[40] Fluorescent Labeling of Recombinant Proteins in Living Cells with FlAsH

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Introduction

Chemical labeling of specific sites in proteins is usually achieved by reaction of single cysteine residues (either native or introduced by site-targeted mutagenesis) with appropriate thiol-reactive derivatives.[1] Other reactive amino acids such as lysines or glutamates are generally too abundant in proteins to allow specific reaction with appropriately reactive probe. This general approach has, however, been limited to *in vitro* modification of purified proteins. Biological studies of such labeled proteins in cells require their reintroduction by disruptive techniques such as microinjection or electroporation, which often greatly limits the scope of such experiments. Labeling of single cysteine residues in specific proteins in living cells is precluded by the millimolar concentrations of competing thiol from glutathione and other proteins.

Alternative approaches generally require fusion of the desired protein (at the DNA level, followed by transfection of cells) with intrinsically fluorescent proteins such as green fluorescent protein (GFP) or proteins to which specific small molecules can be targeted (reviewed in Tsien and Miyawaki[2]). However, such proteins are large (e.g., 30 kDa for GFP), often larger than the target protein, and are restricted in their fusion sites and in the functionalities that can be targeted (e.g., GFP is limited to fluorescence). Incorporation of unnatural amino acids has been achieved by *in vitro* translation methods[3] or in ion channels expressed in oocytes[4] but cannot yet be applied more generally. *In vitro* protein ligation[5] of fluorescently labeled peptides or polypeptides to construct full-length proteins is a promising new approach that may be applicable to living cells.

[1] G. T. Hermanson, "Bioconjugate Techniques." Academic Press, San Diego, California, 1996.
[2] R. Y. Tsien and A. Miyawaki, *Science* **280,** 1954 (1998).
[3] V. W. Cornish, D. Mendel, and P. G. Schultz, *Angew. Chem. Int. Ed. Eng.* **34,** 621 (1995).
[4] M. W. Nowak, J. P. Gallivan, S. K. Silverman, C. G. Labarca, D. A. Dougherty, and H. A. Lester, *Methods Enzymol.* **293,** 504 (1998).
[5] T. W. Muir, D. Sondhi, and P. A. Cole, *Proc. Natl. Acad. Sci. U.S.A.* **95,** 6705 (1998).
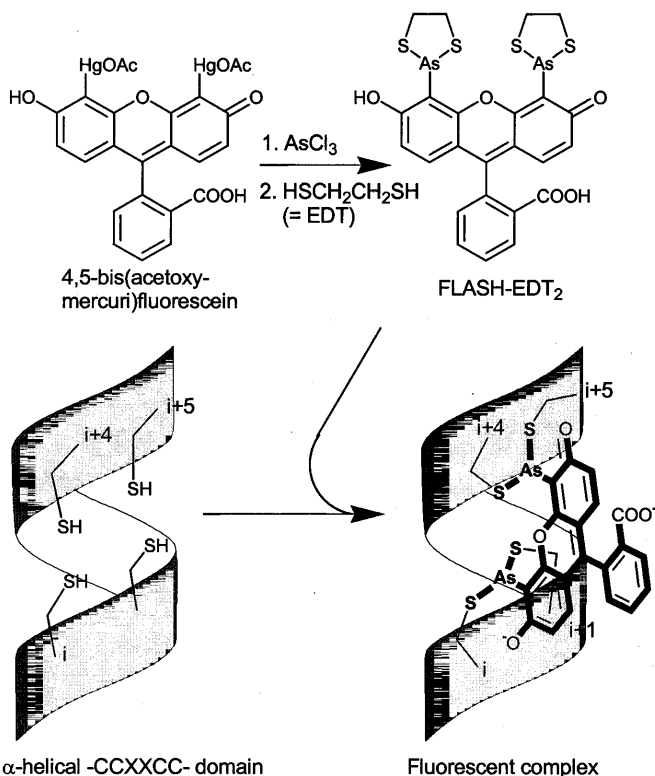
FIG. 1. The synthesis of FlAsH–EDT$_2$ from fluorescein mercuric acetate and arsenic trichloride, followed by reaction with 1,2-ethanedithiol (EDT). The fluorescent complex of FlAsH with an α-helical peptide containing the CCXXCC FlAsH site is shown schematically as the two arsenic atoms bridging the i, $i+1$ and $i+4$, $i+5$ thiols, respectively. An alternative conformation involving i, $i+4$ and $i+1$, $i+5$ binding of the arsenics is also possible.

Our approach[6] to site-specific labeling of proteins in living cells has been to utilize the well-known affinity of arsenoxides (R—As=O) for a pair of closely spaced cysteines. To prevent labeling of such endogenous cellular sites (and the associated toxicity), a fluorescein containing two arsenoxides (FlAsH) was designed that has a much higher affinity for four appropriately spaced cysteines (CCXXCC, where X is any amino acid other than cysteine) in an α-helical conformation (Fig. 1). Such motifs are sufficiently uncommon in naturally occurring proteins to permit specific modification of the target protein incorporating the introduced FlAsH site

[6] B. A. Griffin, S. R. Adams, and R. Y. Tsien, *Science* **281,** 269 (1998).

in living cells. By labeling in the presence of the arsenoxide antidote 1,2-ethanedithiol (EDT), nonspecific labeling and toxicity can be minimized because EDT forms more stable complexes with arsenic than do pairs of cysteines. Furthermore, FlAsH complexed with two EDT molecules, (FlAsH–EDT$_2$) is membrane permeable and nonfluorescent yet becomes brightly fluorescent on binding the CCXXCC site, thereby decreasing background signal from unbound dye during labeling. The tetracysteine site can be attached as an N- or C-terminal tag or incorporated into a known α-helical structure. Addition of a high concentration (millimolar) of EDT reverses the binding of FlAsH to the tetracysteines, permitting reversible labeling. Chemical modification of the fluorescein moiety allows incorporation of different photochemical properties (e.g., different colors for multicolor analysis or fluorescence resonance energy transfer) or use as a handle to target other small molecules to proteins modified with the FlAsH site.

FlAsH may also be used to label purified proteins *in vitro* as an alternative to fluorescein iodoacetamide or maleimide reagents, with the advantage that the tetracysteine-binding site can be labeled without affecting single cysteines in the molecule. In addition, the restricted rotational mobility and fixed orientation of FlAsH bound to the FlAsH site (L. Gross and S. R. Adams, unpublished results, 1999), compared with conventional fluorescein labeling reagents, may be advantageous for studies of protein mobility.

Synthesis of FlAsH and Its Derivatives

FlAsH–EDT$_2$ can be synthesized by a one-pot two-step synthesis from commercially available fluorescein mercuric acetate (Fig. 1). A similar method has been used to make analogs of FlAsH containing additional functionalities (e.g., –CO$_2$H, –NH$_2$, and –Cl) with similar yields (our unpublished results, 1999).

Synthesis of FlAsH–EDT$_2$

Fluorescein mercuric acetate (85 mg, 0.1 mmol; Aldrich, Milwaukee, WI) was suspended in dry N-methyl pyrrolidinone (NMP; 1.5 ml) under argon. Arsenic trichloride (167 μl, 2 mmol) (*CAUTION:* Highly toxic! Use fumehood!) was added followed by palladium acetate (a few milligrams) and dry N,N-diisopropylethylamine (140 μl, 0.8 mmol). The resulting pale yellow solution was stirred at room temperature for 3 hr. The reaction mixture was poured into 50 ml of a stirred 1:1 (v/v) mixture of acetone and 0.25 M pH 7 phosphate buffer (to give a final pH of about 4–5), and

1,2-ethanedithiol (EDT, 99%; 285 μl, 3.4 mmol; Fluka, Buchs, Switzerland) was added immediately. The mixture rapidly turned cloudy. CHCl$_3$ (25 ml) was added with continual stirring. After 15 min, the mixture was diluted with water (50 ml) and separated, the aqueous layer was further extracted with CHCl$_3$ (two 25-ml volumes), and the combined extracts were dried over anhydrous sodium sulfate and evaporated to near dryness, using a water aspirator only. Any precipitates formed during extraction were ignored and removed during filtration of the drying agent. The oily orange residue was dissolved in toluene (50 ml) and washed with brine (three times, 50 ml each), dried, and evaporated to near dryness. This step removed NMP and could be omitted if care was taken not to overload the column during the subsequent chromatography. Alternatively, residual NMP (and EDT) can be removed from the toluene extract by a final (careful!) evaporation under oil vacuum-pump pressure (<1 mmHg). The product was purified by column chromatography on silica gel 60 (230–400 mesh, 20 g packed in toluene; E. Merck, Darmstadt, Germany), as the first orange band eluted with 1:9 (v/v) ethyl acetate–toluene. Fractions containing FlAsH–EDT$_2$ should not be completely concentrated to dryness, because the solid does not redissolve if left under vacuum for even a short time. (A possible explanation could be that vacuum removal of EDT might leave behind the free arsenoxide; arsenoxides are known to be prone to polymerization when concentrated.) Trituration with 95% ethanol overnight at 4° gave an off-white solid, melting point 155° with decomposition. Yield, 24 mg (36%). Keep at −20° protected from light. Solutions of FlAsH–EDT$_2$ in dimethyl sulfoxide (DMSO) or ethanol have been kept frozen for several months without significant deterioration, although some precipitation can occur with samples containing additional EDT.

^{1}H NMR (200 MHz, CDCl$_3$ with a trace of CD$_3$OD): 2.3 (broad singlet, OH), 3.57 (multiplet, 8 protons, -SCH$_2$CH$_2$S-), 6.60 (doublet, J = 8.8Hz, 2 protons, H-2' and H-7'), 6.69 (d, J = 8.8 Hz, 2 protons, H-1' and H-8'), 7.19 (d, 1 proton, H-7), 7.66 (m, 2 protons, H-5, 6), 8.03 (d, 1 proton, H-4). Electrospray mass spectroscopy in negative ion mode indicated a monoisotopic mass for the −1 ion of 663.0 Da (theoretical 662.85). The extinction coefficient was 4.1 × 10^4 M^{-1} cm^{-1} at the absorbance maximum of 507.5 nm in pH 7 buffer.

In Vitro Peptide and Protein Labeling with FlAsH–EDT$_2$

Labeling of tetracysteine peptides by FlAsH in vitro is easily accomplished and can be conveniently monitored by the concomitant increase in fluorescence. Successful labeling of proteins has been achieved at the N or C termini by addition of the sequence of a model peptide that binds FlAsH

(EAA ARE ACC REC CAR A). (The N-terminal tryptophan included in the original model peptide for convenient quantification is not necessary for FlAsH binding.) To date only a few examples of different proteins have been tried, so a minimal FlAsH site has yet to be defined. An internal FlAsH site in an existing α helix of calmodulin was generated by mutation of four amino acids to cysteine and successfully labeled with FlAsH *in vitro* and in living cells.[6]

For labeling *in vitro* it is important that the cysteines that bind to the probe be completely reduced because FlAsH will not react with disulfides. If reduction of cysteines is required, we usually treat a concentrated stock solution of the peptide (millimolar) with either dithiothreitol (DTT) or triscarboxyethylphosphine (TCEP), using standard methodologies. The reduced solution is then diluted to micromolar concentrations for labeling and study. High concentrations of DTT (tens of millimolar) may compete with the tetracysteines for FlAsH and decrease labeling and so should be avoided in the final labeling solution. The rate of labeling with FlAsH is pH sensitive, as reaction requires the cysteine to be in the thiolate form. Adequate reaction rates occur at pH 7 for labeling at micromolar concentrations but lower pH values may be used with higher concentrations of reactants and longer reaction times.

Efficient labeling generally requires the presence of small monothiols (1 mM) such as mercaptoethanol (2-ME) or 2-mercaptoethanesulfonic acid (MES). Monothiols have a weak affinity (millimolar) for arsenoxides and may aid in shuttling the arsenics into the correct position for good binding. In the absence of a small monothiol the labeling proceeds more slowly. Addition of the nonthiol reductant TCEP does not increase the rate of labeling, indicating that it is not the reductive power of 2-ME that is responsible for the enhanced reactivity. Addition of 2-ME to a FlAsH solution can result in the development of some fluorescence even in the absence of a target peptide and the presence of excess dithiols. The source of this fluorescence has not been determined but may be due to formation of 2-ME adducts with the arsenics, which quench the fluorescein less effectively than EDT. The fluorescence intensity of these adducts is low compared with that produced when FlAsH binds to the target peptide. A moderate fluorescent enhancement on labeling with FlAsH (particularly when using an excess), may be indicative of decomposition or of significant fluorescent impurities left over from synthesis. Incubating the FlAsH–EDT$_2$ with a slight excess of EDT prior to reaction can often decrease such background, suggesting that slow hydrolysis of the EDT group from the FlAsH occurs with time. Excess FlAsH may be removed after completion of labeling by standard techniques such as reversed-phase high-performance liquid chromatography (HPLC) or gel filtration.
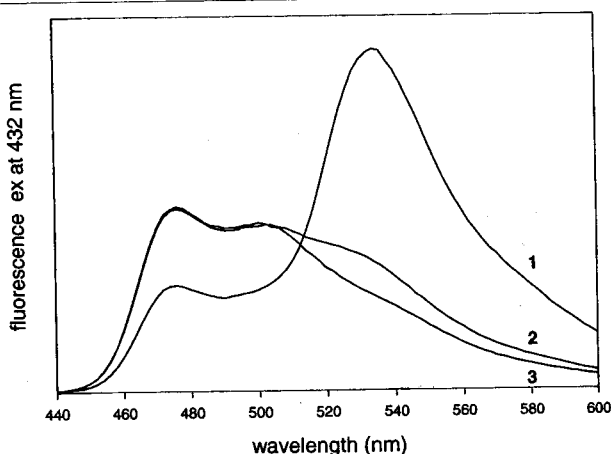
FIG. 2. Fluorescence emission spectra of cyan fluorescent protein (CFP) linked to a FlAsH target sequence by a protease recognition site. (1) Intact fusion protein labeled with FlAsH–EDT$_2$, showing FRET from CFP to FlAsH. (2) FRET is disrupted when fusion protein is cleaved by trypsin. (3) Addition of 5 mM BAL (British Anti-Lewisite, 2,3-dimercaptopropanol) removes FlAsH from the tetracysteine target sequence, making it nonfluorescent. The fusion of CFP (GFP residues 1–227; F64L, S65T, Y66W, N146I, M153T, V163A) to the amino acid sequence RMRPPGPGDEVDGVDEVAKKSKEPGELAEAAAREACCRECCAREAA-AREAAAR was accomplished by standard molecular biology techniques. The recombinant protein was expressed in *E. coli* and purified by means of a polyhistidine tag. It was desalted with a Millipore Ultrafree-4 filter with a 10-kDa cutoff, and then resuspended in 100 mM HEPES. The fusion protein at a concentration of 100 μM (determined with a Bio-Rad DC protein assay kit) was reduced overnight in PBS, pH 7.3, containing 1 mM DTT. Labeling was accomplished by adding 1 μM FlAsH–EDT$_2$ and 10 μM EDT to 1 μM reduced protein in 2 ml of PBS containing 1 mM 2-ME. The labeling took about 2 hr for completion. Trypsin (GIBCO-BRL; final concentration, 0.4 mg/ml) quickly cleaved the FlAsH peptide adduct from CFP. Addition of 1 μl of BAL dissociated the FlAsH probe from its target sequence.

Cyan Fluorescent Protein–FlAsH Construct

FlAsH can be used to label recombinant proteins containing the FlAsH target sequence *in vitro*. Again it is necessary that the four cysteines in the FlAsH target sequence be in the reduced state so that they are available to bind the probe. Purification of recombinant proteins containing reduced tetracysteine-binding sites can be facilitated by including reducing agents (such as 2-ME, DTT, and TCEP) at all stages in the work-up. Figure 2 shows the spectra of a recombinant protein in which a FlAsH target sequence is linked via a peptidase cleavage site to CFP.[7] Cyan fluorescent protein (CFP) is a mutant GFP with blue-shifted excitation and emission spectra that

[7] R. Y. Tsien, *Annu. Rev. Biochem.* **67,** 509 (1998).

undergoes fluorescence resonance energy transfer (FRET) to FlAsH if sufficiently close in space (<5 nm) and orientated appropriately. After labeling with FlAsH but before the addition of trypsin, there is efficient energy transfer between CFP and the FlAsH fluorophore as evidenced by reduced fluorescence at 475 nm compared with the fluorescence at 528 nm (spectrum 1, Fig. 2). On cleavage with trypsin, the 528-nm signal is greatly reduced while that at 475 nm is increased, indicating direct emission from CFP and lack of FRET (spectrum 2, Fig. 2). After cleavage was complete, a 5 mM concentration of the dithiol (British Anti-Lewisite BAL, or 2,3-dimercaptopropanol) was added to dissociate the FlAsH fluorophore from the target sequence. Spectrum 3 in Fig. 2 shows that this reagent reduced the fluorescent signal of the FlAsH–peptide conjugate but had no effect on the CFP emission at 475 nm. This demonstrates that the peptide cleavage was indeed complete and that the residual fluorescence of the FlAsH–peptide conjugate was due to direct excitation of the fluorophore and not to FRET caused by incomplete cleavage by the peptidase. This is a control that cannot be easily performed when using other methods of protein labeling.

FlAsH Labeling in Cells

One of the most useful features of the FlAsH labeling system is the ability of the probe to label recombinant proteins in living cells. The nonfluorescent reagent is applied to the outside of cells, crosses the plasma membrane, finds its target within the cell, binds, and becomes fluorescent. The specificity of FlAsH binding is improved by the addition of EDT to the loading solution. The empirically determined concentration ratio of 10 μM EDT to 1 μM FlAsH–EDT$_2$ decreases staining of endogenous site while still allowing FlAsH to bind to the designed motif.

Typically the loading solution for labeling cells is 1 μM FlAsH–EDT$_2$ and 10 μM EDT in HEPES-buffered saline (HBS; containing either glucose or, for reduction of background fluorescence, 1 mM sodium pyruvate). The empirically found effect of pyruvate may result from a change in the redox state of the cells. The FlAsH–EDT$_2$ and EDT, both in DMSO (1 μl each of stock solutions of 1 mM FlAsH–EDT$_2$ and 10 mM EDT), are mixed first and then diluted with the buffer (1 ml). It is important to use freshly made EDT solutions because oxidation can occur readily. The solution can be incubated at room temperature for 15 min to ensure that any FlAsH–EDT$_2$ that may have become unprotected during storage rebinds EDT before application to cells. The cells to be stained are rinsed with HBS to remove serum proteins that may slow labeling by binding FlAsH–EDT$_2$ (see below). The labeling solution is then added and the cells incubated

for about 1 hr at room temperature. Some labelings may take longer and can be conveniently monitored by fluorescence microscopy. It is useful to label a control of mock-transfected cells to be sure that the fluorescence seen is indeed due to labeling of the desired protein containing the tetracysteine-binding site. Nontransfected cells are not an ideal control, because transfection can introduce artifactual fluorescence staining by increasing cellular debris.

Suppression of Background Staining in Cells

The ratio of reagents outlined above may not sufficiently lower background staining to allow straightforward use of the FlAsH labeling technique. This is especially evident when cells other than HeLa are used (e.g., ECV 304, HEK 293, CHO, 3T3, 3T6) or when the protein to be labeled is expressed at low levels. Increasing the ratio of EDT to FlAsH–EDT$_2$ may further reduce the background staining, but at the expense of desired binding. The appropriate ratio may have to be determined empirically for each protein construct and cell type.

The addition of two nonfluorescent compounds, one membrane permeant and one membrane impermeant, to the loading solution greatly reduces the undesired background fluorescence in cells. *In vitro,* low millimolar concentrations of EDT (or the more water-soluble BAL) are sufficient to completely reverse the binding of FlAsH to a tetracysteine model peptide. In cells, however, some FlAsH staining persists even at high concentrations of EDT (up to 30% of the background staining in wild-type HeLa). The idea that an uncharged molecule might help reduce this non-dithiol-responsive background arose from the observation of the fluorescent interaction of FlAsH–EDT$_2$ with bovine serum albumin (BSA) *in vitro*. A solution of FlAsH–EDT$_2$ becomes significantly more fluorescent when BSA is added (with an estimated quantum yield of 0.1 compared with 0.5 for the FlAsH–peptide complex). This fluorescence is not reduced by the addition of millimolar EDT. An extremely high affinity of the FlAsH arsenics for BSA is unlikely because all cysteines in BSA with the exception of one are oxidized to disulfides, and thus BSA presents no consensus site for tight binding of FlAsH. The most likely explanation is that FlAsH–EDT$_2$ remains intact when binding to a hydrophobic pocket on BSA, but becomes more fluorescent because of a change in environment. BSA is well known for binding hydrophobic dyes and boosting their fluorescence. Quenching of fluorescence in free FlAsH–EDT$_2$ is believed to result from deactivation of the excited state by electron transfer from the arsenic atom to the xanthene fluorophore and/or by vibrational rotation of the As–EDT groups. Hydrophobic binding of FlAsH–EDT$_2$ to BSA probably partially

prevents these modes of quenching. Binding is reversible, addition of a tetracysteine-containing peptide to the complex results in formation of peptide–FlAsH complex, although at a slower rate than in the absence of BSA.

Similar hydrophobic sites in cells may bind FlAsH–EDT_2 and give fluorescence that is unresponsive to high concentrations of dithiol. Such unwanted fluorescence might be reduced if molecules could be found that would preferentially occupy such greasy cellular binding sites, either by higher affinity or by mass action, thus displacing FlAsH–EDT_2. We screened 30 FlAsH–EDT_2 analogs and nonfluorescent dyes (including fluorescein derivatives, EDT adducts of phenylarsenoxides, and hydrophobic dyes) for their ability to reduce FlAsH background staining in untransfected HeLa cells. Of the compounds screened, the commercially available dye Disperse Blue 3 (Fig. 3; Aldrich sample further purified by recrystallization from toluene) was the most effective at reducing background fluorescence (Fig. 4). Typically, 20 μM dye added to the loading buffer is more than sufficient to remove virtually all of the background nonresponsive to dithiols. Other mechanisms may also be operating when uncharged compounds reduce background staining by FlAsH–EDT_2. For example, the background reducing agent may bind close to the FlAsH–EDT_2 molecule and thereby reduce fluorescence by quenching rather than by displacement.

Dead or dying cells are brightly stained (even untransfected cells) by the standard FlAsH loading solution (1 μM FlAsH–EDT_2 and 10 μM EDT), probably by exposure of hydrophobic sites that bind the dye. In the study of single cells by fluorescence microscopy, these bright cells can often be ignored. However, when populations of cells are to be studied these bright cells may overwhelm the desired signal.

Part of the fluorescence in the bright, rounded up cells is removed by the addition of an uncharged dye such as Disperse Blue 3. Suppression of the bulk of the remaining background in these dead cells can be accom-
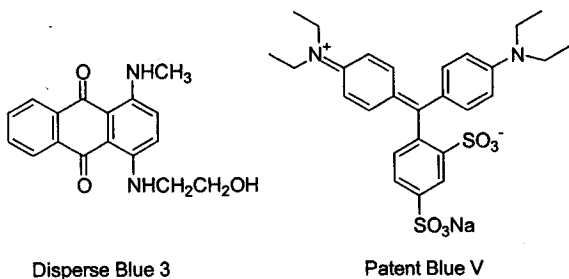

Disperse Blue 3   Patent Blue V

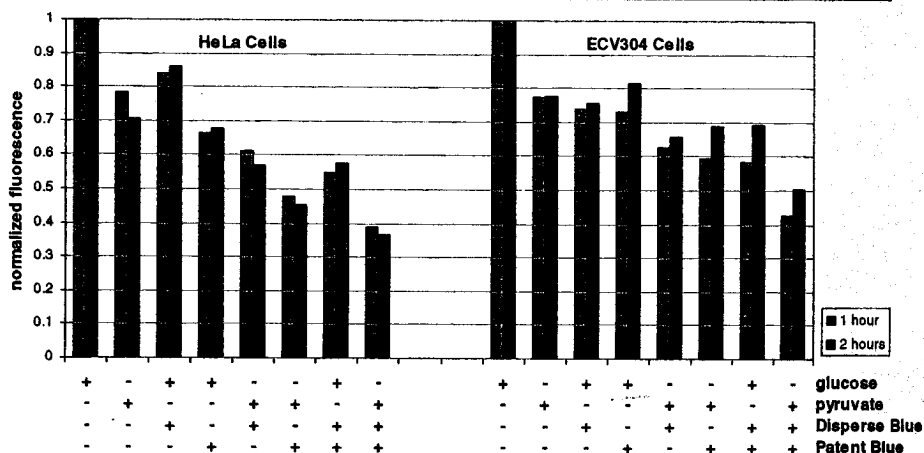FIG. 3. Structures of FlAsH background suppression dyes.

FIG. 4. Demonstration of the additive effect of background suppression techniques. Cells, either HeLa or EVC304, were split into 96-well plates and allowed to grow to confluence overnight in DMEM supplemented with 10% (v/v) FBS. Medium was removed and the cells rinsed once with 200 μl of HBS per well. The indicated components, along with 1 μM FlAsH–EDT$_2$ and 10 μM EDT, in 100 μl of HBS were applied to cells, which were then incubated at room temperature. Fluorescence was measured with a CytoFluor multiwell plate reader 1 and 2 hr after adding the FlAsH solutions. Filters: excitation, 485DF20; emission, 530DF25. Concentrations: glucose, 10 mM (1.8 g/liter); sodium pyruvate, 1 mM; Disperse Blue 3, 20 μM; Patent Blue V, 1 mM.

plished by flooding the cells with quenchers at high concentration. Membrane-impermeant dyes have access to the interior of cells whose cytoplasmic membrane has been compromised, but not to the interior of living cells. We found that the commercially available dye Patent Blue V (Fluka; Fig. 3) is effective, while some dyes such as trypan blue are not. Other charged dyes or combinations may reduce this source of background further.

Demonstration of Background Suppression in Transfected Cells

The additives that were developed with untransfected cells were demonstrated to be effective in transfected cells. Figure 5[6,8,9] (top) illustrates the

[8] M. J. B. van den Hoff, A. F. M. Moorman, and W. H. Lamers, *Nucleic Acids Res.* **20,** 2902 (1992).

[9] G. Zlokarnik, P. A. Negulescu, T. E. Knapp, L. Mere, N. Burres, L. Feng, M. Whitney, K. Roemer, and R. Y. Tsien, *Science* **279,** 84 (1998).

effect in HeLa cells as an example of a cell line that shows low background staining. Figure 5 (bottom) demonstrates the greater effect achieved in 3T6 cells, which have higher background staining. In these experiments the background suppression agents were added with the FlAsH–EDT$_2$ loading solution and remained on the cells for the duration of labeling. The uncharged compound and the membrane-impermeant dyes may be added after FlAsH–EDT$_2$ labeling if it is found desirable to limit the exposure of cells to these agents, although no toxic effects were apparent. After labeling is complete, the FlAsH–EDT$_2$ solution is removed from the cells and replaced with a rinse solution. For best results, the rinse solution should contain Disperse Blue, Patent Blue, and 10 μM EDT to minimize retention of the unbound and less membrane-permeable FlAsH–EDT$_2$ by cellular hydrophobic sites during rinsing.

FlAsH staining of intact bacteria expressing proteins with FlAsH target sites requires higher concentrations of FlAsH–EDT$_2$ (10–20 μM) in the presence of 2-ME (1–5 mM) for several hours. Bacterial lysis (by freeze–thawing) increases the rate of labeling, suggesting decreased permeability of bacteria cell walls to FlAsH compared with mammalian cells (S. R. Adams and A. Miyawaki, unpublished results, 1999). Similarly, preliminary experiments with yeast indicate that FlAsH does not have access to the interior of the cell until yeast spheroblasts are formed by removal of the cell wall (B. A. Griffin and G. Odorizzi, unpublished results, 1999).

Summary and Outlook

FlAsH labeling of recombinant proteins for cellular localization studies can be considered an alternative to the popular method using GFP fusions,[7] with the FlAsH method having some advantages. The size of the fluorescent tag is considerably smaller: Bound FlAsH has a molecular weight of less than 600 and the addition of a FlAsH target site can be as small as four introduced cysteines (with negligible change in molecular weight) with an appended peptide adding less than 2 kDa. This compares with a molecular weight of 30,000 for GFP, which is therefore more likely to perturb the native structure and function of the tagged protein. Both FlAsH and GFP tagging generate a fluorescent protein with similar brightness (the product of the extinction coefficient and fluorescence quantum yield). However, multiple FlAsH sites could be introduced into a protein so that considerably brighter labeling would aid in detecting low-abundance proteins.

Fusions with GFP are generally limited to the C and N termini of proteins, although insertion between some domains may be tolerated (e.g.,

Loading conditions: **1**   **2**   **3**

HeLa with
FLASH site

HeLa mock
transfected

Loading conditions: **1**   **2**   **3**

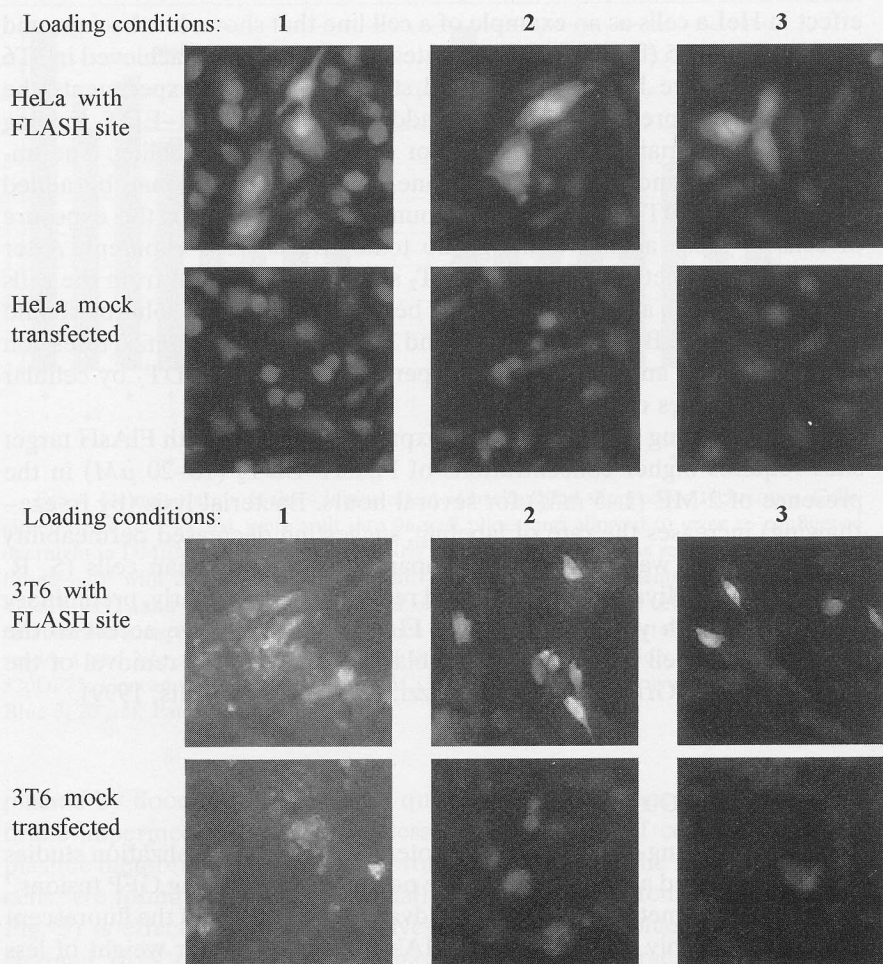3T6 with
FLASH site

3T6 mock
transfected

FIG. 5. Comparison of FlAsH labeling in cells with and without background suppression techniques. A construct encoding the FlAsH target peptide AEAAAREACCRECCARA appended to the C terminus of *Xenopus* calmodulin was inserted into pcDNA3 by standard molecular biology techniques.[6] Cells, either HeLa or 3T6, were dissociated from cell culture dishes, using a low calcium buffer, spun down, and then resuspended in a buffer[8] that resembles intracellular ionic concentrations. Ten micrograms of plasmid was added to 1 ml of the above, containing about 10^7 cells. The mixture was electroporated at 0.324 kV with a Bio-Rad GenePulser II equipped with a Bio-Rad Capacitance Extender Plus. Immediately after electroporation the mixture was diluted into medium [DMEM–10% (v/v) FBS without antibiotics] and split into several culture flasks. A control plasmid encoding β-lactamase[9], which does not contain a FlAsH target sequence, was similarly transfected into cells. The next day the cells were stained in HBS using: conditions 1 (1 μM FlAsH–EDT$_2$, 10 μM EDT and 10 mM glucose), or conditions 2 (1 μM FlAsH–EDT$_2$, 10 μM EDT, 1 mM pyruvate, 20 μM Disperse Blue 3) or conditions 3 (conditions 1 with 1 mM Patent Blue V added). In the HeLa

in the Shaker potassium channel[10]) and insertion of proteins into GFP[11] (at specific tolerant sites) without loss of fluorescence has been achieved. Internal FlAsH sites may be tolerated in numerous surface α-helical regions of a protein, more readily permitting location of the fluorophore at nonperturbing sites.

Color mutants of GFP span the blue to yellow range of the spectrum; FlAsH currently is limited to green emission although a red variant has been developed that is a good FRET acceptor for GFP and YFP (S. R. Adams and J. L. Llopis, unpublished results, 1999). In contrast, FRET between color mutants of GFP has been limited to BFP–GFP and CFP–YFP pairs, necessitating excitation with ultraviolet or violet light, respectively, with its inherent drawbacks of higher autofluorescence and less convenient laser lines. The description of red fluorescent proteins[12] from coral may eventually allow use of the more favorable GFP–RFP or YFP–RFP pairs.

A major advantage of the FlAsH system is the comparative ease of chemical modification of FlAsH. Coupled with the synthetic versatility of organic chemistry, this enables the incorporation of functionalities other than fluorescence into targeted proteins or peptides. Some modifications already investigated include addition of photosensitizing groups, magnetic resonance imaging agents, membrane-impermeant groups, cross-linking groups, fluorescent Ca^{2+} sensors, or reactive groups for immobilization (our unpublished results, 1999).

Disadvantages of FlAsH include the requirement of FlAsH binding for reduced cysteines. Labeling of proteins in oxidizing environments (such as the secretory pathway or extracellular) requires *in situ* reduction prior to labeling (S. R. Adams and Y. Yao, unpublished results, 1999). More importantly, higher background staining is generally seen in the FlAsH labeling technique compared with GFP chimeras. This can limit the usefulness of the technique for protein localization (especially of proteins

[10] M. S. Siegel and E. Y. Isacoff, *Neuron* **19**, 735 (1997).

[11] G. S. Baird, D. A. Zacharias, and R. Y. Tsien, *Proc. Natl. Acad. Sci. U.S.A.* **96**, 11241 (1999).

[12] M. V. Matz, A. F. Fradkov, Y. A. Labas, A. P. Savitsky, A. G. Zaraisky, M. L. Markelov, and S. A. Lukyanov, *Nature Biotechnol* **17**, 969 (1999).

experiment, the staining solution was replaced with solutions containing all components except FlAsH–EDT_2, before images were collected from a cooled CCD with Axon Imaging Workbench software. Filters: excitation, 495DF10, 505 dichroic; emission, 535DF50, In the 3T6 experiment, the staining solution was not removed before images were recorded on a Pixera digital camera. Filters: excitation, 450DF50, 480 dichroic; emission, 485 long pass.

expressed in low abundance) although the suppression techniques described above help decrease this problem significantly. The use of FlAsH as an FRET acceptor from CFP (or the red version from GFP) suffers much less from this problem as only specifically bound FlAsH is excited through FRET. Background FlAsH staining is not significantly excited by the wavelengths used for CFP. Further optimization of the FlAsH target site, perhaps through screening of peptide libraries, should still allow strong binding at concentrations of dithiol that minimize background staining.

[41] Ubiquitin Fusion Technique and Its Descendants

By Alexander Varshavsky

The ubiquitin (Ub) fusion technique was developed in 1985–1986, through experiments in which a segment of DNA encoding the 76-residue Ub was joined, in frame, to DNA encoding *Escherichia coli* β-galactosidase (βgal).[1,2] When the resulting protein fusion was expressed in the yeast *Saccharomyces cerevisiae* and detected by radiolabeling and immunoprecipitation with an anti-βgal antibody, only the moiety of βgal was observed, even if the labeling time was short enough to be comparable to the time (1–2 min) required for translation of the Ub-βgal open reading frame (ORF). It was found that in eukaryotic cells the Ub moiety of the fusion was rapidly cleaved off after the last residue of Ub (Fig. 1).[1] The proteases involved are called deubiquitylating[3] enzymes (DUBs) or Ub-specific processing proteases (UBPs).[4–7] A eukaryotic cell contains more than 10 distinct DUBs, all of which are highly specific for the Ub moiety. The *in vivo*

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[2] A. Varshavsky, *Proc. Natl. Acad. Sci. U.S.A.* **93**, 12142 (1996).

[3] Ubiquitin whose C-terminal (Gly-76) carboxyl group is covalently linked to another compound is called the *ubiquityl* moiety, the derivative terms being *ubiquitylation* and *ubiquitylated*. The term *Ub* refers to both free ubiquitin and the ubiquityl moiety. This nomenclature, which is also recommended by the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology,[19] brings Ub-related terms in line with the standard chemical terminology.

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