Supporting Information

Azido Push–Pull Fluorogens Photoactivate to Produce Bright Fluorescent Labels

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Movie S1. Single molecules activated in PMMA. See Figure 2B of main text for more information. Several activations take place in the movie; the frames during the activations were removed. The movie is sped up 4× real time. The original integration time was 100 ms per frame with an electron-multiplication gain setting of 300.

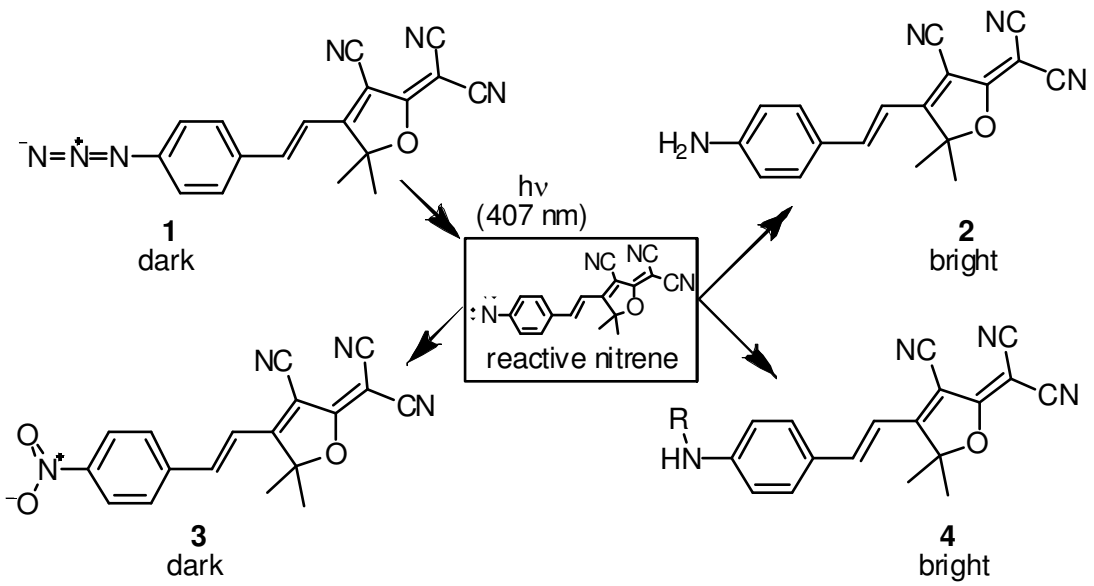
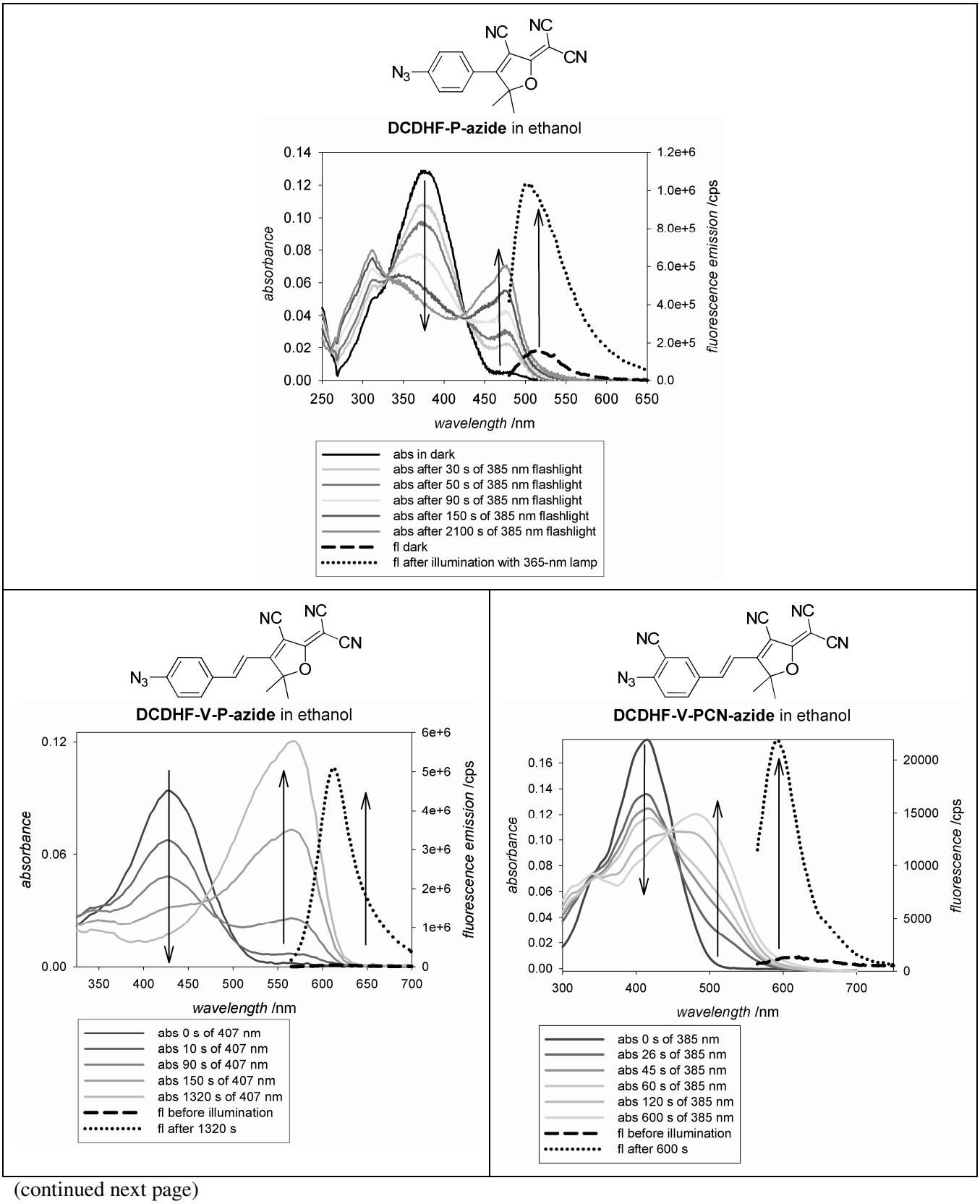


Figure S1. Various products resulting from photochemical conversion of the **DCDHF-V-P-azide** fluorogen. Compounds **1**–**3** have been identified in reference S1; compound **4** is hypothetical. (Adapted with permission from *J. Am. Chem. Soc.* **2008**, *130*, 9204–9205. Copyright 2008 American Chemical Society.)



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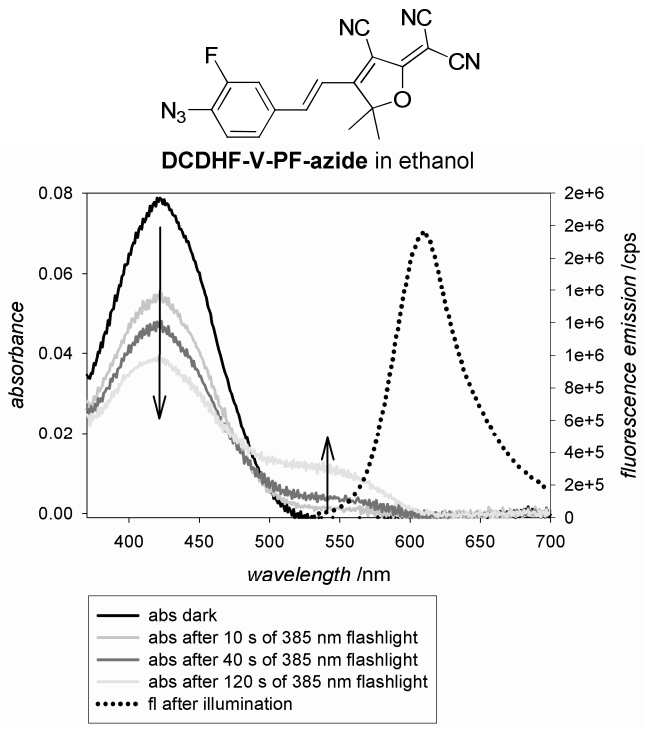
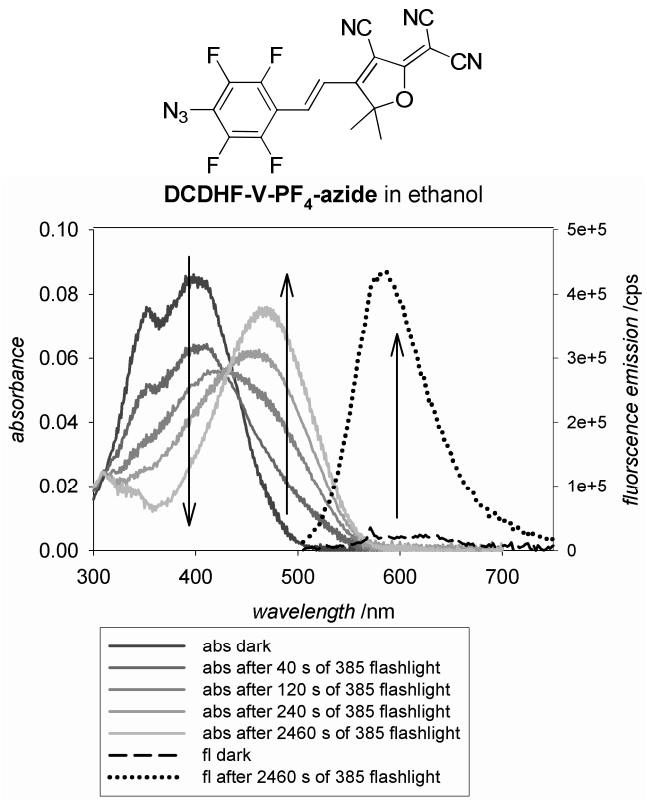
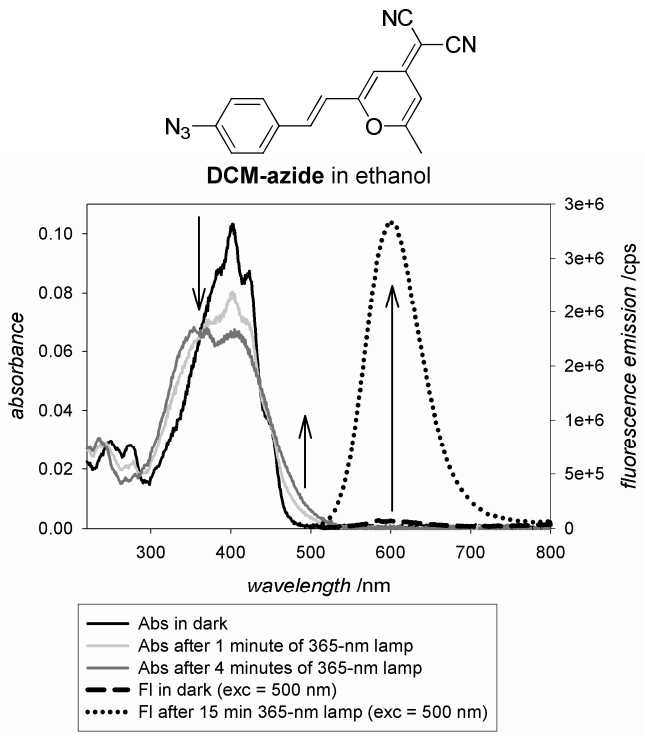
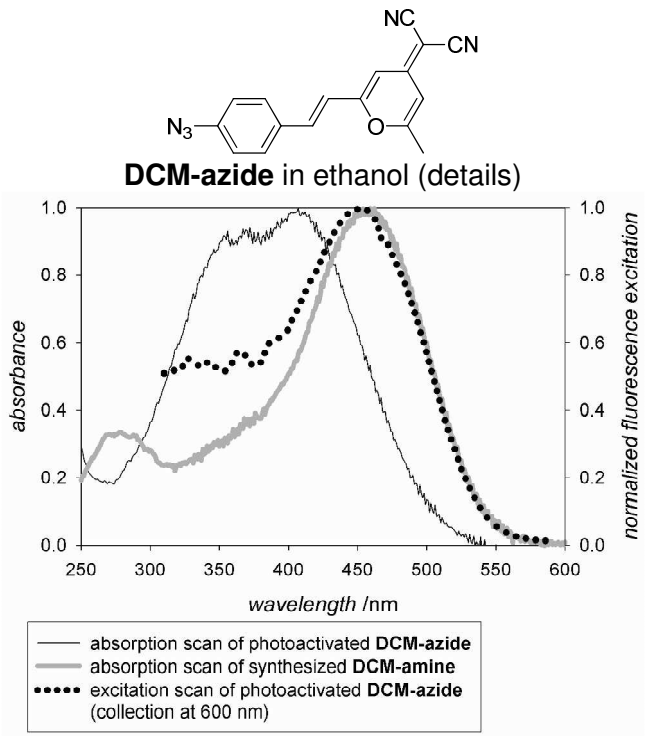






(see next pane for more information)



(Spectra of **DCM-azide** and synthesized DCM-amine corroborate that the fluorescing species in the photoreaction mixture is the **DCM-azide**.)

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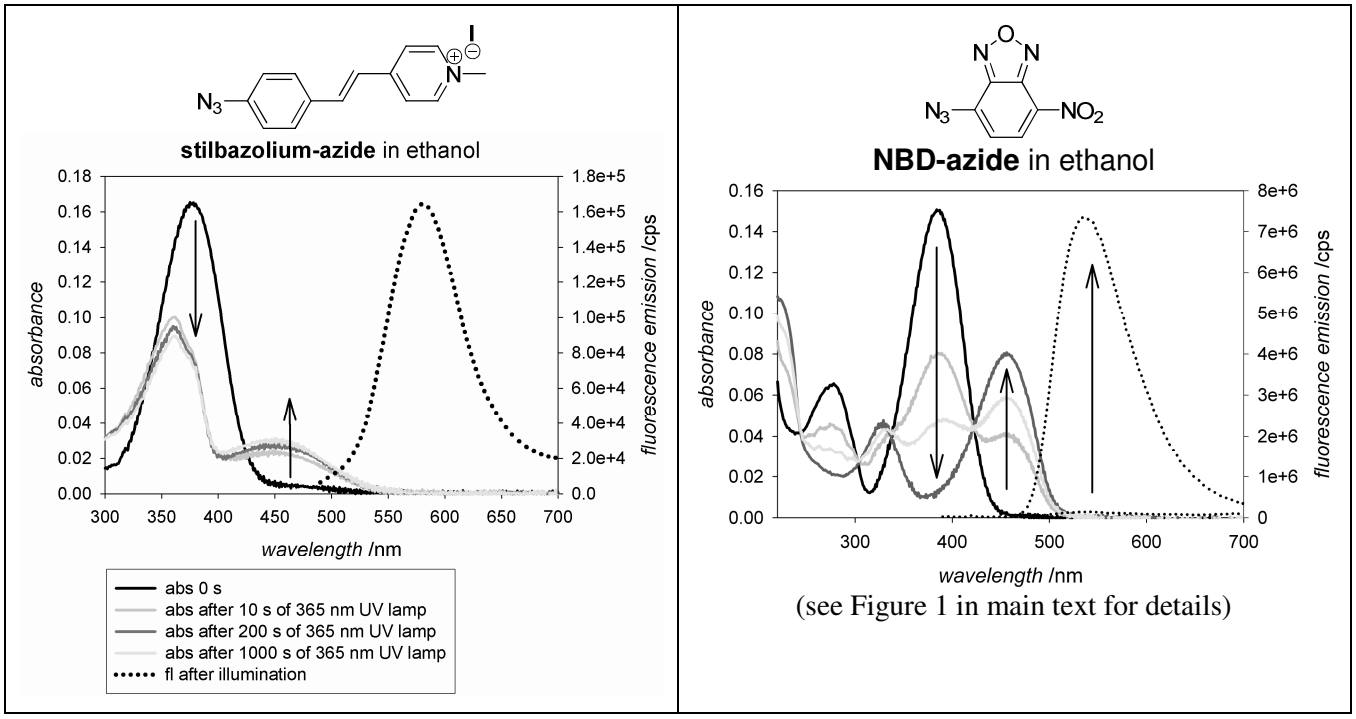

(see Figure 1 in main text for details)

Figure S2. Spectra of azido push–pull fluorogens, their photoreaction mixtures and fluorescence spectra of the azido fluorogens and amino photoproducts. Refer to Table 1 in main text for details. (The spectra for **DCDHF-V-PF$_4$-azide** were previously reported in the supporting information of reference S2.)

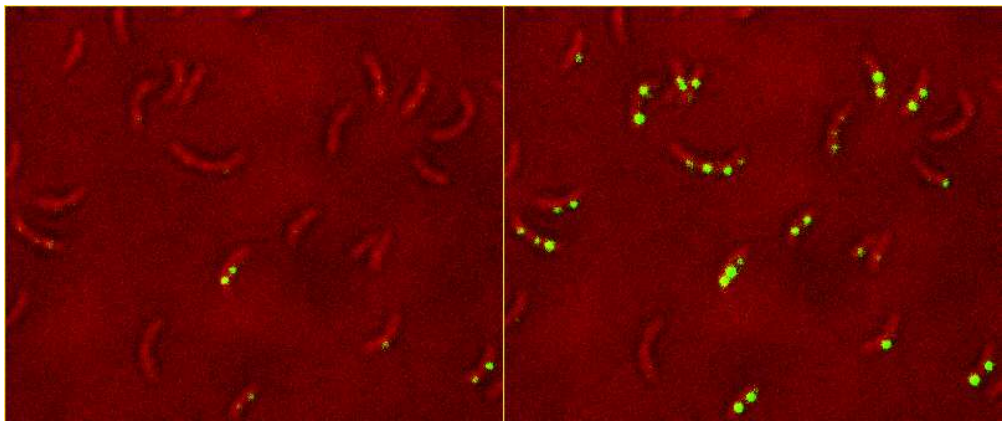

Figure S3. Imaging and photoactivation in living *Caulobacter crescentus* cells. Cells were incubated with **DCDHF-V-P-azide**, photoactivated using 407 nm and imaged at 594 nm. The left frame is immediately before photoactivation (showing some preactivation fluorescence); the right frame is immediately after. The figure is false color: red is the white-light transmission image; green is the superimposed fluorescence images. The green dots are fluorophores collected in ill-defined regions in cells and are likely aggregated, because the probe was not targeted to a specific location or biomolecule in the cells. Each frame is approximately 15×15 μm.

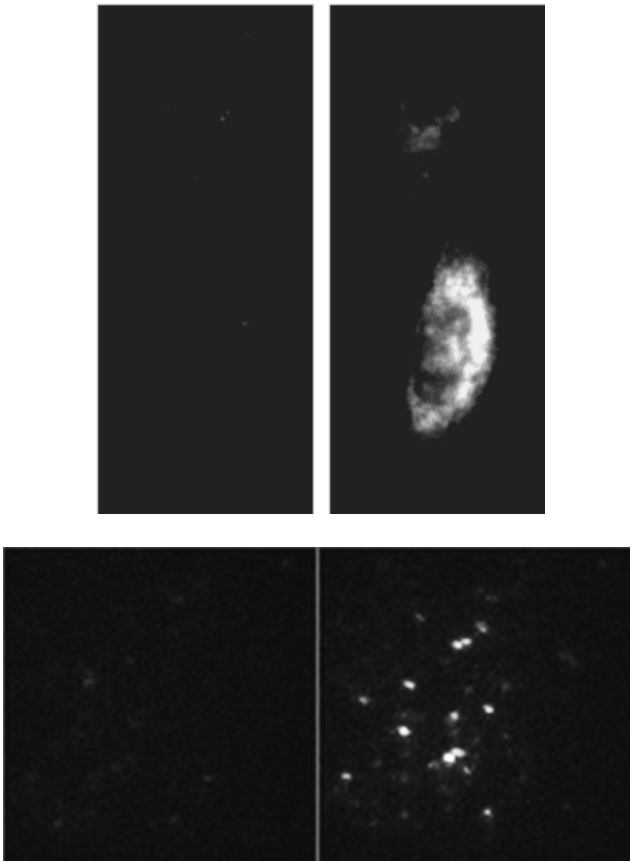

Figure S4. Raw fluorescence images of CHO cells and single-molecule activation images in Figure 2 of main text.

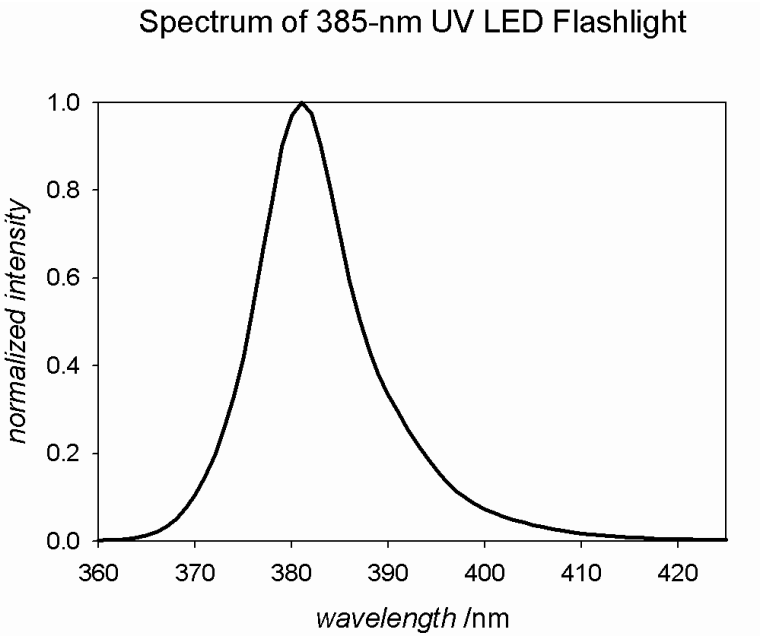

Figure S5. Spectrum of 385-nm UV LED flashlight used for photoactivating some of the fluorophores in Table 1.

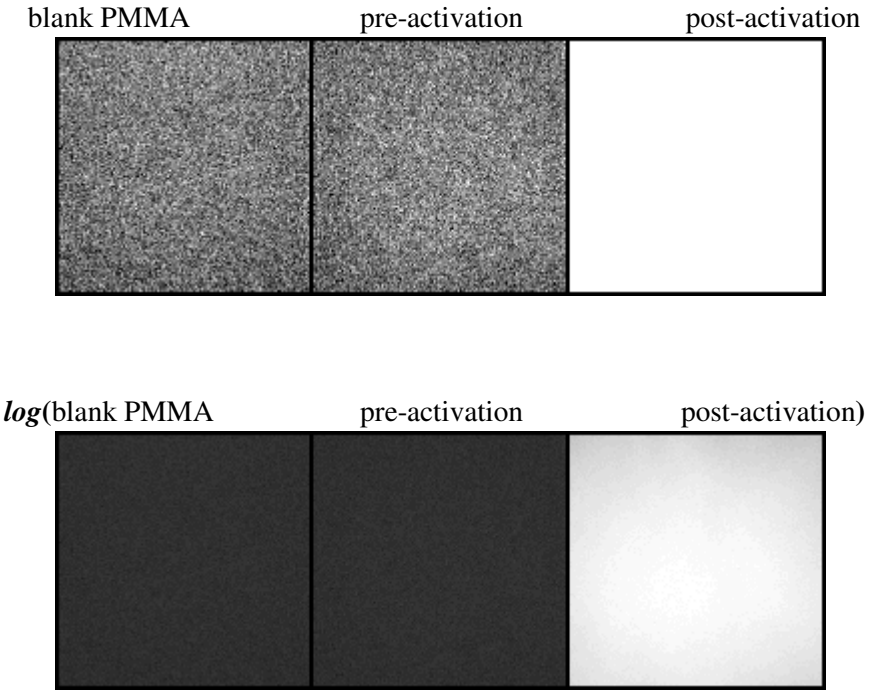

Figure S6. Images of an undoped thick PMMA film and an identical film doped with **DCDHF-V-P-azide** before and after photoactivation. The top row shows the three images with the same contrast level, and it is easy to compare the intensity levels of the dark fluorogen and the blank PMMA film. However, the post-activation fluorescence intensity is saturated on this scale. To better compare the intensities before and after photoactivation, the bottom row is the logarithmic intensity (also with the three images were set to the same contrast level). Even on the logarithmic intensity scale, the dark fluorogen and blank are just barely visible before the intensity of the activated frame saturates. The samples were imaged using 594-nm (~100 W/cm^2), and activated using a 385-nm (~1 mW/cm^2) flashlight for about an hour. Illumination of the blank PMMA film with the UV flashlight did not change the background emission signal when imaged at 594 nm. The average background signal is 106.98 ± 0.23 counts per pixel; the average signal before activation is 107.91 ± 0.09; and the average signal in first frames after photoactivation 392.31 ± 12 counts/10 ms. This is just one of multiple experiments performed to measure the turn-on ratio.

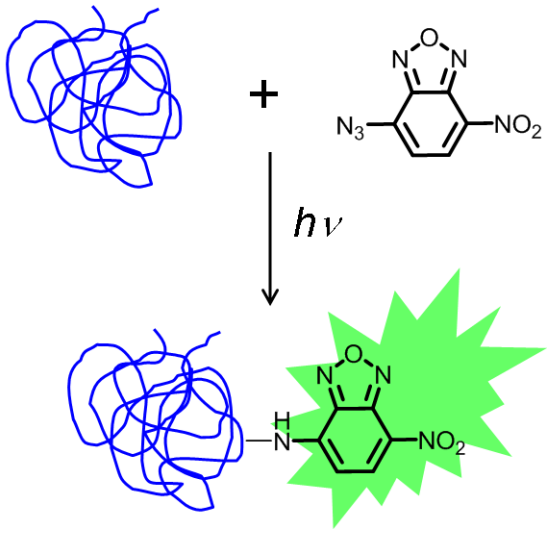

Figure S7. A schematic of fluorogenic photoaffinity labeling (PAL) of proteins. The nitrene intermediate resulting from the photoconversion of an azide to an amine is reactive enough to insert into bonds of nearby biomolecules. The reaction simultaneously turns-on fluorescence and covalently links the probe to the biomolecule.

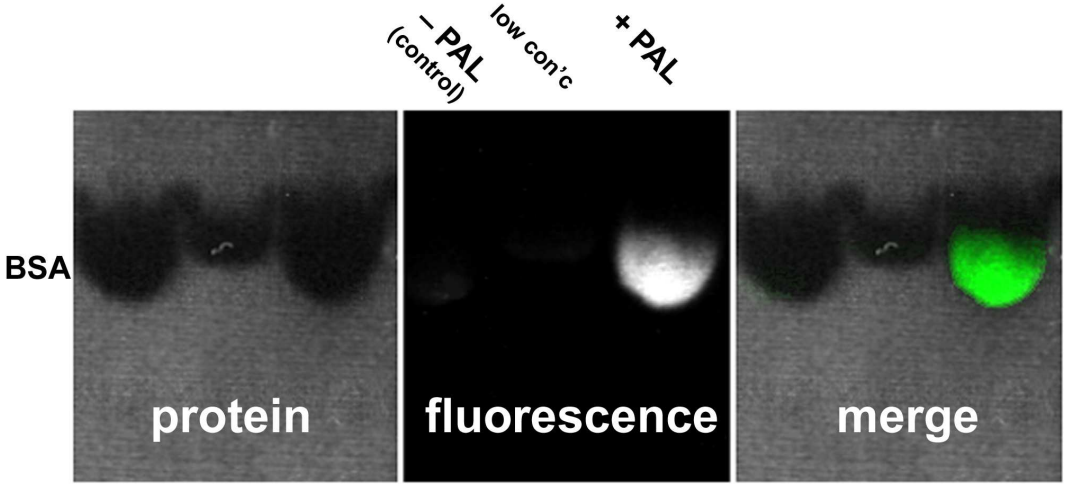

Figure S8. Gel electrophoresis demonstrating fluorogenic photoaffinity labeling (PAL) of BSA protein using **NBD-azide**. The left panel shows the stained protein; the middle panel is the fluorescence from photoactivated NBD (excited at 365 nm); the right panel is a grey-green (false-color) merge of the two left panels. The right column in each panel (+PAL) is the protein covalently labeled with NBD. The left column in each panel (–PAL) corresponds to a control obtained by mixing into the BSA solution preactivated NBD, which cannot participate in the covalent PAL bioconjugating photoreaction. The central column in each panel is a lower-concentration PAL sample, and is not as fluorescent; high protein concentration was required to image the fluorescence, because the labeling ratio was low. The labeling ratio could be increased by a higher concentration of azido fluorogen (the dye:BSA molar ratio in the labeling solution was only 1:25) or by using a biomolecule with higher binding affinity for the fluorogen.

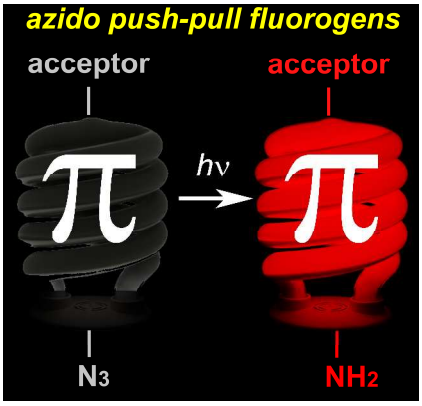

Figure S9. Table of contents artwork.

Supplemental Methods

For Figure S3, *Caulobacter crescentus* cells were grown in 5 mL of M2G buffer. Approximately 50 μL fluorophore stock solution in ethanol was added to 5 mL cells in buffer, then incubated for 30 minutes in the dark. Cells were washed by centrifuging at 10,000 RPM for 90 s, the supernatant exchanged with clean M2G, and repeated. Agarose pads were prepared by heating 2% (by mass) agarose in buffer, pipeting 700 μL of agarose solution onto a clean 35×55-mm coverslip, then sandwiching with another coverslip. After cooling, one coverslip was slid off the agarose pad. A 2-μL sample of cells was placed on the agarose pad and a clean small coverslip was placed over the cells. Excess agarose was cut away and wax was used to seal the two coverslips.

For Figure S8, PAL on pure protein was performed under red lights by mixing 20 μL of ~100-μM NBD-azide stock solution in ethanol (kept in the dark to prevent preactivation) with 50 μL of 1-mM bovine serum albumin (BSA) in 18.2-MΩ water. The mixed solution was photoactivated using a 365-nm handheld Hg UV lamp (0.4 mW cm^{-2}) for several minutes. The nitrene intermediate inserted into bonds of the protein and was covalently linked to the protein. For a control, an aliquot of the NBD-azide stock solution was preactivated before mixing 20 μL into 50 μL of BSA solution; the preactivated dye is unable to participate in the covalent PAL bioconjugating photoreaction. To separate the unbound fluorophores from BSA, denaturing polyacrylamide gel electrophoresis (PAGE) was performed. To 15 μL of 100-μM sample and control protein solutions, 4 μL of LDS denaturing sample buffer (Invitrogen, catalog # NP0007) was added. Samples and controls were heated for 10 min at 65 ºC, then loaded into the gel (Invitrogen NuPAGE, 4-12% Bis-Tris, 1.0 mm). The gels were run at 130 V for 45 min, with a MES SDS running buffer (Invitrogen, catalog # NP0002). The completed gels were imaged under UV light to detect fluorescence from bound fluorophores. To confirm that the fluorescent bands contained protein, the gels were stained for 30 min using Coomassie Brilliant Blue (BioRad R-250 stain) and washed overnight (BioRad R-250 destain).

REFERENCES

(S1) Lord, S. J.; Conley, N. R.; Lee, H. D.; Samuel, R.; Liu, N.; Twieg, R. J.; Moerner, W. E. A Photoactivatable Push–Pull Fluorophore for Single-Molecule Imaging in Live Cells. *J. Am. Chem. Soc.* **2008**, *130*, 9204–9205.

(S2) Pavani, S. R. P.; Thompson, M. A.; Biteen, J. S.; Lord, S. J.; Liu, N.; Twieg, R. J.; Piestun, R.; Moerner, W. E. Three-Dimensional, Single-Molecule Fluorescence Imaging Beyond the Diffraction Limit by using a Double-Helix Point Spread Function. *Proc. Nat. Acad. Sci. U. S. A.* **2009**, *106*, 2995–2999.