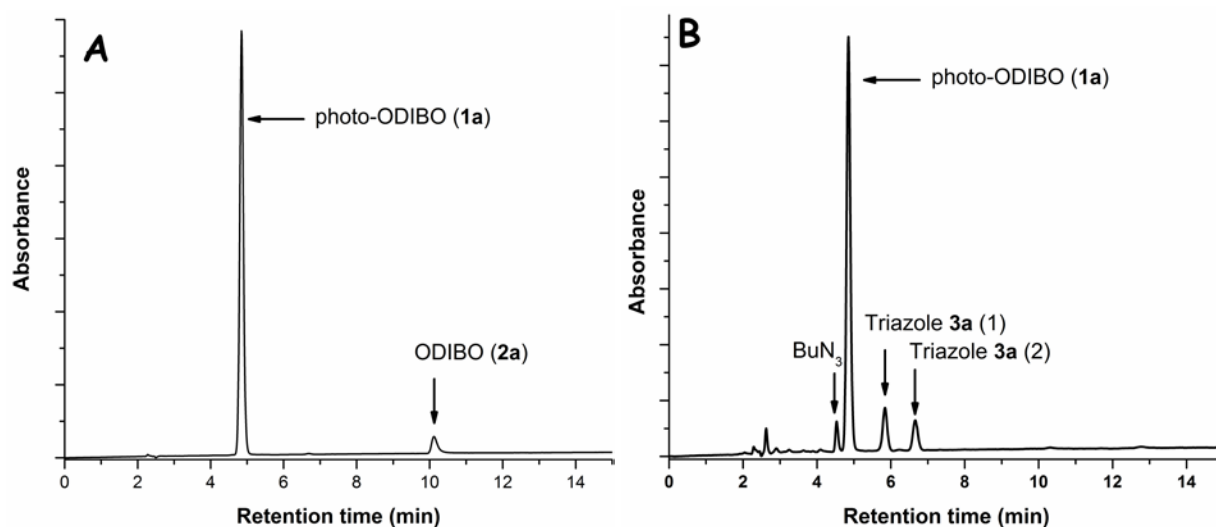


Figure S1. HPLC traces of reaction mixtures of 800 nm pulsed laser irradiation of 200 μ M solution of photo-ODIBO (**1a**): panel A, neat; panel B, in the presence of 400 μ M of butyl azide.

* The duration of irradiation has been scaled to account for the difference between irradiated and total volume (*vide supra*)

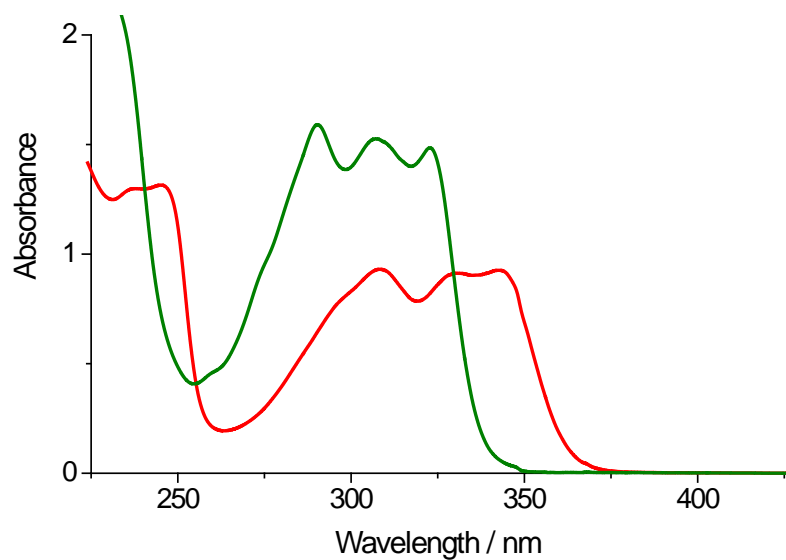


Figure S2. Spectra of ca. 0.1 mM methanol solutions of photo-ODIBO (**1a**, red line) and ODIBO (**2a**, green line).

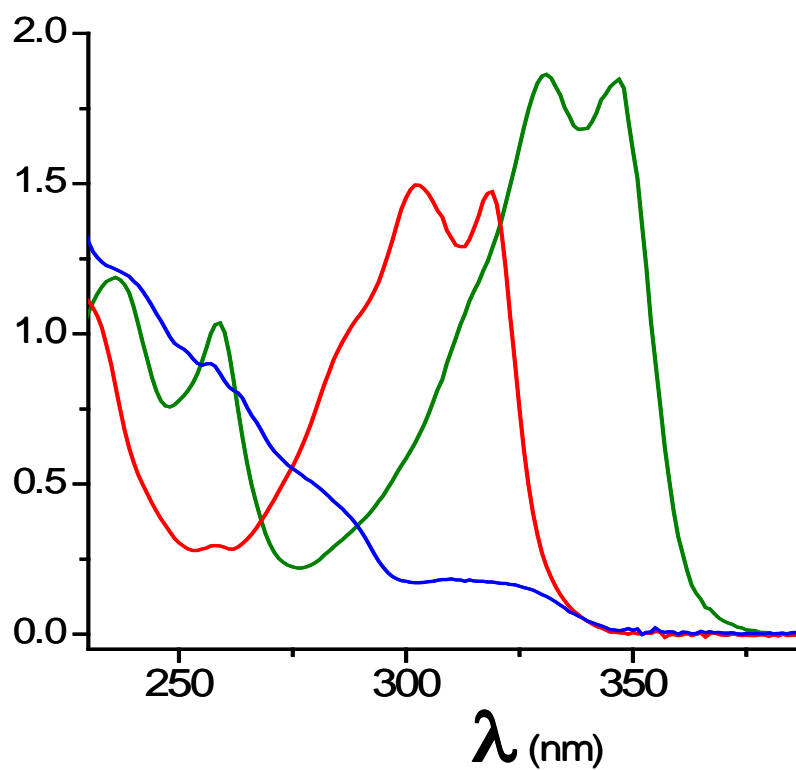


Figure S3. Spectra of ca. 0.1 mM methanol solutions of photo-DIBO (**1c**, green line), DIBO (**2c**, red line), and triazole **3c** (blue line).

Two-Photon patterning of the PAG-photo-ODIBO gel. Slides with a slab of the gel were mounted on the stage of an Olympus FVMPE-RS microscope equipped with a Spectra-Physics InSight® DeepSee™ laser (80 MHz pulse rate, ~100 fs pulses). The upper surface of the slab was brought into the focus of a water immersion 25X 1.05 N.A. objective. The focus was then adjusted into the slab about 20-50 μm from the upper surface. The femtosecond laser was set to the desired wavelength, power, and exposure duration using the Olympus control software. Resolution tests were performed using a 100X 1.4 N.A. oil immersion objective, focused 10-20 μm below the upper surface of the slab. The laser was set to 690 nm wavelength, attenuation at 10% of laser power, and the decarbonylation and imaging were performed using a 100X oil immersion objective, N.A. 1.4. The slabs were reacted with Rhodamine B azide, washed and imaged using 2PE at 830 nm with attenuation set to 3% laser power.

Multiphoton imaging of the slides was performed using the Olympus microscope described above, or a Nikon A1R MP+ microscope equipped with a Coherent Chameleon Vision II femtosecond laser. The excitation wavelength was set at 830 nm. Adjustments of image background and contrast were made using Image J.¹ The 3D image stack was processed using Nikon software.

Materials. All organic solvents were dried and freshly distilled before use; Tetrahydrofuran was distilled from sodium/benzophenone ketyl and dichloromethane was distilled from CaH_2 . Other reagents were obtained from Aldrich or VWR and used as received unless noted. Photo-ODIBO-EG₄-OH (**1a**),² photo-ODIBO-EG₄-NHS (**1b**),² photo-DIBO-EG₄-OH (**1c**),² and Rhodamine B azide³ were prepared using previously reported procedures.

Triazoles 3a (R' = Bu). Butyl azide (9 mg, 0.092 mmol) was added to a solution of ODIBO-EG₄-OH (40 mg, 0.88 mmol) in MeOH (2 mL). The reaction mixture was stirred overnight, concentrated in vacuo, and purified via flash chromatography (1:1 hexanes: acetone) to afford two isomeric triazole as colorless oils (total yield: 28 mg, 58 %).

Triazole 1 (7 mg, 15%): ¹H-NMR: 7.60-7.61 (d, J = 2.5 Hz, 1H), 7.25 (d, J = 2.3 Hz, 1H), 7.18-7.21 (dd, J = 8.7, 2.5 Hz, 1H), 7.10 (d, J = 2.5 Hz, 1H), 7.00-7.03 (dd, J = 8.5, 2.6 Hz, 1H), 6.83-6.85 (d, J = 8.7 Hz, 1H), 4.90-5.35 (d, J = 179.3 Hz, 2H), 4.33-4.35 (m, 2H), 4.17-4.20 (t, J = 4.6 Hz, 2H), 3.85-3.88 (t, J = 4.7 Hz, 2H), 3.65-4.74 (m, 10H), 3.58-3.60 (m, 2H), 1.82 (s, 2H), 1.27 (s, 11H), 0.84-0.87 (t, J = 7.4 Hz, 3H). ¹³C-NMR: 159.85, 152.26, 145.17, 144.24, 138.51, 132.01, 130.03, 129.91, 127.06, 120.10, 119.98, 116.91, 116.71, 116.00, 72.69, 71.03, 70.86, 70.78,

70.51, 69.77, 69.70, 67.85, 61.93, 48.40, 34.31, 32.33, 31.56, 19.89, 13.63. ESI HRMS: calcd. (M+H⁺): C₃₁H₄₄N₃O₆⁺ 554.3225, found 554.3222.

Triazole 2 (21 mg, 23%). ¹H-NMR: 7.64 (d, J = 8.6 Hz, 1H), 7.34-7.36 (dd, J = 8.7, 2.5 Hz, 1H), 7.07-7.09 (d, J = 8.7 Hz, 1H), 7.05-7.06 (d, J = 2.4 Hz, 1H), 6.95-6.98 (dd, J = 8.6, 2.6 Hz, 1H), 6.87 (d, J = 2.6 Hz, 1H), 5.22 (s, 1H), 4.39-4.43 (t, J = 7.5 Hz, 2H), 4.14-4.16 (t, J = 4.8 Hz, 2H), 3.84-3.86 (t, J = 4.8 Hz, 2H), 3.66-3.74 (m, 10H), 3.59-3.62 (m, 2H), 1.85-2.62 (m, 1H), 1.89-1.97 (m, 2H), 1.33-1.37 (m, 2H), 1.28 (s, 9H) 0.87-0.91 (t, J = 7.4 Hz, 3H). ¹³C-NMR: 158.82, 153.85, 145.71, 144.79, 136.86, 132.98, 132.06, 128.33, 127.12, 124.07, 121.51, 116.62, 115.09, 114.75, 73.77, 72.68, 71.03, 70.87, 70.81, 70.55, 69.88, 67.71, 61.95, 48.77, 34.50, 32.49, 31.54, 19.99, 13.64. ESI HRMS: calcd. (M+H⁺): C₃₁H₄₄N₃O₆⁺ 554.3225, found 554.3224.

Photo-ODIBO derivatized polyacrylamide slabs. Amine-doped polyacrylamide hydrogel slabs were prepared as previously described.² Briefly, the amine source allyamine was added to a standard TEMED-ammonium persulfate catalyzed acrylamide/bisacrylamide polymerization reaction in 0.1 M sodium citrate buffer at pH 5, which was degassed and then poured into a mold made from layered glass microscope slides. The spacers used to define the resulting slab thickness were either 1 or 0.07 mm thick. The hydrogel slabs were washed with pH 5 citrate buffer, and then photo-ODIBO-EG₄-NHS (**1b**) dissolved in anhydrous DMSO was added to bring the concentration to 0.3 mM and the slabs were incubated overnight with continuous rocking. The slabs were then immersed in PBS with 20 mM sodium azide to quench spurious ODIBO, washed with PBS, placed onto microscope slides and covered with 0.17 mm thick glass coverslips supported on spacers matching the slab thickness.

General Procedure for Determination of Two-Photon Cross-Section. 1.2 mL of methanol solutions of either photo-ODIBO-EG₄-OH (**1a**, ~210 μM) or photo-DIBO-EG₄-OH (**1c**, ~120 μM) in MeOH (1.2 mL) were irradiated with a Ti:Sapphire laser (800, 690, 660 nm). The conversion of cyclopropenone to alkyne was measured by following the decay of the characteristic cyclopropenone peak in UV spectra (348 nm for **1a** and 350 nm for **1c**) after various time intervals.

Table S1. Two-photon induced decarbonylation of **1a** and **1c** under 800 nm pulsed irradiation.

Photo-ODIBO-EG ₄ -OH (1770 μ J/pulse)			Photo-DIBO-EG ₄ -OH (1800 μ J/pulse)		
Time of Irradiation (s)	Concentration (M)	Conversion (%)	Time of Irradiation (s)	Concentration (M)	Conversion (%)
0	2.10×10^{-4}	0	0	1.18×10^{-4}	0
2673	1.97×10^{-4}	6	1782	1.09×10^{-4}	8
5347	1.86×10^{-4}	12	3565	1×10^{-4}	16
8020	1.75×10^{-4}	17	5347	9.2×10^{-5}	22
10694	1.65×10^{-4}	22	7129	8.44×10^{-5}	29
13367	1.55×10^{-4}	26	8912	7.69×10^{-5}	35
16041	1.43×10^{-4}	32	10694	7.03×10^{-5}	41
18715	1.35×10^{-4}	36	12476	6.4×10^{-5}	46
21388	1.28×10^{-4}	39			
23170	1.24×10^{-4}	41			

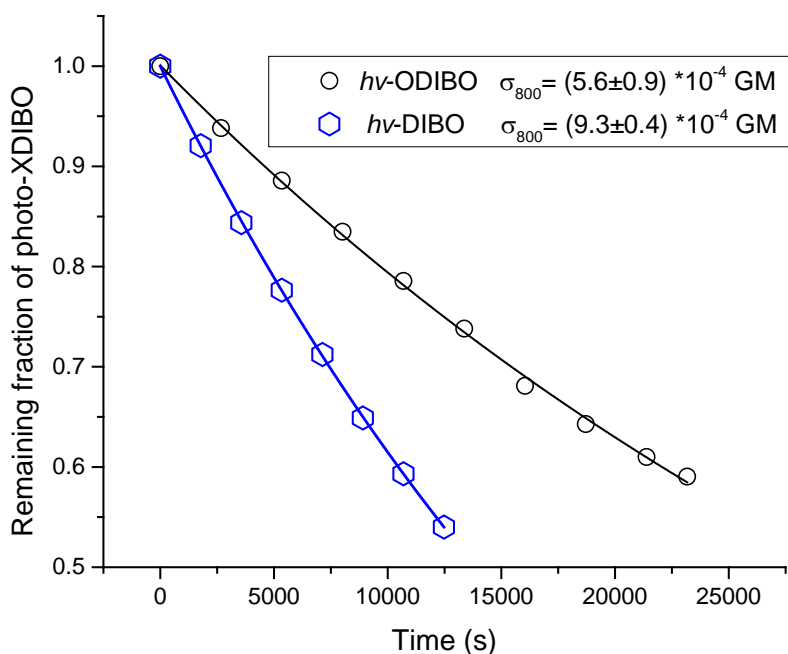


Figure S4. Decarbonylation of photo-ODIBO (**1a**, black circles) and photo-DIBO (**1c**, blue hexagons) in methanol solution under 800 nm pulsed irradiation. The trend-lines are least squares fits of equation 1 to the experimental data.

Table S2. Two-photon induced decarbonylation of **1a** and **1c** under 690 nm pulsed irradiation

Photo-ODIBO-EG₄-OH (85 μJ/pulse)			Photo-DIBO-EG₄-OH (85 μJ/pulse)		
Time of Irradiation (s)	Concentration (M)	Conversion (%)	Time of Irradiation (s)	Concentration (M)	Conversion (%)
0	2.14×10^{-4}	0	0	1.15×10^{-4}	0
713	1.84×10^{-4}	11	535	1.02×10^{-4}	11
1425	1.65×10^{-4}	20	1069	9.22×10^{-5}	20
2138	1.48×10^{-4}	28	1604	8.37×10^{-5}	27
2994	1.31×10^{-4}	37	2138	7.61×10^{-5}	33
3706	1.17×10^{-4}	43	2673	6.92×10^{-5}	40
			3267	6.23×10^{-5}	46

Table S3. Two-photon induced decarbonylation of **1a** and **1c** under 660 nm pulsed irradiation

Photo-ODIBO-EG₄-OH (112 μJ/pulse)			Photo-DIBO-EG₄-OH (114 μJ/pulse)		
Time of Irradiation (s)	Concentration (M)	Conversion (%)	Time of Irradiation (s)	Concentration (M)	Conversion (%)
0	2.14×10^{-4}	0	0	1.16×10^{-4}	0
178	2.07×10^{-4}	3	59	1.13×10^{-4}	3
713	1.9×10^{-4}	11	178	1.08×10^{-4}	7
1425	1.71×10^{-4}	20	356	1.01×10^{-4}	13
2138	1.52×10^{-4}	29	653	9.04×10^{-5}	22
3207	1.29×10^{-4}	40	1069	7.7×10^{-5}	34
3741	1.21×10^{-4}	46	1603	6.14×10^{-5}	47

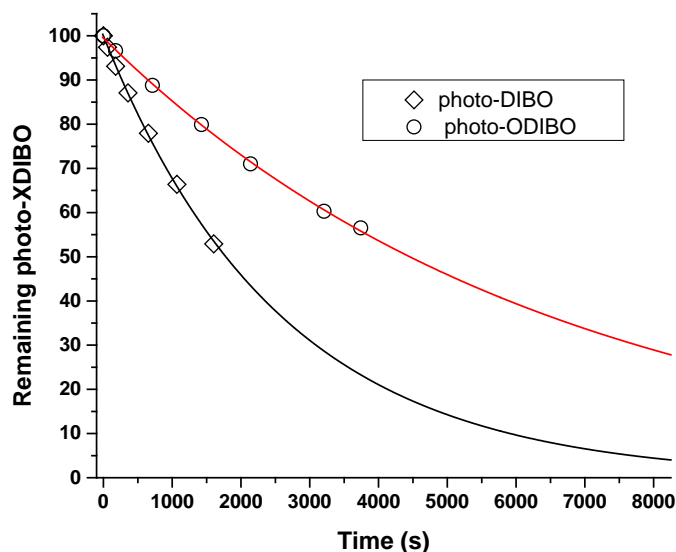


Figure S5. Decarbonylation of photo-ODIBO (**1a**, circles) and photo-DIBO (**1c**, hexagons) in methanol solution under 660 nm pulsed irradiation. The trend-lines illustrate the results of the least squares fitting of the equation 1 to experimental data.

Analysis of the dependence of the rate of decarbonylation on the laser power. A solution of photo-ODIBO-EG₄-OH (**1a**, ~200 μ M) or photo-DIBO-EG₄-OH (**1c**, ~110 μ M) in MeOH (1.2 mL), was irradiated with a Ti:Sapphire laser, at 660 nm, for three hours. The pulse energy was varied using neutral density filters (THOR LABS) and the conversion of the cyclopropanone to alkyne was followed by UV absorbance spectroscopy.

Table S4. Conversion of **1a** and **1b** at after 3h of 660 nm irradiation with variable pulse energies

Photo-ODIBO-EG ₄ -OH			Photo-DIBO-TEG ₄ -OH		
Pulse Energy (μ J/pulse)	Concentration (M)	Conversion (%)	Pulse Energy (μ J/pulse)	Concentration (M)	Conversion (%)
0	2.05×10^{-4}	100	0	1.14×10^{-4}	100
35	1.99×10^{-4}	97	31	1.11×10^{-4}	97
44	1.96×10^{-4}	96	41	1.09×10^{-4}	96
54	1.95×10^{-4}	95	55	1.05×10^{-4}	92
68	1.85×10^{-4}	90	71	9.43×10^{-5}	83
91	1.62×10^{-4}	79	85	6.78×10^{-5}	59

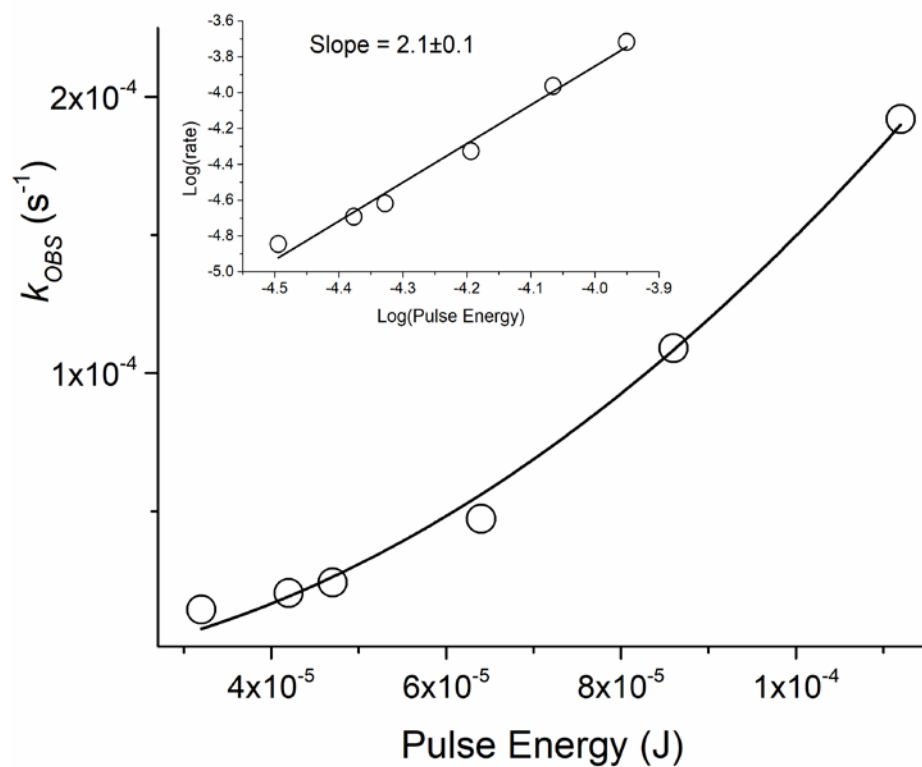


Figure S6. The observed rates (k_{obs}) of photo-decarbonylation of photo-ODIBO (**1a**, 210 μ M in MeOH) under 660 nm pulsed irradiation of variable pulse energy. The trend-line represents the least squares fit of equation 2 to the experimental data. Insert shows logarithmic representation of the rate versus pulse energy relationship.

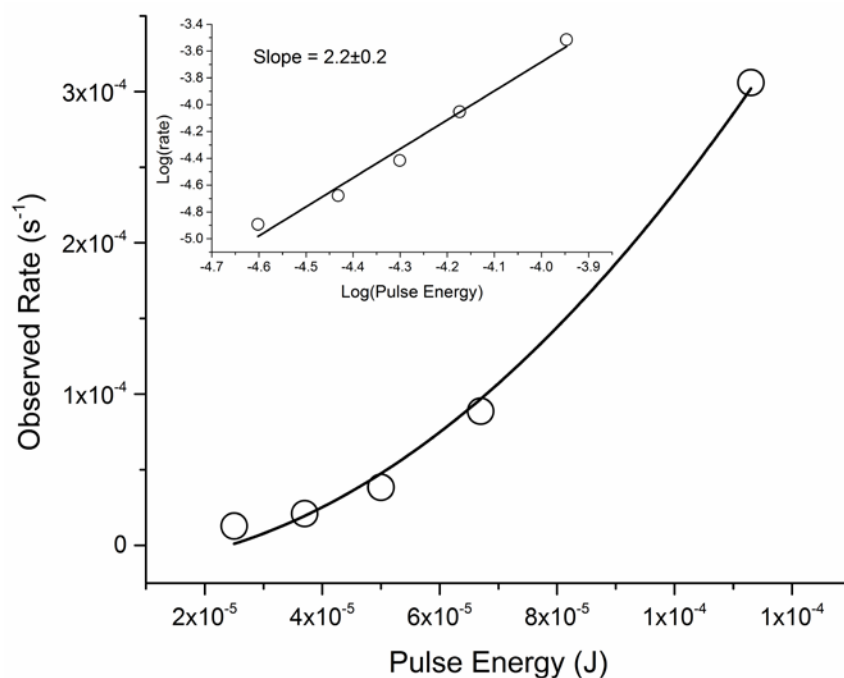


Figure S7. The observed rates of photo-decarbonylation of photo-DIBO (**1c**, 114 μM in MeOH) under 660 nm pulsed irradiation of variable pulse energy. The trend-line is the least squares fit of equation 2 to the experimental data. Insert shows logarithmic representation of the rate versus pulse energy relationship.

References

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