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Photochemical control of FlAsH labeling of proteins

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ABSTRACT

Spatiotemporal control of protein fluorescence is a powerful tool in tracking protein movements within cells. Here we report an approach to using genetically encoded photo-caged amino acids to control labeling protein tetracysteine tags with biarsenical fluorescein dyes (FlAsH).

The discovery of green fluorescent protein (GFP) and its relatives has revolutionized cellular biology[1–4] With a wonderful palette of mutant colors, researchers can tag and discriminate different proteins as genetic fusions within the same cell. More recently, this collection of GFPs has been expanded to include those that change optical properties upon irradiation with light resulting in photo-activatable GFPs (PA-GFPs). These modified versions exhibit either a significant increase in intensity or color of fluorescence when stimulated to their active conformation.[5–7] This provides spatiotemporal control over protein fluorescence, and therefore one can activate these fluorescent tags at will. Provided an appropriate microscopy platform, one can track the movements of single protein molecules within a cell at sub-diffraction resolution by photo-activation followed by fluorescence tracking.[8,9] Of course, there are limitations to GFPs including brightness, rate of chromophore formation, and significant size (GFP contains 238 amino acids). There is always the concern that a genetic fusion of a tag this large might alter the function of the protein that is being studied.

As an alternative to fluorescent proteins, Tsien and co-workers introduced the biarsenical pro-fluorescent dyes 4,5-bis(1,3,2-dithiasolan-2-yl)fluorescein and 4,5-bis(1,3,2-dithiarsolan-2-yl)resorufin (FlAsH and ReAsH, respectively). These compounds specifically bind to, and are activated by the small amino acid sequence, CCPGCC.[10,11] Key to this technology is that the nonfluorescent molecules exchange 1,2-ethanedithiol (EDT) for the tetracysteine motif generating a distinctly fluorescent protein-bound complex. This small genetically encoded sequence (TC tag) is orthogonal to cellular proteins and can be labeled extremely selectively and efficiently in vivo. The nominal size (~700 Da) has shown less protein perturbation as compared to the relative size of GFP fusions.[12,13] Whereas these dyes show advantages to GFP fusions, there is no mechanism for the spatiotemporal control of protein labeling, and therefore all proteins within the cell displaying the CCPGCC motif are labeled.

Recently there has been a significant interest in controlling biological phenomena with light.[14,15] Intriguingly, the tetracysteine sequence is critical for the function of these dyes and single amino acid changes cause a major decrease in fluorescence due to destabilization of the biarsenical complexes.[10] We chose to take advantage of this requirement and extend a level of control to the labeling and fluorescence events by temporally masking these cysteine residues. The genetically encoded unnatural amino acid *p*-nitrobenzylcysteine can be site-specifically introduced into proteins in yeast cells in response to amber stop codons.[16] This 'caged' unnatural amino acid becomes cysteine upon irradiation with UV light (365 nm) and therefore tag labeling and activation of fluorescence should only occur after irradiation (Fig. 1). Due to the fast

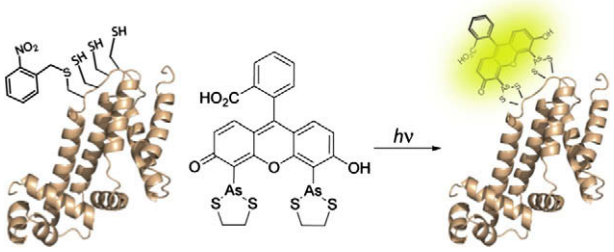

Figure 1. Process of UV-induced activation of labeling.

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association rate for these complexes (~310,000 M^{-1} s^{-1}, fluorescence observed in seconds)[10] photo-activation of fluorescence is rapid and therefore might serve as a supplement to PA-GFP. In the case of precise light delivery, this might allow individual protein molecules to be selectively fluorescently labeled.

To test this hypothesis we PCR amplified the *Schistosoma japonicum* gene encoding glutathione-*S*-transferase (GST) such that it contains an N-terminal TC-tag and a C-terminal 6X-histidine tag for purification purposes. In addition, we created synthetic genes that contain amber stop codons (TAG) in place of either Cys 1, 2, 5, or 6 of the TC-tag (using amino acid numbering in the actual tag). These five genes were then expressed from the yeast expression vector pYES2 in yeast carrying an aminoacyl-tRNA synthetase/tRNA pair specific for *p*-nitrobenzylcysteine.[16] Expressions of the mutants were carried out in the presence of 2 mM *p*-nitrobenzylcysteine and galactose for induction, and the protein isolated by nickel affinity chromatography. Because FlAsH-protein complexes are stable under denaturing gel electrophoresis conditions, we first confirmed fluorescent binding events via SDS–PAGE and subsequent fluorescence imaging. Any FlAsH-protein binding, and thus fluorescence, should be visible at the correct molecular mass of the protein (~28 kDa).

As can be seen in Figure 2, fluorescence is observed for GST-TC carrying the 'normal' tag with four cysteines. No labeling is seen in protein bearing tags that are masked with *p*-nitrobenzylcysteine indicating that these mutants are not effectively labeled with FlAsH, despite possessing three of the four cysteine residues. When these same samples are irradiated with 365 nm light for 15 min however, clear labeling of the proteins can be seen. The differences of intensity seen among the mutants are negligible, indicating that the activation ratio of the four mutants is essentially the same. If any difference can be identified, it is that the protein containing the mutation in place of Cys2 which expresses at a higher level than the others, potentially due to codon context effects.

In order to quantify this activation of protein labeling, we used purified protein to measure the fluorescence intensity derived from the TC-biarsenical complexes following the thiol exchange reaction. Protein (0.5 μM total concentration) that had or had not been irradiated for 15 min was added to an MOPS buffer solution (100 mM, pH 7.2) containing 10 μM EDT and 1 μM FlAsH compound. The specificity of FlAsH binding is improved by the addition of EDT to the solution.[11] The labeling was allowed to incubate at room temperature for one hour as fluorescence increased rapidly but required this to reach maximum levels. Concurrent with the data observed for the SDS–PAGE, there was a significant increase in fluorescence upon decaging (~1000-fold at these concentrations, Fig. 3), for those proteins containing the unnatural amino acid. Again we see no apparent difference in the activation ratio between the four positional mutants, indicating that the presence of four thiols is an absolute requirement and that each of the four mutants decages equally well. Moreover, the activation of the caged tags under these conditions is nearly quantitative when compared to the wild-type tags.

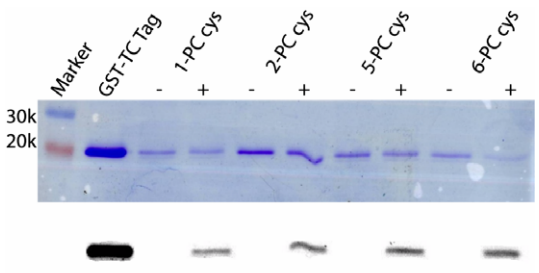

Figure 2. SDS–PAGE analysis of FlAsH labeling. Top panel shows a Coomasie stained gel. Bottom panel shows the same gel imaged for fluorescence (excitation 450 nm). Protein samples contain a TC tag or a caged tag with the location of the unnatural amino acid mutation shown. −/+ indicates the absence/presence of irradiation at 365 nm for 15 min.

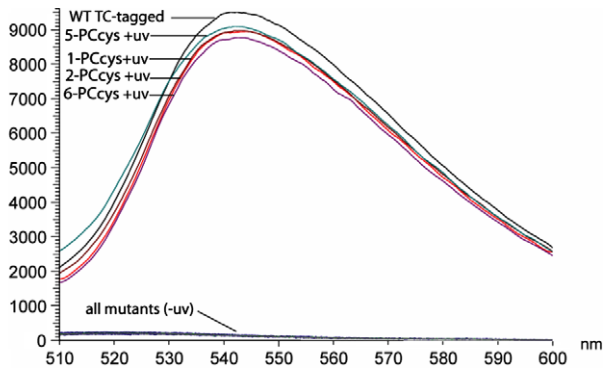

Figure 3. Emission scan of TC-tagged proteins (excitation 508 nm). The baseline traces are those of no protein (dye only), and all four mutants (with dye) in the absence of UV irradiation.

In summary, we have shown that the ubiquitous FlAsH labeling event can be controlled with photo-caged unnatural amino acids. This represents an attractive alternative to PA-GFP tagging that is smaller, and less likely to disrupt protein function. In addition to FlAsH, several other derivatives of biarsenical dyes have been reported including the red dye ReAsH[10], opening the possibility for multicolor imaging. Indeed this approach might facilitate sequential labeling of multiple TC tags on the same protein or within a cell. Furthermore, biarsenical chemistry has recently been used for purposes outside of simple fluorescence labeling such as site-specific bioconjugations and even orthogonal small-molecule control of enzyme function.[17] The use of photo-caged TC-tags may now bring an unprecedented level of control to these applications as well.

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Supplementary data

Supplementary data (detailed experimental procedures) associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2009.05.072.

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