Published in final edited form as:

Nat Protoc. 2010 September ; 5(10): 1666–1677. doi:10.1038/nprot.2010.129.

Fluorescent labeling of tetracysteine-tagged proteins in intact cells

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Abstract

In this paper, we provide a general protocol for labeling proteins with the membrane-permeant fluorogenic biarsenical dye fluorescein arsenical hairpin binder–ethanedithiol (FIAsH-EDT₂). Generation of the tetracysteine-tagged protein construct by itself is not described, as this is a protein-specific process. This method allows site-selective labeling of proteins in living cells and has been applied to a wide variety of proteins and biological problems. We provide here a generally applicable labeling procedure and discuss the problems that can occur as well as general considerations that must be taken into account when designing and implementing the procedure. The method can even be applied to proteins with expression below 1 pmol mg⁻¹ of protein, such as G protein–coupled receptors, and it can be used to study the intracellular localization of proteins as well as functional interactions in fluorescence resonance energy transfer experiments. The labeling procedure using FIAsH-EDT₂ as described takes 2–3 h, depending on the number of samples to be processed.

INTRODUCTION

Fluorescent labeling of proteins in intact cells

Studying biomolecules in their native environment in living cells has become a major field of interest in biomedical research. Although lipids or nucleic acids mainly require chemical labeling strategies¹, cellular proteins can be visualized by genetically encoded fluorescent tags or labels. Ideally, these tags should enable labeled proteins to be studied in intact cells and to preserve their biological functions. Over the past few years, the use of genetically encoded fluorescent proteins such as green fluorescent protein (GFP) or its color variants has become the most popular method to label cellular proteins. The cDNA for GFP can be fused to that of another protein, permitting the expression of a fluorescent fusion protein. Numerous variants of GFP and other fluorescent proteins with distinct fluorescent properties have been described; they permit various fluorescence techniques for studying the

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AUTHOR CONTRIBUTIONS C.H., G.G., A.Z., S.R.A. and S.T. conducted experiments; C.H. and G.G. drafted the paper; and M.H.E., R.Y.T. and M.J.L. finalized the paper.

COMPETING FINANCIAL INTERESTS The authors declare competing financial interests: details accompany the full-text HTML version of the paper at <http://www.natureprotocols.com/>.

localization and interaction of proteins in intact cells². However, sometimes problems can result from modifying the target protein with these relatively large (~27 kDa) proteins³. Genetically encoded alternatives to GFP have been developed, such as the O⁶-alkylguanine-DNA-alkyltransferase (AGT, also known as SNAP-Tag). The AGT enzyme can be covalently labeled with O⁶-benzylguanine derivatives that are chemically coupled with various fluorescent compounds; thus, it offers the generation of highly fluorescent labels. Depending on the dyes used and their membrane permeability, this method can be used to label cell surface proteins and intracellular proteins^{4–7}. However, the AGT tags are also of considerable size (~20 kDa). Further fusion protein approaches include dihydrofolate reductase (size ~18 kDa) in combination with fluorescent trimethoprim derivatives⁸; a modified haloalkane dehalogenase (size ~30 kDa) capable of covalently binding synthetic ligands⁹; and the acyl carrier protein (size ~10 kDa), which can be labeled with 4'-phosphopantetheine from coenzyme A¹⁰.

Attempts to reduce the still-considerable size of these labels led to the development of alternatives based on smaller genetically encoded labels involving metal chelation strategies^{11,12}. These methods combine an oligohistidine tag with Ni²⁺ complexed to fluorescent derivatives of nitrilotriacetate or Zn²⁺ complexed to a fluorescein-based chelator HisZiFIT with Zn²⁺. The metal and fluorescent chelator can form a ternary complex with the oligohistidine genetically inserted into the protein of interest. Fluorescence resonance energy transfer (FRET) measurements have been done with purified proteins by combining different fluorescently labeled nitrilotriacetate variants¹³. With sizes of 2–3 kDa, these complexes are much smaller than the GFP variants or the AGT tag, but their low affinity results in a relatively low stability of the complex and, thus, of the labeling¹¹. Furthermore, these fluorophores do not cross the plasma membrane; thus, in intact cells, only labeling of proteins at the extracellular face has been achieved^{12,14}. Another technology uses optimized peptide sequences of triple or quadruple repeats of the sequences KIAALKE and EIAALEK, which form coiled-coil interactions based on negative charges in one coil matching positive charges in the second coil¹⁵. The peptide sequences can be added to a target protein, whereas the second motif is chemically modified with a fluorescent dye. This strategy has been applied to cell surface labeling of target proteins¹⁶, but, because of the high number of charged amino acids, it is also limited to extracellular applications.

Development of the tetracysteine tag technology

The first and currently most successfully applied genetically encoded technology involving a designed peptide/small molecule pair is the tetracysteine tag technology^{17,18}. This method is based on the binding of a small fluorescein derivative, called fluorescein arsenical hairpin binder (FAsH), to a short peptide sequence of the general structure Cys-Cys-Xaa-Xaa-Cys-Cys (CCXXCC, in which X denotes any amino acid). FAsH is used as a nonfluorescent complex with ethanedithiol (EDT), and it becomes fluorescent on binding to this amino acid sequence. The latter property results in relatively low background fluorescence. After its first description¹⁷, other laboratories had difficulties using this method¹⁹. Since then, the technology has significantly matured through improvements to the labeling procedure and identification of sequences with improved binding properties. In a first step, optimal amino acids for the two central positions were identified, leading to the motif CCPGCC²⁰. Subsequently, flanking sequences were optimized, and in particular two 12-amino-acid motifs, FLNCCPGCCMEP and HRWCCPGCCCKTF, were introduced²¹. Additional endogenous low-affinity FAsH-binding sequences have been discovered²² but have not been of practical utility.

In addition to FAsH, a set of color variants with different spectral properties and enhanced photostability (e.g., fluorinated variants of FAsH) has been reported^{11,23,24}. The most extensively used alternative is the red-shifted analog ReAsH (resorufin arsenical hairpin

binder). The two variants FIAsh and ReAsH are commercially available from Invitrogen but can also be synthesized using a recently described procedure²⁴. The structure of the motif FLNCCPGCCMEP bound to ReAsH has been solved by NMR²⁵, revealing that the peptide sequence forms a hairpin, the details of which explain why Phe1, Pro6 and Gly7 are particularly important for the four cysteines to bind the biarsenical dye with high affinity.

Labeling of proteins with FIAsh and ReAsH can be achieved in intact cells; it is stable and lasts for hours, but it can be quickly reversed if desired. The method has been applied in a large variety of cellular systems and with many different types of proteins. Using common cell lines, labeling with FIAsh and/or ReAsH has been achieved in HeLa, HEK293, COS-7 and CHO cells^{26–30} as well as in primary cortical neurons³¹. It has also been used successfully in Gram-negative bacteria such as *Shigella flexneri*³² and *Escherichia coli* strains³³. The small size (< 0.7 kDa) of FIAsh and ReAsH has also permitted the study of fluorescently labeled viral proteins, whereas the larger GFP tag would hinder virus packing^{34,35}. Successful labeling has even been reported *in vivo* in *Drosophila* eyes³⁶.

In addition to straightforward labeling experiments, FIAsh and ReAsH have also been used for more complex investigations. For example, sequential pulse labeling of proteins with FIAsh and ReAsH has been developed for selective labeling of newly synthesized proteins^{18,31}. Chromophore-assisted light inactivation of ReAsH- or FIAsh-labeled proteins has permitted rapid photochemical inactivation of specific proteins in intact cells^{37,38} and also *in vivo* in *Drosophila* eyes³⁶. Furthermore, FIAsh has been used in combination with a second fluorophore in FRET studies. Fluorescent proteins, such as the cyan fluorescent protein (CFP) as well as ReAsH, have been used as FRET partners. These FRET studies have allowed the investigation of conformational changes in individual proteins and of protein-protein associations^{17,28,29,39–42}. Alternatively, conformational changes of proteins as well as protein-protein interactions can also be studied by measuring the fluorescence intensity of FIAsh binding to split versions of the binding sequence⁴³. This approach is based on the fact that fluorescence intensity increases on binding to its target sequence¹⁷; because of conformational changes, the split sequences approach closely and allow FIAsh to bind to the sequence⁴³. Several low- and high-affinity FIAsh-binding sequences have been recently described^{21,22}. The group of Squier and Meyer presented a variant tetracysteine motif, CCKACC, with different binding rates and affinities for FIAsh and ReAsH compared with the CCPGCC motif. This approach allowed sequential labeling, but it has so far been used only with purified proteins⁴⁴. A very recent addition to these protocols has been the development of a selective labeling strategy that permits the orthogonal labeling of two different tetracysteine motifs with FIAsh and ReAsH⁴⁵. Such selective labeling allows the simultaneous study in intact cells of two proteins of interest, each labeled with its own color, as well as the investigation of their interactions by colocalization and FRET experiments.

Comparison of FIAsh and GFP labeling

FIAsh labeling aims for a 1:1 stoichiometry of FIAsh:tetracysteine motifs with low background. Using the protocol described here, such complete labeling can be achieved within a short time. Background staining is then reduced by a washing procedure; this washing procedure can also reduce specific labeling if too-high antidote concentrations are used. This potential loss is minimized if the newer 12-residue motifs are used. Specificity of labeling can be ascertained by several controls. The simplest strategy is to compare cells expressing the tetracysteine-harboring protein with nontransfected control cells or—even better—with cells expressing the same protein but lacking this motif²⁸. A second strategy—used here for illustration purposes—involves fusion of tetracysteine-harboring proteins with a GFP variant. Although this approach negates the advantages of the small FIAsh label, it permits exact colabeling controls. Otherwise, this approach should only be used when a second label is required, e.g., for FRET experiments or to excite FIAsh by means of a

second fluorophore. It has been reported that direct excitation of ReAsH had a 90% reduced contrast compared to GFP alone²¹; however, in combination with GFP and excitation by FRET, the contrast was only decreased by 50% (ref. 21). Generally, the contrast should be better when using the high-affinity 12-amino-acid motif compared with the 6-amino-acid motif, as the wash buffer includes higher concentrations of the antidote^{21,45}. Multiple repeats of the tetracysteine motif resulted in a modest increase in brightness⁴⁶, but the labeling did not achieve the 1:1 stoichiometry seen for single motifs. When multiple repeats of the motif were added to β -tubulin, protein function was less conserved; with two repeats, the protein still functioned, but three repeats ablated the protein function⁴⁷.

Concerns about the cytotoxic effects of reagents, in particular of the washing procedure, have been raised^{19,48} and should therefore be addressed. Although damage to individual cells can occasionally be observed after the labeling procedure, our experience with the technique does not support the notion of a general cytotoxicity. For example, cell signaling pathways remain functional after labeling with FAsH. This includes the activation of G protein-coupled receptors^{28,29,42}, as well as the activation of PKC pathways, as a cytosolic PKC-FRET probe was phosphorylated normally after FAsH labeling⁴¹. GPCRs labeled with FAsH or ReAsH have been shown to continue to internalize after agonist stimulation^{30,45}, indicating that the endocytotic machinery remains functional, and the receptor recruits β -arrestin to the plasma membrane⁴⁵. Similarly, this labeling approach allows the transport of newly synthesized proteins in dendrites³¹, indicating that targeting of proteins remains functional. Ion channels (L-type calcium channels^{37,49} and P2X₂ channels²⁷) have been labeled in live cells, and patch-clamp experiments have been successfully performed on these FAsH-labeled cells to study channel functions. Labeling of connexin-43 or AMPA (α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) receptor subunits with FAsH did not impair labeling of the same cells 4–8 h later with ReAsH. During this time, protein synthesis had occurred, as demonstrated by the labeling of newly synthesized proteins by ReAsH compared with older proteins that had been labeled with FAsH^{18,31}. ReAsH-labeled cells were also shown to undergo normal cell division, as demonstrated by long-term imaging of HeLa cells⁵⁰. Taken together, these data suggest that many cellular functions remain normal after labeling with biarsenical dyes. However, notwithstanding this general point, it seems useful to ascertain the functionality of the specific biological process after this labeling procedure.

Experimental design

This method seems to be widely applicable to different biological systems and proteins. However, a detailed protocol on how to use and optimize it has been lacking. Therefore, the following sections describe these basic procedures in a step-by-step manner and emphasize potential problems and their solutions.

The full procedure involves modifying a protein of interest by insertion of a tetracysteine site, followed by monitoring of its expression in cells (or intact organisms) and, finally, labeling the protein with FAsH or ReAsH. The first step generally requires some knowledge of the protein of interest and the identification of sites where a tetracysteine motif may be placed without disturbing protein function. In general, we prefer to replace an endogenous stretch of amino acids with the tetracysteine motif. To genetically encode the core tetracysteine motif, CysCysProGlyCysCys, we generally use the codon sequence 5'-TGTTGCCCGGGCTGCTGT-3'. This codon sequence contains a *SmaI/XmaI* restriction site (underlined sequence) and thus allows easy screening for positive clones. Once the tetracysteine-tagged protein has been cloned and its expression, distribution and function have been tested in the cell line of interest, biarsenical dyes are used to fluorescently label the protein and thus visualize it. In general, one should try to minimize excess light exposure of the FAsH reagent. The preparation for labeling can be done under normal room light, but

direct sunlight should be avoided. The 1-h incubation time and later storage of cells should be done in a cell incubator, thereby automatically occurring in the dark. The entire labeling procedure must be done in a fume hood. EDT and the less-odorous BAL (2,3-dimercaptopropanol or British anti-Lewisite) are both hazardous and smell unpleasant. However, these dithiol antidotes are important components of the tetracysteine-biarsenical method and need to be used. BAL is somewhat less noxious but hinders labeling because its complexes with biarsenical dyes are tighter and less membrane permeant; thus, it should not be used for the labeling step but rather should be limited to the washing steps.

The following standard protocol has been established and tried with several different proteins, including some with very low levels of expression. We have found the same protocol useful for all proteins that we have labeled and thus believe that it is generally applicable. To demonstrate its feasibility, most of the images we show here as examples (see PROCEDURE and ANTICIPATED RESULTS) relate to G protein-coupled receptors. In particular, we focus on the A_{2A}-adenosine receptors, which are examples of cell surface proteins and are expressed at densities below 1 pmol mg⁻¹ of cellular protein, as well as their regulatory proteins, the β -arrestins, which are examples of cytosolic proteins and are expressed at similarly low levels. Thus, for proteins expressed at higher levels, the results, particularly the signal-to-noise ratios, should exceed those presented here.

MATERIALS

REAGENTS

- *Xma*I (NEB, 5,000 U, cat. no. R0180S)
- *Sma*I (NEB, 2,000 U, cat. no. R0141L)
- TC-FIAsh (Invitrogen, labeling kit, cat. no. T34561) ▲ **CRITICAL** Invitrogen is the only commercial supplier, but we have successfully used self-synthesized reagents (our FIAsh-EDT₂ stock is 1 mM in DMSO; stock solutions are stored at – 20 °C)
- TC-ReAsH (Invitrogen, labeling kit, cat. no. T34562) ▲ **CRITICAL** Invitrogen is the only commercial supplier, but we have successfully used self-synthesized reagents.
- EDT (1,2-ethanedithiol, grade for synthesis >99%; Merck, cat. no. 8.00795.0100)
! **CAUTION** The compound has a bad odor. Handle in a well-ventilated fume hood.
- BAL (2,3-dimercapto-1-propanol, grade purum p.a. 97%; FLUKA, cat. no. 38520)
! **CAUTION** The compound has a bad odor. Handle in a well-ventilated fume hood.
- DMSO (dimethylsulfoxide, cell culture grade; AppliChem, cat. no. A3672.0250)
! **CAUTION** DMSO is irritating to eyes, respiratory system and skin; wear suitable gloves and eye and skin protection.
- Ethanol (absolute, p.a. >99.8%; Sigma-Aldrich, cat. no. 33205)
- Sodium chloride (NaCl)
- Potassium chloride (KCl)
- Sodium phosphate, dibasic (Na₂HPO₄)
- Potassium phosphate, monobasic (KH₂PO₄)
- Sodium bicarbonate (NaHCO₃)
- Calcium chloride (CaCl₂)
- Magnesium chloride (MgCl₂)

- Magnesium sulfate (MgSO_4)
- Glucose
- HEPES
- Reagents for cell culture (these will vary according to desired cells and culture conditions)

EQUIPMENT

- Fume hood
- Fluorescence spectrofluorometer (LS50B, Perkin Elmer)
- Confocal microscope (Leica SP2, Leica)
- Leica software (version 2.61 (or later), Leica)
- Laser line of an argon laser (514 nm)
- Laser line (430 nm, Melles Griot)
- Laser line (405 nm, UV-laser diode)
- Attofluor cell chamber (Invitrogen, cat. no. A-7816)
- Silanized tubes (Eppendorf, safe-lock tubes 1.5 ml, cat. no. 0030120.086)
- Falcon tubes (Blue Max; Becton Dickinson, cat. no. 352070)
- Glass coverslips (general supplier, microscope cover glass, 24 mm)
- Glass-bottomed cell dishes (35 mm, MaTek, cat. no. P35G-1.5-10-C)
- Microscope for fluorescence measurements (including FRET)
  - Zeiss inverted microscope (Axiovert 200, Zeiss)
  - Dual-emission photometric system (Till Photonics)
  - Light source polychrome IV (Till Photonics)
  - Clampex software version 9.0
  - Origin software version 6.1 (OriginLab)
  - Dishes for cell culture

REAGENT SETUP

Labeling buffer—Standard commercial HBSS ('complete', no phenol red; see below) can be used for the labeling reaction. Instead of HBSS, one may also use the imaging buffer (see below) for labeling, and presumably many other buffers will also work. It is important, however, that no plasma or serum be added to the buffer because albumin binds the biarsenical dyes. Buffers can be prepared in advance in larger quantities and stored at 4 °C for several weeks. To improve storage time of HBSS, glucose should be added freshly before use.

Hank's balanced salt solution—To make HBSS (pH 7.4), combine 137 mM NaCl, 5.4 mM KCl, 0.3 mM Na_2HPO_4 , 0.4 mM KH_2PO_4 , 4.2 mM NaHCO_3 , 1.3 mM CaCl_2 , 0.5 mM MgCl_2 , 0.6 mM MgSO_4 and 5.6 mM glucose.

Imaging buffer—To make imaging buffer (pH 7.3), combine 140 mM NaCl, 5.4 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂ and 10 mM HEPES.

Cell culture—Cells may be cultured under any conditions before labeling, depending on their specific needs. For example, HEK cells are cultured in DMEM (4.5 g per liter of glucose) supplemented with 10% (vol/vol) FCS, 100,000 U per liter of penicillin and 100 mg per liter of streptomycin at 37 °C and 7% CO₂.

Cells and protein expression—Cells used for labeling need to express the protein of interest carrying the tetracysteine motif. This motif can be either the 6-amino-acid motif CCPGCC²⁰ or, preferably, one of the more recently described 12-amino-acid motifs (such as FLNCCPGCCMEP21), which display higher affinity for FAsH (see INTRODUCTION). The choice will depend on the insertion site and the ability of the modified protein to maintain its function. In general, we tend to replace the native stretch with the binding sequence rather than insert the sequence; we hope that this will affect the overall structure of the modified protein to a lesser extent.

Ideally, stably transfected cell clones should be used if correct targeting of the expressed protein is a problem (e.g., targeting to the cell surface). However, transient transfections have the advantage of speed, together with usually higher expression levels, and they avoid possible clonal artifacts. Routinely, we use HEK293 cells transiently transfected with any standard transfection method using either calcium phosphate or transfection reagents such as Effectene (Qiagen), Lipofectamine (Invitrogen) or GeneJuice (Novagen). Other cell lines such as HeLa, PC12 or CHO have also been used, as have primary neurons. Generally, cells are used 48 h after transfection (transfections are done at 40–50% confluence). Cells should be plated on polylysine-coated coverslips (24 mm in diameter) or on similar devices (such as MaTek dishes); they are handled on these coverslips throughout the procedure. We routinely grow the cells in six-well plates, with each well containing a 24-mm-diameter coverslip.

PROCEDURE

Preparation of dyes for labeling

- 1 In a silanized polypropylene tube, prepare a 25 mM EDT solution in DMSO by mixing 2.1 µl of EDT with 1 ml of DMSO. **▲ CRITICAL STEP** Stock solutions of dithiols should be prepared freshly before use, as they are prone to air oxidation. EDT has only limited solubility in aqueous solutions; hence, DMSO or absolute ethanol should be used for preparing concentrated solutions (above millimolar concentrations).
- 2 In a separate silanized polypropylene tube, add 1 µl of freshly prepared 25 mM EDT and 1 µl of FAsH stock for each sample to be labeled.
▲ CRITICAL STEP For a final concentration of FAsH, we use a 1 mM FAsH-EDT₂ stock in DMSO, whereas the stock currently provided by Invitrogen is 2 mM FAsH-EDT₂ in DMSO/H₂O (TC-FAsH, cat. no. T34561). This is important to note, as one may need to recalculate the appropriate volumes. The described protocol is given for a final 2-ml incubation volume. Our final working concentration for the FAsH labeling is 500 nM in a six-well tissue culture plate. In our hands, this has proven to be a good starting concentration for labeling of new constructs modified with the core sequence CCPGCC.
- 3 Incubate at room temperature (20–25 °C) for 5–10 min. This step ensures that all FAsH is in the FAsH-EDT₂ form. While incubating the EDT/FAsH mix, prepare the cells as described in Steps 4 and 5 below.

Labeling

- 4 Wash the tissue culture dish twice with 2 ml of 1× HBSS/glucose.
▲ CRITICAL STEP The complete removal of medium is necessary, as the remaining serum proteins may interfere unpredictably with the labeling reaction. Biarsenical dyes, like many synthetic dyes, bind reversibly to hydrophobic pockets on proteins such as BSA and may become fluorescent⁵¹. To decrease such competitive binding, which hinders the desired labeling, we generally wash off the cell growth medium before labeling and perform the labeling step in HBSS/glucose, PBS or the specific cell growth medium without serum. The labeling buffer should always contain Ca^{2+} and Mg^{2+} to prevent cells from detaching. We previously introduced Patent Blue and Disperse Blue to quench FIAsh bound to both extracellular and intracellular hydrophobic sites, respectively⁵¹. However, they only give a modest increase in contrast (twofold) and do not seem to be useful for ReAsH. We routinely use phenol red–free buffer to avoid any unnecessary dye for fluorescence imaging.
- 5 Add 1 ml of HBSS/glucose solution to the dish. If several samples need to be treated, carry out Steps 4 and 5 sequentially on smaller batches of samples to prevent the cells from being left dry for long periods of time.
- 6 For each sample to be labeled, add 1 ml of HBSS/glucose into a Falcon tube.
- 7 Take a small aliquot (50–100 μl per sample) from this HBSS/glucose and add it to the EDT/FIAsh solution prepared in Step 2.
- 8 Mix well by pipetting up and down and let the EDT/FIAsh/HBSS solution incubate for 5 min. This step ensures that the FIAsh/EDT in DMSO will mix homogeneously in a small volume and that it will later mix without problems in the larger volume of HBSS/glucose.
- 9 Add the solution from Step 8 to the Falcon tube containing the remaining HBSS/glucose from Step 6 and mix thoroughly by repeatedly inverting the Falcon tube to ensure a homogeneous solution.
- 10 Add 1 ml of this solution to each dish. Gently swirl the labeling solution to ensure initial mixing and incubate the tissue culture dish for 1 h at 37 °C (with a humidified atmosphere and CO_2 as appropriate for the buffer and cell type used). Thus, the concentrations during labeling are 500 nM FIAsh/EDT₂, 12.5 μM EDT and 0.1% (vol/vol) DMSO.
▲ CRITICAL STEP The incubation time that is necessary to reach full labeling of all sites with FIAsh may vary. In Figure 1, binding FIAsh to CFP-labeled $\text{A}_{2\text{A}}$ -adenosine receptors was monitored by quenching of the CFP fluorescence²⁸. With this approach it can be seen that, in living HEK293 cells, the binding motif CCPGCC is saturated using 500 nM FIAsh in less than 1 h. No further decrease in CFP fluorescence was observed after full saturation, as seen by reaching a plateau after 35–45 min. Therefore, an incubation time of 1 h includes a safety margin to ensure full occupancy of all available sites. We recommend using 10–15 μM EDT during the labeling step; higher concentrations increase specificity but slow down reaction rates. EDT is used during the labeling step, as BAL appears to decrease the membrane permeability of the biarsenical dyes, probably because the arsenic-BAL complex is more stable, rapidly displaces the EDT bound to each arsenic group and introduces two polar alcohol groups.

Reaction with biarsenical dyes requires that the cysteine thiols of the tetracysteine tag be reduced. Cellular redox mechanisms maintain cytoplasmic tetracysteine in this form, but

oxidizing cellular compartments such as the Golgi apparatus and extracellular space requires acute reduction with stronger reducing agents such as phosphines⁵⁰.

Removal of excess and nonspecifically bound dye

- 11 Prepare washing solution by mixing 42 μ l of EDT with 1 ml DMSO (500 mM EDT in DMSO).

▲ **CRITICAL STEP** Stock solutions of dithiols should be prepared freshly before use as they are prone to air oxidation. EDT has only limited solubility in aqueous solutions; hence, DMSO or ethanol should be used for making concentrated solutions (above millimolar concentrations).
- 12 Add 25 μ l of the 500 mM EDT in DMSO to 50 ml of HBSS/glucose solution, resulting in an HBSS/glucose solution containing 250 μ M EDT (washing solution, room temperature).
- 13 Aspirate the supernatant from the cells and add 2–3 ml of 250 μ M EDT washing solution. Incubate the cells for 10 min at 37 °C (humidified atmosphere and CO₂ as necessary for the buffer and cell type used).

▲ **CRITICAL STEP** The effect of this washing step is illustrated in Figure 2 with transiently transfected HEK cells. The concentration of EDT used to compete for nonspecific binding is crucial. If the concentration used is too low, the background fluorescence may remain unnecessarily high, whereas using too high concentrations will diminish the specific binding.

We currently use EDT for standard applications, but we have also used the less-odorous BAL. Figure 3 illustrates the BAL-resistance of the newly described motifs (FLNCCPGCCMEP or HRWCCPGCCKTF) in comparison with the CCPGCC motif. BAL has an approximately threefold higher potency in displacing FIAsh from its specific binding motif than does EDT (see Fig. 4). Figure 3 shows that, for the motif CCPGCC²¹, specific binding starts to be affected when washing is done with BAL concentrations higher than 100 μ M. At 250 μ M BAL, up to 30% of the specifically bound FIAsh is displaced. The Invitrogen kit currently provides a new wash buffer using 250 μ M BAL instead of 250 μ M EDT to reduce the odor. However, for the protein shown here, this concentration appears to be too high. Figure 3 also shows that the new motifs have a much better resistance to displacement of specific binding by BAL. Therefore, higher BAL concentrations can be used with the new motifs to reduce nonspecific binding while leaving the specific binding unaffected.
- 14 Aspirate the supernatant and wash the cells with 2–3 ml of HBSS/glucose solution without EDT.
- 15 Repeat Step 14 one or two additional times to reduce remnants (and odor) of EDT. Cells are now ready to be imaged (see Box 1 for details). In the case of larger sample numbers, we add cell culture medium used to culture the particular cell line instead of buffer and store cells at 37 °C (humidified atmosphere and CO₂ as appropriate for the medium and cell type used) until use. Thereafter, before imaging, wash cells with the imaging buffer as described in Step 4 (to remove phenol red); all measurements should be done in this buffer. We have studied cells up to 8 h after labeling and have not observed changes in cell morphology during this time.

● TIMING

The entire labeling procedure (Steps 1–15) using FAsH-EDT₂ as described takes 2–3 h, depending on the number of samples to be processed as well as on the experience of the researchers in conducting the labeling. There is no pause point within the procedure, but the 1-h incubation time in Step 10 can be used to prepare the microscopes for later sample imaging.

? TROUBLESHOOTING

This procedure does not allow the assessment of the success of labeling at any intermediate step; however, Table 1 provides advice for troubleshooting should no labeling or nonspecific labeling be observed at the end of the PROCEDURE. In general, we strongly recommend the use of control constructs that allow the potential source of the problem to be narrowed down. Cells expressing recombinant, nontetracycline-tagged host protein or, more simply, nontransfected cells are good negative controls. Careful choice and use of negative controls will help determine the baseline and potential signal/noise problems related to the specific cell type. Similarly, positive controls are valuable tools in the fine tuning of labeling with biarsenical compounds. We have found that adding other molecular tags to the tetracycline fusion is a simple, efficient way to generate internal positive controls (see use of CFP in Fig. 2).

Box 1 IMAGING CELLS

Confocal microscopy

Direct excitation of FAsH can be done using the 514-nm wavelength of an argon laser with the standard settings for visualizing yellow fluorescent protein.

Fluorescence emission of FAsH can be recorded using the manufacturer's settings for yellow fluorescent protein.

Laser intensity should be kept low to minimize photobleaching of the fluorophore.

Cells can be observed with objectives of high magnification ($\times 63$ or $\times 100$) for analysis of single cells, or with low magnification ($\times 10$ or $\times 20$ objectives) for analysis of cell ensembles.

FRET recordings

FRET recordings are made with a fluorescence microscope. Use objectives with high magnification ($\times 63$ or $\times 100$) for analysis of single cells, or use objectives with low magnification ($\times 10$ or $\times 20$) for analysis of cell ensembles.

FRET recordings can be done using either photodiodes or CCD cameras to record the emissions of the donor and the acceptor.

Cells labeled as described (Steps 1–15) are mounted on an Attolfluor holder and placed on an inverted microscope (e.g., Zeiss Axiovert 200) equipped with a dual-emission photometric system (e.g., Till Photonics) to record separately the two emission wavelengths.

Excitation for standard recording is done at the optimum wavelength for the donor.

Shutters or a tunable light source (e.g., Polychrome IV, Till Photonics) are essential. Switch on the excitation light only when observations are being made in order to minimize photobleaching and phototoxicity, as FAsH bleaches with similar kinetics as fluorescein.

Illumination and recording times of 5–40 ms for each data point are generally sufficient.

Emission levels of donor and acceptor wavelengths are recorded simultaneously using a beam splitter that divides the emissions of the donor and acceptor fluorophores (e.g., 480 nm for CFP and 535 nm for FAsH).

FRET can then be monitored as the emission ratio of FAsH over CFP¹⁴.

Examples for filters and beam splitters in the emission path for such experiments are 535 ± 15 nm (FAsH) and 480 ± 20 nm (CFP), using a dichroic long-pass beam splitter DCLP 505 nm.

Excitation can be done at 436 ± 10 nm (e.g., for CFP) with a dichroic long-pass beam splitter DCLP 460 nm.

The respective spillover between channels (i.e., direct excitation of FAsH by 436-nm light and emission of CFP into the 535-nm channel) needs to be corrected by the respective measurements of controls labeled with CFP alone or with FAsH alone before a corrected F^*_{535}/F^*_{480} ratio (F^* denotes corrected fluorescence) can be calculated.

The factors for such corrections need to be frequently determined, as they vary not only with the age of light sources but also with the type of instrumentation and other specific influences.

To monitor acute changes in FRET with such a system, cells should be continuously superfused with buffer, and compounds can then be applied using a computer-assisted solenoid valve-controlled rapid superfusion device (e.g., ALA-VM8, ALA Scientific Instruments; solution exchange in 5–10 ms).

Signals can be detected by photodiodes or with CCD cameras.

ANTICIPATED RESULTS

If the tetracysteine tag is intended to be used for labeling and visualization of the protein of interest, cDNA construction may only take a few days, especially if the tag can be placed in a region known to be tolerant of concatenation or substitution. When the placement of the motif must be varied to maximize FRET signals or minimize perturbation of physiological function, a thorough optimization may be required and this process can take significantly longer. For general strategies on FRET-probe design, we refer to a recent publication⁵². As the probe design is a very individual process and depends on biological application, it is impossible to give an estimated timeline for probe generation. As already mentioned, we find it useful to encode the desired CCPGCC motif with the nucleotide sequence 5'-TGTTGCCCGGGCTGCTGT-3'. This results in an additional cut by the restriction enzymes *Xma*I or *Sma*I and makes it easy to distinguish modified versus nonmodified DNA sequences by the appearance of distinct patterns in gel electrophoresis.

Once the desired construct has been generated and expressed in cells, the labeling protocol described here can be applied. The results shown here were obtained with proteins expressed at low levels, such as cell surface receptors (Fig. 2) or cytosolic β -arrestins (Fig. 5); both were expressed at levels below 1 pmol mg^{-1} of cellular protein. Better results can be expected with more abundantly expressed proteins.

In Figure 2, we expressed a previously published cell surface receptor construct²⁸ and labeled the cells until Step 10. Next, we placed the cells on a confocal microscope and continued the procedure on the microscope. The pictures in the top row show the results of the labeling protocol before the washing steps (Steps 11–13), whereas the bottom row shows the same cells when EDT washing solution was added to the cells while they remained on the microscope (i.e., this represents a very incomplete washing procedure). With this

experiment, we can simulate what we refer to as insufficient washing, and this likely reflects a situation that many researchers have observed when they started with this technique. From the top row of images, one might conclude that no specific labeling had been achieved. However, this is clearly not the case, as demonstrated by the images in the bottom row. It can be seen that the construct had been specifically labeled, but when no washing was performed, the nonspecific background was too high to visualize the specific label. The nontransfected cells in the panels, which can be easily distinguished here by the CFP fused to the protein of interest, serve well as internal controls for background staining and they help in judging the quality of the washing procedure. Obviously, such additional CFP-fusion should only be used as a control, given the size advantage of the small FIAsh label (unless a second label is wanted).

As different binding motifs can be used^{20,21}, it is important to analyze their individual affinity carefully. Figure 3 demonstrates the difference of motifs FLNCCPGCCMEP and CCPGCC in the stability of their FIAsh binding with respect to removal by BAL⁴⁵. This different stability of bound FIAsh has consequences for the washing procedure of differently tagged proteins. If a protein tagged with the sequence FLNCCPGCCMEP is washed with 250 μ M BAL, background labeling will be reduced, but specific labeling will not be reduced until BAL concentrations exceed 1 mM. However, if the same construct is made using the short CCPGCC motif, washing with 250 μ M BAL will already reduce specific labeling by 25–30% and 1 mM BAL will reduce specific labeling to almost background. This needs to be kept in mind when working with these tags. A special case is demonstrated in Figure 4. The initial antidote for washing was EDT and this can be exchanged for BAL to reduce the unpleasant odor during the labeling process. However, Figure 4 demonstrates that EDT and BAL are not equal with respect to displacing FIAsh from its binding motif. It can be seen that BAL is approximately threefold more potent in displacing FIAsh compared with EDT (see also Step 12 and TROUBLESHOOTING).

The same protocol can be applied to cytosolic proteins^{41,45}. Figure 5 demonstrates the labeling with FIAsh of the cytosolic protein β -arrestin-2 according to this protocol, again expressed at a low level of ~ 1 pmol mg^{-1} of cellular protein; better results can be expected for more abundantly expressed cytosolic proteins. Nontransfected cells indicate background staining. Functionality of the labeled β -arrestin-2 is demonstrated by the fact that it can be recruited to the cell surface by stimulation of a cotransfected receptor⁴⁵.

FIAsh labeling can be done alone to visualize proteins, or with a second label for FRET studies. The second label may be a fluorescent protein (such as CFP, together with FIAsh^{28,29,41,42,53}) or a small second label such as ReAsH^{38,45,54}. Details on how to perform FRET measurements are described in Box 1. Figure 6 shows a FRET recording as described in Box 1 using FIAsh and CFP as labels to monitor stimulus-dependent conformational changes in the α_2A -adrenergic receptor²⁹. The top panel shows the individual channels (after corrections for bleed-through and wrong excitation), and the bottom panel shows the normalized ratio of the two channels.

Up to 20% changes in FRET ratio have been observed in several systems employing FIAsh with CFP. Obviously, these effects depend not only on the labels but more so on the biological system. Using FIAsh and ReAsH as labels, we have recently obtained a change in the FRET ratio of about 4% for a protein-protein interaction⁴⁵. Successful labeling requires that the cysteine thiols of the tetracysteine tag be reduced. In oxidizing cellular compartments as the Golgi apparatus or the extracellular milieu, acute reduction with stronger reducing agents such as phosphines may be required (see also Figure 7 and TROUBLESHOOTING).

Acknowledgments

Research in the authors' laboratories is supported by the US National Institutes of Health, the Howard Hughes Medical Institute, the Deutsche Forschungsgemeinschaft (German Research Foundation) and the European Research Council.

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Figure 1.

Time dependence of FIAsh binding to the target sequence. The figure is modified with permission from Hoffmann *et al.*²⁸. HEK293 cells transiently transfected with the construct A_{2A}-CFP-Flash-C (containing the CCPGCC sequence) were incubated at the indicated time points with 500 nM FIAsh. Binding of FIAsh to this sequence was monitored by the quenching of the emission of CFP, which was measured at 450–515 nm every 3 min. After the decrease in CFP fluorescence had reached a plateau, cells were washed with 250 μM EDT to reduce nonspecific binding. Addition of 5 mM BAL completely reversed nonspecific binding of FIAsh within 10 min, with little effect on specific binding (as seen from the continued quenching of CFP fluorescence).

Figure 2.

Specific labeling with FAsH can only be seen after appropriate washing. Confocal images of a human A_{2A} -adenosine receptor construct with the tetracysteine motif CCPGCC in intracellular loop 3 and CFP at the C-terminus at position Gly-340 (A_{2A} -Flash3-CFP²⁸) transiently expressed in HEK293 cells. Cells were imaged 48 h after transfection. The top row shows cells after labeling with 500 nM FAsH according to the protocol without washing (thus, without Steps 11–13). The bottom row shows the same cells 10 min after addition of 250 μ M EDT according to the protocol. The left column shows images excited at 430 nm and emission from 470–550 nm (i.e., CFP fluorescence); the middle column shows the same cells excited at 514 nm and emission measured from 530–600 nm (i.e., FAsH fluorescence). After addition of EDT, the fluorescence intensity of FAsH-labeled cells was 15- to 20-fold over background of nontransfected neighboring cells. Note that the mere addition of EDT does not represent the full washing procedure and that better background reduction is obtained with the full procedure. The right column represents the transmission images of cells. The white scale bar = 10 μ m.

Figure 3.

Reduction of FIAsh binding to different tetracysteine motifs by BAL. Removal of label from tetracysteine motifs was achieved by addition of BAL. The experiment was done with two constructs, derived again from the human A_{2A} -adenosine receptor. Both constructs carried a CFP in the terminus to quantify FIAsh binding and were stably expressed in HEK-293 cells. The construct termed CCPGCC is identical to the one used in Figure 2; the CCPGCC motif in this construct is flanked by the normal A_{2A} sequence environment, finally reading ESQCCPGCCARS. In the construct termed FLNCCPGCCMEP, the binding motif was introduced N-terminally to the CFP, replacing the corresponding amino acids of the A_{2A} receptor. Cells were labeled with FIAsh, and membranes from these labeled cells were prepared as described⁴². Fluorescence spectra of homogenized membranes were measured in a fluorescence spectrophotometer. Thereafter, BAL was added at increasing concentrations, and for each concentration, fluorescence spectra were recorded after 10 min of incubation time. Spectra were recorded using 436-nm excitation (excitation bandwidth 5 nm) and emission was recorded from 460 to 700 nm (bandwidth 5 nm, scan speed 200 nm min⁻¹). The ratios of the fluorescence emission peaks at 480 nm (CFP) and 528 nm (FIAsh) were calculated and plotted against increasing BAL concentrations to determine the fraction of FIAsh bound (as percentage of the prewash value)⁴⁵. Squares represent the basic CCPGCC motif, whereas circles represent data for the motif FLNCCPGCCMEP. Note that the optimized motifs exhibit a much greater (10- to 30-fold) resistance to displacement by BAL. The figure is modified with permission from Zürn *et al.*⁴⁵.

Figure 4.

Comparison of BAL and EDT for removing FLaSH from its target sequences. HEK293 cells stably expressing α_{2A} -adrenergic receptors and carrying the CCPGCC motif (in the third intracellular loop²⁹) were incubated with FLaSH as described in the above protocol, and then membranes from these cells were prepared. Fluorescence spectra of homogenized membranes were measured in a fluorescence spectrophotometer as in Figure 3. Thereafter, BAL or EDT was added at increasing concentrations; at each concentration, fluorescence spectra were recorded after 10 min of incubation. BAL had an approximately threefold higher potency than EDT in displacing FLaSH from its specific binding motif.

Figure 5.

Labeling of the cytosolic protein β -arrestin-2 with FAsH. A β -arrestin-2-CCPGCC construct and the human PTH (parathyroid hormone) receptor⁴⁵ were transiently expressed in HEK293 cells. At 48 h after transfection, the cells were labeled according to this protocol. Top row: cells labeled with FAsH were excited at 514 nm and emission was measured from 530–600 nm (i.e., FAsH fluorescence). The right column represents transmission images of the cells. The white scale bar = 10 μ m. Bottom: the same cells were monitored 9 min after addition of 1 μ M PTH (1–34). After PTH (1–34) addition, a clear translocation of FAsH-labeled β -arrestin-2-CCPGCC to the membrane is visible, indicating that cytosolic proteins such as β -arrestin-2 can be specifically labeled and maintain their function⁴⁵. The figure is modified with permission from Zürn *et al.*⁴⁵.

Figure 6.

Dynamic FRET measurements between CFP and FAsH. HEK293 cells stably expressing an α_{2A} -adrenergic receptor construct, carrying the CCPGCC motif in the third intracellular loop and CFP at the C-terminus (α_{2A} -Flash3-CFP adrenergic receptor²⁹), were labeled with FAsH according to the protocol. The cells were placed on the FRET setup and perfused with buffer alone or with saturating conditions of norepinephrine or dopamine. The top panel shows the corrected CFP and FAsH channels of a typical experiment (A.U., arbitrary units), whereas the bottom panel shows the trace of the normalized FRET ratio of the same experiment.

Figure 7.

Labeling of extracellular tetracysteine-tagged proteins by membrane-impermeant biarsenical dyes. Wide-field images of HEK293 cells transiently transfected with FLNCCPGCCMEP-Myc-V2R-CeFP vasopressin receptor and stained for 10 min with the following: (top row) 2.5 μ M sulfo-FIAsH (sFIAsH)-EDT₂ (ref. 20) and 10 μ M EDT after a 30-min preincubation with 5 mM 2-mercaptoethanesulfonate (MES); (middle row) 2.5 μ M sFIAsH-EDT₂ and 10 μ M EDT after a 30-min preincubation with 0.5 mM tris(carboxyethyl)phosphine (TCEP) and 5 mM MES; (bottom row) 2.5 μ M ReAsH-EDT₂ and 5 μ M 2,3-dimercaptopropane-sulfonate (DMPS) after a 30-min preincubation with 0.5 mM TCEP and 5 mM MES. Reducing agents were retained during staining and removed with unbound dye by washing with 100 μ M DMPS for 10 min before imaging. All procedures were carried out at 37 °C in HBSS/glucose. DIC, differential interference contrast. The white scale bar = 10 μ m.

TABLE 1

Troubleshooting table.

Problem	Possible reason	Solution
No labeling observed	Inappropriate design of the protein construct	There are different motifs that can be used for labeling of a host protein (see Fig. 3 and references); choose motifs to minimize perturbation of host protein and background staining <i>CCPGCC</i> : shorter length, less resistant to EDT or BAL treatment, potentially more background staining left. We recommend this motif if an insertion into the host protein is desired. If N- or C-terminal tagging is possible, longer motifs are recommended <i>FLNCCPGCCMEP</i> : longer sequence, more resistant to EDT or BAL treatment, most likely less background staining. When this sequence is inserted, it may eventually be more perturbing to the host protein than the CCPGCC motif, but this needs to be verified
	Cells are not transfected	If cells are difficult to transfect or if a protein of interest does not express well, it may be necessary to identify transfected cells with a second label. This label may be in a separate protein or fused to the protein of interest (e.g., CFP, see above). This will help to verify that transfected cells are investigated and makes it possible to judge whether the labeling procedure works for the desired protein of interest
	Low-expressing protein	Obviously, better labeling is obtained with abundantly expressed proteins. We demonstrate in this protocol that it can be used to label proteins with expression levels below 1 pmol mg ⁻¹ of protein, both for membrane proteins and cytosolic proteins. Low-expressing proteins may not produce a good signal-to-noise ratio. We recommend using 12-amino-acid tetracysteine motifs to minimize background staining by stronger washing
Yellow fluorescence observed but no specific labeling (see Fig. 2)	Incomplete washing	Nonspecifically bound FIAsh remains in the cells that will mask the signal. We recommend repetition of Steps 13 and 14 for a second round of washing
	Inappropriate incubation time	The incubation time is critical to allow complete labeling of the protein. As can be seen in Figure 1 , the target protein showed exponential saturation kinetics for labeling. The time periods given here assure virtually complete labeling with both FIAsh and ReAsH. Accurate determination of labeling time courses is best achieved as described in Figure 1. Further increases in incubation time while using high FIAsh concentrations most likely increases only nonspecific binding. A decrease in incubation time may result in unoccupied binding motifs. We are currently testing whether longer labeling time periods with lower concentrations of FIAsh (50–100 nM) may produce higher signal-to-noise ratios
	Inappropriate concentration of the wash solutions	The correct wash procedure is a major issue for this technique. Figure 2 illustrates results with and without washing. Figure 3 illustrates that the CCPGCC motif can be washed with up to 100 µM BAL without displacing specifically bound FIAsh, whereas the FLNCCPGCCMEP resists up to 1 mM BAL, a concentration that would already displace 75% of the specific binding from the 6-amino-acid motif
	Inappropriate use of EDT or BAL as antidotes for washing	Because BAL is less odorous than EDT, one can use BAL instead for washing. ! CAUTION the two reagents are not equal with respect to their strength in displacing FIAsh from the specific binding motif: BAL is apparently threefold more effective in displacing FIAsh (Fig. 4) The recent change in the protocol supplied by Invitrogen might be of concern. Until recently, the kit was sold with 250 µM EDT for washing. Now the kit is supplied with a washing solution containing 250 µM BAL instead. This should be kept in mind, if one plans to use the 6-amino-acid CCPGCC tag. Figure 4 illustrates that with 250 µM BAL the bound fraction is displaced in part and thus the specific signal is reduced by 25%. This is not the case for the improved 12-amino-acid tag
	Tetracysteine is localized in an oxidizing environment such as the Golgi apparatus or on the extracellular side of the plasma membrane	Successful labeling requires that the cysteine thiols of the tetracysteine tag be reduced. In oxidizing cellular compartments, acute reduction with stronger reducing agents such as phosphines may be required. We have used membrane-permeant phosphines such as tributylphosphine (TBP) or triethylphosphine (TEP) to label targets in oxidizing environments inside organelles in living cells. Both TEP and TBP are prepared fresh in ethanol (1 M solutions) and diluted 1:1,000 directly into the labeling mixture with the biarsenical in Step 8. Phosphines react with DMSO and cannot be stored for any length of time in that solvent

Problem	Possible reason	Solution
		For tetracysteines facing the extracellular milieu, the best conditions so far (S. Terrillon, unpublished results) for live mammalian cells have been: reduction of extracellular disulfides with the membrane-impermeant reducing agents MES (5 mM) and TCE (0.5 mM) for 30 min at 37 °C; labeling with 2.5 µM ReAsH or sulfo-FLAsH ²⁰ in the presence of the reducing agents used in the previous step and 5 µM DMPS (a membrane-impermeant analog of EDT) for 10 min at 37 °C; and removal of nonspecific binding with 100 µM DMPS for 10 min at 37 °C. DMPS prevents ReAsH from crossing the plasma membrane and would probably do the same with FLAsH as well, which would avoid the requirement for sulfo-FLAsH, which is not commercially available (see also Fig. 7)