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Light-Mediated Spatial Control via Photolabile Fluorescently Quenched Peptide Cassettes

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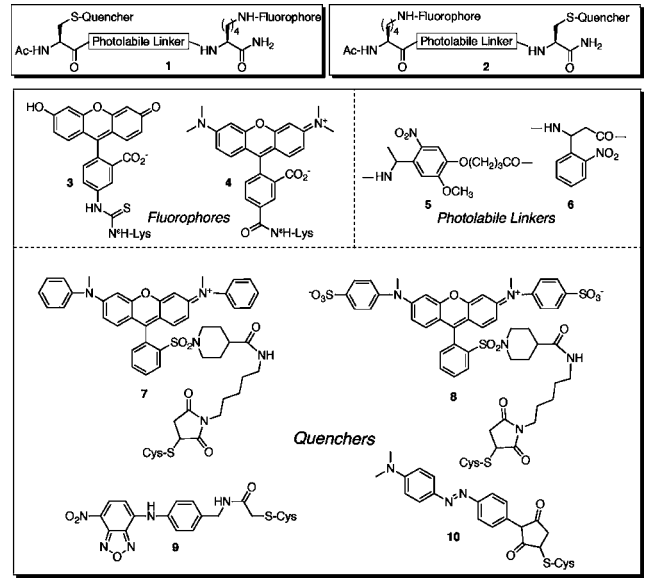
Light-regulatable compounds are finding increasing utility as spatial and temporal probes of biological behavior.[1] Although these compounds are presumed to undergo photolysis (or photoisomerization) in response to illumination, the observed biological response is typically taken as the only evidence that a photoinitiated event has transpired. An independent measure of successful light-induced structural change is possible when alteration (e.g., activation, deactivation, etc.) of the bioprobe can be directly linked to a fluorescent readout. Furthermore, such a design element not only would allow one to quantify the amount of photoaltered bioprobe that had been generated but also could be used to visualize the intracellular localization of the bioprobe after illumination. To the best of our knowledge, Muir and co-workers[2] were the first to put this concept into practice. These investigators prepared a protein construct (Smad2) via expressed protein ligation that generates a 26-fold fluorescence enhancement upon photolysis. Subsequently, Kutateladze and co-workers[3] described a thioxanthone-based system that furnishes an up to 17-fold enhancement in response to photocleavage.

Unlike enzyme-catalyzed reactions, in which readouts (e.g., fluorescence) are continuously amplified as a function of time, photolysis produces a fixed amount of product. A large fluorescence change in response to illumination reduces the amount of bioprobe required for visualization, which in turn reduces the likelihood of undesired "observer effect"-induced alterations in cellular biochemistry.[4] A case in point is the mitochondrion, the so-called energy factory of the cell, which contains several suborganelle compartments that can be targeted using specific amino acid sequences. However, because of the small size of these organelles, targeting-sequence oversaturation of these compartments is possible if large quantities are required for visualization. With this concern in mind, we initiated a program to evaluate an array of structural motifs in order to identify quenched fluorescent cassettes that furnish a large fluorescent response upon photolysis.

A library of 32 modularly designed tripeptides having the general structures **1** and **2** was prepared (Chart 1). Fluorescein (**3**) and tetramethylrhodamine (**4**; TAMRA) analogues were evaluated as the fluorophore component since they are commonly employed in cell-based studies. The other variables constituting the library included two photolinkers (**5** and **6**), four different quenchers (**7**−**9**), and two sequences [with the fluorophore at the C-terminus (**1**) or the N-terminus (**2**)]. The library was prepared via solid-phase synthesis followed by stepwise modification of the Lys and Cys side chains with the fluorophore and quencher, respectively. All of the library members were HPLC-purified (Figure S-1 in the Supporting Information) and subsequently characterized. Fluorescence readings were obtained prior to and following photolysis (Figures S-2−S-6). The fluorescein- and TAMRA-derivatized library members are listed in Table 1. Our leads [Ac−Lys(Fl)−photolinker−Cys(Q)−amide, where Lys(Fl) = **4**, photolinker = **5**, Cys(Q) = **7** (**4**−**5**−**7**), **8** (**4**−**5**−**8**)] display a greater than 300-fold fluorescent enhancement upon photolysis.

Chart 1. Quenched Fluorescent Cassette Library (**1** and **2**) Derived from Fluorophores **3** and **4**, Photolabile Linkers **5** and **6**, and Quenchers **7**−**10**



The most impressive light-induced fluorescence enhancements appear to be a consequence of two structural features: First, **7** and **8** quench the fluorescence of fluorescein and TAMRA more deeply than do **9** or **10** [which may be a consequence of efficient

Table 1. Light-Induced Fluorescence Changes (in *italics*) of the Quenched Fluorescent Cassette Library (see Chart 1 for Structures); The Fluorescein (Upper Half of Table) and TAMRA (Lower Half of Table) Cassettes Are Segregated (See Figures S-4 and S-5)

7−**5**−**3**	**8**−**5**−**3**	**9**−**5**−**3**	**10**−**5**−**3**
25 ± 1	*6.5 ± 1*	*3.0 ± 0.2*	*4.0 ± 0.3*
7−**6**−**3**	**8**−**6**−**3**	**9**−**6**−**3**	**10**−**6**−**3**
11 ± 1	*22 ± 1*	*2.8 ± 0.1*	*14 ± 2*
3−**5**−**7**	**3**−**5**−**8**	**3**−**5**−**9**	**3**−**5**−**10**
75 ± 1	*48 ± 15*	*17 ± 3*	*35 ± 1*
3−**6**−**7**	**3**−**6**−**8**	**3**−**6**−**9**	**3**−**6**−**10**
75 ± 7	*57 ± 13*	*6.0 ± 0.1*	*12 ± 1*
7−**5**−**4**	**8**−**5**−**4**	**9**−**5**−**4**	**10**−**5**−**4**
160 ± 4	*290 ± 20*	*32 ± 2*	*8.0 ± 0.1*
7−**6**−**4**	**8**−**6**−**4**	**9**−**6**−**4**	**10**−**6**−**4**
55 ± 10	*56 ± 2*	*31 ± 1*	*28 ± 1*
4−**5**−**7**	**4**−**5**−**8**	**4**−**5**−**9**	**4**−**5**−**10**
340 ± 10	*360 ± 10*	*71 ± 7*	*86 ± 2*
4−**6**−**7**	**4**−**6**−**8**	**4**−**6**−**9**	**4**−**6**−**10**
100 ± 20	*120 ± 10*	*63 ± 2*	*30 ± 3*

10.1021/ja907427p

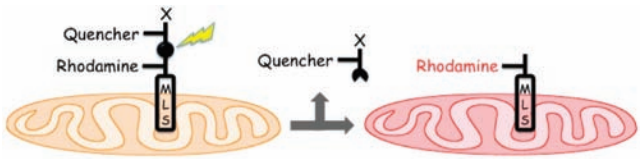
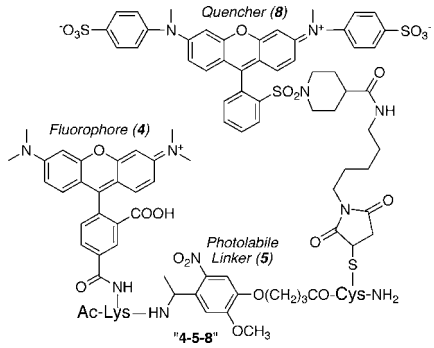


Figure 1. Mitochondrial-targeted light-mediated release strategy. Photolysis releases the quencher (and any associated biochemically active agent "X") from the mitochondrial surface into the cytoplasm.

fluorescence resonance energy transfer (FRET) and collisional quenching] and thus deliver a larger fluorescent response. Second, nitrobenzyl derivatives are modest quenchers of fluorescence as well.[5] Cassettes in which photolysis detaches the nitrobenzyl-based photolinkers from the fluorophore-appended segment (e.g., **4**−**5**−**8**) produce larger fluorescence changes than the corresponding cassettes in which the nitrobenzyl linker remains associated with the fluorophore (e.g., **8**−**5**−**4**). These large fluorescence changes can be easily observed using a handheld UV−vis lamp (see the movie in the Supporting Information), which both photolyzes the linker and excites the fluorophore.



The construction of "caged" compounds commonly relies upon transformation of a biologically active species into an inert derivative via covalent modification of an essential functional group with a light-sensitive moiety.[1] However, direct modification of a single key site for complete biological caging purposes is not always feasible. It occurred to us that an alternative approach for manipulating activity would be light-driven spatial control of the cellular distribution of the biological entity. For example, mitochondrial localization sequences (MLSs), as well as related species, could be used to deliver activators (or inhibitors) to mitochondria, thereby triggering (or inhibiting) an organelle-specific biochemical cascade.[6] If the bioactive species were linked to the MLS via a photocleavable moiety, then photolysis could potentially be used to switch off mitochondrial-specific activity via release of the active reagent from the MLS, leading to its subsequent dilution in the cytoplasm (Figure 1). We used cassettes from Table 1 to examine this "organelle targeted/light-mediated release strategy".

The peptide *MLALLGWWWFFSRKK*−PEG−**4**−**5**−**8**−amide ("MLS−PEG−**4**−**5**−**8**"), where the italicized component is based on an MLS that targets the mitochondrial outer membrane[7] and PEG = $-NHCH_2(CH_2CH_2O)_3(CH_2)_3NHCO-CH_2OCH_2CO-$, was prepared via solid-phase peptide synthesis. Photolysis of MLS−PEG−**4**−**5**−**8** splits the quencher (**8**) from the TAMRA (**4**)-appended peptide, which furnishes an 11-fold fluorescence enhancement (Figure S-7). The latter is dramatically reduced from the fluorescence yield observed for the **4**−**5**−**8** cassette itself, which we ascribe to the presence of the MLS Trp (a known fluorescence quencher[8]) triplet. The mauve-colored MLS−PEG−**4**−**5**−**8** is rapidly taken up by isolated bovine heart mitochondria (Figures S-12 and S-13) with little remaining in the supernatant, as assessed by visual inspection as well as absorbance and fluorescence spectroscopy. Photolysis produces a sharp increase in supernatant absorbance, consistent with the release of the colored fluorescence quencher **8** from the mitochondria. Mitochondrial fluorescence quantitation was assessed via flow cytometry,[9] which revealed a light-mediated 13.4-fold fluorescence enhancement (Figure S-13).

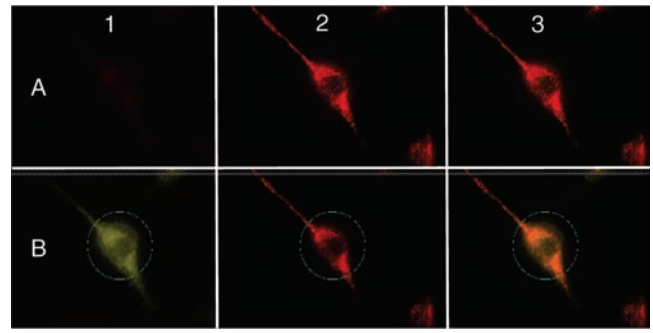


Figure 2. HeLa cells exposed to CPMLS−**4**−**5**−**7** and mitotracker FarRed: row A (unphotolyzed), row B (photolyzed); column 1 (Cy 3 window, green, TAMRA-labeled peptide), column 2 (Cy 5.5 window, red, mitotracker FarRed), column 3 (merged columns 1 and 2, orange signifies regions of overlap, Pearson coefficient 0.88 ± 0.03); green circle (laser focus).

Unfortunately, the Trp-induced dim fluorescence of photolyzed MLS−PEG−**4**−**5**−**8** precluded single-cell imaging experiments. Consequently, we synthesized alternative constructs that utilized the cell-permeable MLS (CPMLS)[10] Ac-Fx-r-Fx-K-Fx-r-Fx-K, where Fx = cyclohexyl-Ala and r = D-Arg); CPMLS−**4**−**5**−**7** furnished the largest light-induced fluorescence change (51-fold; Figure S-8). Exposure of HeLa cells to CPMLS−**4**−**5**−**7** and subsequent photolysis produced an (11.8 ± 0.7)-fold fluorescence increase and subcellular mitochondrial localization as assessed by overlap with mitotracker FarRed (Figure 2). In contrast, photolyzed **4**−**5**−**8** gave a diffuse pattern similar to TAMRA itself (Figure S-16).

We have identified a series of photolabile fluorescently quenched cassettes that display large fluorescence enhancements upon photolysis. Cassette-containing MLS peptides are mitochondrially absorbed and photolyzed in the expected fashion. These reagents are under evaluation as the basis for a new strategy for spatial manipulation of intracellular biochemical activity.

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Supporting Information Available: Details of the quenched fluorophore cassette and MLS peptide syntheses and the photolysis, mitochondrial, and cellular studies; movie (QT) showing fluorescence changes. This material is available free of charge via the Internet at http://pubs.acs.org.

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