Published in final edited form as:

Nat Protoc. 2008 ; 3(9): 1527–1534. doi:10.1038/nprot.2008.144.

Preparation of the membrane-permeant biarsenicals, FIAsh-EDT₂ and ReAsH-EDT₂ for fluorescent labeling of tetracysteine-tagged proteins

Stephen R. Adams¹ and Roger Y. Tsien^{1,2,3}

¹Department of Pharmacology, University of California, San Diego, La Jolla, CA 92093-0647, USA.

²Chemistry and Biochemistry, University of California, San Diego, La Jolla, CA 92093-0647, USA.

³Howard Hughes Medical Institute, University of California, San Diego, La Jolla, CA 92093-0647, USA.

Abstract

The membrane-permeant, fluorogenic biarsenicals, FIAsh-EDT₂ and ReAsH-EDT₂ can be prepared in good yields by a straightforward two-step procedure from the inexpensive precursor dyes, fluorescein and resorufin respectively. Handling of toxic reagents such as arsenic trichloride is minimized so the synthesis can be carried out in a typical chemistry laboratory, typically taking about 2–3 days. A wide range of other biarsenicals reagents and intermediates that also bind to tetracysteine-tagged (CysCysProGlyCysCys) proteins can be prepared similarly using this general procedure.

INTRODUCTION

The introduction of genetically-encoded tags such as Green Fluorescent Protein (GFP) for visualizing proteins in living cells, tissues and organisms has been a major advance in cell biology^{1,2}. Fluorescent proteins however, are large (~30 kD) and can perturb the activity or localization of the protein being studied. We have introduced an alternative and much smaller genetically-encoded tetracysteine tag (< 1 kD) that has a high affinity for membrane-permeant, fluorogenic biarsenicals such as FIAsh-EDT₂ and ReAsH-EDT₂ (Figure 1)^{3,4}. The minimal tetracysteine sequence is CysCysProGlyCysCys although optimized motifs with higher affinity are preferable⁵. A unique feature of the biarsenicals is that their fluorescence is quenched until bound by the tetracysteine tag which allows the protein to be imaged without exhaustive washing to remove unbound dye³.

In addition to fluorescent marking of the tagged protein, biarsenicals have enabled many other techniques such as the determination of protein age by two-color pulse chase⁶, dye-triggered protein aggregation⁷, localized protein inactivation by chromophore-activated light inactivation (CALI)^{8,9}, correlative fluorescent and electron microscopy^{6,10}, and the generation of improved Ca²⁺ and FRET indicators^{11, 12}. The major limitations of the method are its reduced sensitivity compared to FPs because of non-specific staining (although the optimized tetracysteine sequences have reduced this considerably) and restriction (so far) to cultured cells and tissue preparations. The requirement for washing with dithiols after staining limits the methods use in animals although long incubation times with very low (nM)

Correspondence should be addressed to Stephen R. Adams, Department of Pharmacology, University of California, San Diego, 310 George Palade Laboratories 0647, La Jolla, CA 92093-0647, USA, Tel: 858-534-5268, Fax: 858-534-5270, sadams@ucsd.edu, rtsien@ucsd.edu, Lab Home page: <http://tsienlab.ucsd.edu/>.

concentrations of biarsenicals without any subsequent destaining may be one solution. Surprisingly, toxicity has not been an issue in most cellular applications. Many similar genetically-targeted labeling strategies have been described² but the genetically- encoded tag is either large (protein)¹³ or if small (peptide) can only be used for extracellular labeling^{14, 15}.

FlAsH emits green-yellow fluorescence and is a good FRET acceptor from CFP (spectrally it is very similar to YFP) and has been used most in cell labeling experiments. ReAsH fluorescence peaks in the red (609 nm), is a FRET acceptor from longer wavelength FPs such as GFP and YFP and is useful for pulse-chase labeling, CALI and correlated electron microscopy. Although FlAsH and ReAsH are commercially available, their straightforward synthesis in two steps from inexpensive starting materials using standard chemical techniques allows the preparation of the substantial amounts required for many experiments. For other biarsenicals (Figure 2), synthesis similar to these basic protocols is often the only option although non-acidic mercuration conditions are required for rhodamines⁴. This procedure for FlAsH-EDT₂ differs from previously published methods^{3,16} in the first step; reaction of fluorescein using mercuric trifluoroacetate proceeds with near quantitative yield to give 4',5'-bis(trifluoroacetoxymethyl)fluorescein and a higher yield of FlAsH-EDT₂ in the next step. Earlier reports used commercially-available 4',5'-bis(acetoxymethyl)fluorescein (fluorescein mercuric acetate). The subsequent transmetallation step appears generally applicable to the synthesis of a wide range of biarsenicals (Figure 2).

MATERIALS

REAGENTS

- Fluorescein (purity >97%; Acros, 11924)
- Resorufin (Free acid, dye content >95%; Aldrich 424455; or sodium salt, dye content 75%; Aldrich 230154)
- Mercuric oxide, HgO (purity >99%; Aldrich 213357). CAUTION Handle with care (extremely toxic)
- Trifluoroacetic acid, TFA (purity 99%; Aldrich T62200 and other suppliers). CAUTION Handle with care (corrosive)
- Phosphorus pentoxide
- 1-Methyl-2-pyrrolidinone, NMP (anhydrous; Aldrich 328634 and other suppliers)
- Arsenic (III) chloride, AsCl₃ (purity > 99.9 %; Aldrich 200077). CAUTION Handle with care in a well-ventilated fumehood (extremely toxic, carcinogenic)
- N,N-diisopropylethylamine, DIEA (purity >99.5%; Aldrich 387649)
- Palladium (II) acetate, Pd(OAc)₂ (purity 98%; Aldrich 205869)
- Acetone. CAUTION Inflammable
- Phosphate buffer, pH 6.9 prepared from equal volumes of 1M solutions of the monobasic and dibasic potassium salts, KH₂PO₄ and K₂HPO₄
- 1, 2-Ethanedithiol, EDT (purity >98%; Fluka 02390). CAUTION Stench. Handle in a well-ventilated fumehood
- Chloroform. CAUTION Toxic
- Brine (saturated aqueous solution of sodium chloride)

- Sodium sulfate (anhydrous, Fisher S429)
- Toluene
- Ethyl acetate. CAUTION Inflammable.
- Molecular sieve 4A beads 8–12 mesh (Aldrich 208604)
- Sand
- Silica gel 60 (230–400 mesh ASTM, EMD)
- 95% ethanol
- TLC plates (silica gel 60 F₂₅₄, EMD)
- Nanopure water
- 2-mercaptoethanesulfonate, sodium salt (Sigma M1511)
- Dimethylsulfoxide, DMSO (anhydrous, Aldrich 276855 and other suppliers)
- 4-Morpholinepropanesulfonic acid, MOPS
- Sodium hydroxide, pellets or solution
- Tetracysteine peptide containing the motif, Cys-Cys-Pro-Gly-Cys-Cys. Optimal results are obtained with FLNCCPGCCMEP (H₂N- Phe-Leu-Asn-Cys-Cys-Pro-Gly-Met-Gln-Pro-CONH₂). HPLC-purified or crude peptide is suitable.

CRITICAL All solvents used for reactions should be of the highest quality possible. Solvents for chromatography are HPLC grade.

EQUIPMENT

- 2-necked round bottom flask
- Round bottom flask
- Fumehood
- Rotary evaporator
- Dessicator
- Constant volume pipets (Microman, Gilson, Inc., Middleton, WI) or glass syringes
- Filter funnels, filter paper
- Graduated cylinders
- Heating mantle
- Heat gun or Bunsen burner
- Glass chromatographic columns (air-pressurizable)
- Separatory funnels
- Spatulas
- Teflon-coated magnetic stir bars
- Thermometers
- TLC developing chambers
- UV hand-held lamp

- Pipets

REAGENT SETUP AND HANDLING

- Solvents: Dry NMP and DIEA over 4A molecular sieve for at least several days before use in reactions or use from bottles of anhydrous material kept and withdrawn under inert gas. All reactions, workup and chromatography should be carried out in a well-ventilated fumehood.
- Arsenic trichloride handling: Constant volume pipettes (Microman) are recommended for this toxic and volatile liquid as the plastic pistons and tips are both disposable and can be decontaminated with dilute bleach solution. (Alternatively dry glass syringes with needles may be used). Used reaction glassware can similarly be treated with dilute bleach. Solid and liquid waste generated should be disposed of according to appropriate regulations.
- Solutions: 0.1 M (approx.) potassium phosphate buffer in water-acetone. Make a buffer containing an equimolar (0.25M) concentration of NaH_2PO_4 and Na_2HPO_4 , then add acetone until cloudiness develops.

0.1M MOPS adjusted to pH 7.2 with sodium hydroxide solution

EQUIPMENT SETUP

Glassware for the transmetallation step should be dried before use in an oven or by flame drying.

A convenient but optional way to produce a N_2 atmosphere in a reaction flask is to use a Firestone valve (Aldrich Z103616) attached by plastic or rubber tubing to N_2 (g) tank, house vacuum and one of the necks of the round-bottom flask via a septum-inlet adaptor (e.g. Aldrich Z102288).

PROCEDURE

1. Prepare FIAsh-EDT₂ or ReAsH-EDT₂ by following the steps in Options A or B respectively.

Option A:

- i. **Synthesis of fluorescein 4', 5'-bis(mercuric trifluoroacetate) (Steps 1–4. Timing ~ 24 hrs)** Weigh 1.3 g of mercuric oxide (HgO) (6 mmol) and transfer to a 50 ml round-bottom flask equipped with a Teflon-coated magnetic stir bar

!CAUTION. HgO is poisonous; avoid contact and breathing dust.
- ii. Transfer 15 ml of trifluoroacetic acid to the flask and gently heat the stirred suspension with a heatgun until the solid has dissolved to form a colorless solution. Allow the solution to cool to room temperature.
- iii. Weigh out 1.0 g of fluorescein (3 mmol) and add to the solution of mercuric trifluoroacetate to give a yellow solution. Protection from moisture is not necessary. A heavy yellow precipitate forms over the next few hours.
- iv. After 5 hours, evaporate off the trifluoroacetic acid (bath temperature $< 40^\circ\text{C}$) and suspend the resulting solid in 25 ml of water. Filter off the product, wash several portions of water (10ml),

allow to air-dry, then place overnight in a vacuum desiccator containing a glass beaker of phosphorus pentoxide (~20 g) evacuated using a high vacuum pump. If the P₂O₅ becomes liquid, replace with fresh solid and continue desiccation. The product is a yellow-orange solid. Typical yields are ~2.8 g (over 96 %).

- v. **Synthesis of FIAsh-EDT₂ (4',5'-Bis(1,3,2-dithiarsolan-2-yl)-fluorescein (Steps v–xx; Timing ~8 h.)** Weigh 0.96 g of fluorescein 4',5'-bis(mercuric trifluoroacetate) (1.0 mmol) and transfer to a dried 50 ml two-necked round-bottom flask, equipped with a Teflon-coated magnetic stir-bar, and under N₂ atmosphere (preferably with a slight positive-pressure of N₂ gas) through one neck of the flask. Seal neck with rubber septum.
- vi. Transfer 10 ml of anhydrous 1-methyl-2-pyrrolidinone via a disposable plastic or a glass syringe with attached dry needle into the flask by injecting through the septum.
- vii. Transfer 1.69 ml of arsenic trichloride (20 mmol) into the flask via syringe or positive-displacement pipette by injecting through the septum or by briefly removing the septum if the reaction vessel is under positive N₂ pressure.

CAUTION! Arsenic trichloride is volatile, corrosive, acutely toxic and a carcinogen. Handle only in a well-ventilated fumehood wearing suitable protective clothing (gloves, labcoat and safety glasses). Keep bottle tightly closed. Dispose of residues in syringes, needles and pipette tips, gloves by rinsing with diluted bleach (sodium hypochlorite solution).

- viii. Transfer 1.4 ml of anhydrous diisopropylethylamine (8 mmol) into the flask by syringe or pipette by injecting through the septum or by briefly removing the septum.
- ix. Transfer a few milligrams of palladium (II) acetate into the flask by spatula and by briefly removing the septum.
- x. The dark colored reaction mix is heated at 60 °C for about 3 hours.
- PAUSE POINT The reaction can be left stirring at room temperature overnight under N₂ at this stage.
- xi. Transfer the reaction mixture with a disposable plastic or glass pipet into a 250 ml glass Erlenmeyer containing a well-stirred (with a Teflon-coated magnetic stir-bar) mixture of 100 ml of 1:1 v/v 0.25 M potassium phosphate buffer pH 6.9-acetone to give an orange solution. Note that this amount of buffer is insufficient to maintain the pH at ~7 during quenching of the excess AsCl₃ in the reaction mix but will prevent the solution from becoming too acidic and causing decomposition of the product.
- xii. Transfer ~4 ml of 1,2-ethanedithiol (EDT) to the stirred solution with a disposable plastic or glass pipet. The solution decolorizes, turns milky and a solid deposits. CAUTION Stench from EDT, use only in a well-ventilated fume-hood. Quench the transfer pipet in a diluted bleach solution

- xiii.** Transfer ~50 ml of chloroform to the rapidly stirred solution. Rinse out the reaction vessel by transferring the mixture to and from the flask. Stir for 15–30 min at room temperature (18–25 °C).
- xiv.** Separate the aqueous and chloroform layers in a separatory funnel. Extract the aqueous layer twice with fresh ~50 ml portions of chloroform. Dry the combined organic layers with anhydrous Na₂SO₄. After ~30 min, remove the drying agent by filtration through filter paper. Remove the chloroform by rotary evaporation at 40 °C to give an orange liquid.

Note that a large amount of Na₂SO₄ will be required so add until free solid remains suspended rather than clumped after swirling the mixture. Alternatively, residual NMP can be removed by evaporation on a high-vacuum pump with gentle warming. This avoids the following step but traces of NMP may affect the chromatography step.

- xv.** Dissolve the oil in 100 ml of toluene and wash (three times) with 50 ml of water in a separatory funnel. [See troubleshooting]. One or two back extractions of the pooled aqueous washes with ~20 ml of toluene are recommended. After ~30 min, remove the drying agent by filtration through filter paper. Remove the toluene by rotary evaporation at 40°C to give an orange oil. Dissolve in ~5 ml of toluene.

PAUSE POINT. The crude product can be stoppered and left overnight in the fumehood. As₂(EDT)₃ may precipitate as a white solid; redissolve by gentle heating or by adding a few milliliters of chloroform before proceeding to the next step.

- xvi.** Prepare a chromatography column (1 in. i.d. × 12 in. with 250 ml reservoir) of silica gel (~50 g) packed in toluene. Cover the top with a layer of sand (0.5 cm thick).
- xvii.** Load the column with the crude product from Step 15 and elute the column with toluene (~500 ml) under air pressure (or gravity) until the eluent shows no UV absorbance when spotted on to TLC silica gel plates.
- xviii.** Elute the product with 10–20% ethyl acetate-toluene (~500 ml). An orange band will move down the column and elute but the fractions will be colorless. Identify fractions of the product by eluting TLC silica gel plates eluting with 20% ethyl acetate-toluene (retention factor = 0.48). Gently warming the developed plate with a hot air gun reveals the product as a yellow spot.
- xix.** Remove the solvents from the pooled collected fractions by rotary evaporation (< 40 °C) to yield the product as an off-white solid. Remove immediately as soon as dry.

CRITICAL STEP Continuing to evaporate the solid on the rotary evaporator or by further evaporation on a vacuum pump leads to loss of EDT from the arsenic groups and some polymerization. The solid will no longer dissolve completely in chloroform and toluene and will give a baseline spot under the TLC conditions in step 18. [See Troubleshooting]

- xx. Remove residual traces of toluene by adding absolute ethanol (~10 ml) and reevaporating. Suspend the remaining solid in ~5 ml of 96% ethanol, chill on ice for 30 min, and filter off the product, washing with a little cold ethanol. Dry overnight on filter without vacuum to give the product as a pale yellow or pinkish solid. Yield, ~300 mg. Store in the dark at -20 °C.

Option B:

- i. **Synthesis of resorufin 4, 5-bis(mercuric trifluoroacetate) (Steps i–iv; Timing ~1 day)** Weigh 120 mg of mercuric oxide (HgO) (0.5 mmol) and transfer to a 10 ml round-bottom flask equipped with a Teflon-coated magnetic stir bar

CAUTION. HgO is poisonous, avoid contact and breathing dust.

- ii. Transfer 2 ml of trifluoroacetic acid to the flask and gently heat the stirred suspension with a heatgun until the solid has dissolved to form a colorless solution. Allow the solution to cool to room temperature.
- iii. Weigh out 59 mg of resorufin sodium salt (0.25 mmol) and add to the solution of mercuric trifluoroacetate to give a dark red solution. Equip with a reflux condenser with water cooling and gently reflux the solution overnight. Protection from moisture is not necessary. A dark red precipitate is formed.

Note that an equivalent amount of resorufin free acid can be substituted for the sodium salt.

- iv. After cooling to room temperature, evaporate off the trifluoroacetic acid (bath temperature < 40 °C) and suspend the resulting solid in 5 ml of water. Filter off the product, wash with several portions of water (5 ml), allow to air-dry, then dessicate over phosphorus pentoxide (~5 g) under reduced pressure overnight. If the P₂O₅ becomes liquid, replace with fresh solid and continue dessication. The product is a yellow-orange solid. Typical yields are ~ 132 mg (over 60 %).
- v. **Synthesis of ReAsH-EDT₂ (4,5-Bis(1,3,2-dithiarsolan-2-yl)-resorufin) (steps v–xx; Timing ~8h)** Weigh 73 mg of resorufin 4,5-bis(mercuric trifluoroacetate) (0.087 mmol) and transfer to a dried 5 ml two-necked round-bottom flask, equipped with a Teflon-coated magnetic stir-bar, and under N₂ atmosphere (preferably with a slight positive-pressure of N₂ gas) through one neck of the flask. Seal neck with rubber septum.
- vi. Transfer 1.5 ml of anhydrous 1-methyl-2-pyrrolidinone (NMP) via a disposable plastic or a glass syringe with attached dry needle into the flask by injecting through the septum.
- vii. Transfer 0.17 ml of arsenic trichloride (4 mmol) into the flask via syringe or positive-displacement pipette by injecting through the septum or by briefly removing the septum if the reaction vessel is under positive N₂ pressure.

CAUTION! Arsenic trichloride is volatile, corrosive, acutely toxic and a carcinogen. Handle only in a well-ventilated fumehood

wearing suitable protective clothing (gloves, labcoat and safety glasses). Keep bottle tightly closed. Dispose of residues in syringes, needles and pipette tips, gloves by rinsing with diluted bleach (sodium hypochlorite solution).

- viii.** Transfer 0.14 ml of anhydrous diisopropylethylamine (1.6 mmol) into the flask by syringe or pipette by injecting through the septum or by briefly removing the septum.
- ix.** Transfer a few milligrams of palladium (II) acetate into the flask by spatula and by briefly removing the septum.
- x.** The dark colored reaction mix is heated at 60 °C for about 3 hours.
PAUSE POINT The reaction can be left stirring overnight at room temperature under N₂ at this stage.
- xi.** Transfer the cooled reaction mixture with a disposable plastic or glass pipet into a 100 mL glass Erlenmeyer containing a well-stirred (with a Teflon-coated magnetic stir-bar) mixture of 25 ml of 1:1 v/v 0.25M potassium phosphate buffer pH 6.9-acetone to give an orange solution.
- xii.** Transfer ~0.5 ml of 1,2-ethanedithiol (EDT) to the stirred solution with a disposable plastic or glass pipet. The solution decolorizes, turns milky and a solid deposits. CAUTION Stench from EDT, use only in a well-ventilated fume-hood. Quench the transfer pipet in a diluted bleach solution
- xiii.** Transfer ~25 ml of chloroform to the rapidly stirred solution. Rinse out the reaction vessel by transfer the mixture to and from the flask. Stir for 15–30 min.
- xiv.** Separate the aqueous and chloroform layers in a separatory funnel. Extract the aqueous layer twice with fresh ~20 ml portions of chloroform. Dry the combined organic layers with anhydrous Na₂SO₄. After ~30 min, remove the drying agent by filtration through filter paper. Remove the chloroform by rotary evaporation at 40 °C to give a red liquid.

Note that a large amount of Na₂SO₄ will be required; add until free solid remains suspended after swirling the mixture. Alternatively, residual NMP can be removed by evaporation on a high-vacuum pump with gentle warming. This avoids the following step but traces of NMP may affect the chromatography step.

- xv.** Dissolve the oil in 30 ml of toluene and wash with 10 ml of water in a separatory funnel. One or two back extractions of the pooled aqueous washes with ~5 ml of toluene are recommended... Dry the combined toluene layers with anhydrous Na₂SO₄. After ~30 min, remove the drying agent by filtration through filter paper. Remove the toluene by rotary evaporation at 40°C to give a dark red oil. Dissolve in ~5 ml of toluene.

PAUSE POINT. The crude product can be stoppered and left overnight in the fumehood. As₂(EDT)₃ may precipitate as a white solid; redissolve by gentle heating or by adding a few milliliters of chloroform before proceeding to the next step.

xvi. Prepare a chromatography column (0.5 in i.d. × 12 in. with 100 ml reservoir) of silica gel (~20 g) packed in toluene. Cover the top with a layer of sand (0.5 cm thick).

xvii. Load the column with the crude product from Step xv and elute the column with toluene (~200 ml) under air pressure (or gravity) until the eluent shows no UV absorbance when spotted on to TLC silica gel plates.

xviii. Elute the product with 2.5–5 % ethyl acetate-toluene (~200 ml). A pale orange band will move down the column following a blue band. Identify fractions of the product by spotting TLC silica gel plates eluting with 20% ethyl acetate-toluene (retention factor = 0.53). Gently warming the developed plate with a hot air gun reveals the product as a purple spot. **CRITICAL STEP.** Carefully separate the product from the blue-colored, more non-polar side-product (retention factor ~0.6), ReAsH-EDT₃ as this gives background staining in mammalian cells that is not removable with the usual post-staining washing concentrations of the dithiol arsenic antidotes, EDT or BAL (British anti-Lewisite; 2,3-dimercaptopropanol) and therefore reduces the sensitivity of the method in live cells. Traces of ReAsH-EDT₃ are best identified by TLC or by HPLC.

xix. Remove the solvents from the pooled collected fractions by rotary evaporation (< 40 °C) to yield the product as an off-white pinkish solid. Remove immediately.

CRITICAL STEP: Continuing to evaporate the solid on the rotary evaporator or by further evaporation on a vacuum pump leads to loss of EDT from the arsenic groups and some polymerization. The solid will no longer dissolve completely in chloroform and toluene and will give a baseline spot under the TLC conditions in step 18.

xx. Remove residual traces of toluene by adding absolute ethanol (~5 ml) and reevaporating. Suspend the remaining solid in ~1 ml of 96% ethanol, chill on ice for 30 min, and filter off the product, washing with a little cold ethanol. Dry overnight on filter without vacuum to give the product as a dark red solid. Yield, 5–16 mg. Store in the dark at –20 °C.

2. Quality control of the products by measuring fluorescence before and after addition of tetracysteine tagged peptide

- i.** Prepare 1 mM solutions of FAsH-EDT₂ or ReAsH-EDT₂ in dry DMSO and keep protected from light. Aliquot and keep at –20°C.
- ii.** Prepare stock solutions of 10 mM EDT in dry DMSO (0.84 µL EDT/ml) and 1M 2-mercaptoethanesulfonate in water. **CRITICAL STEP.** Make these solutions up immediately before use.
- iii.** Prepare a 10 mM stock solution of a tetracysteine peptide in 50% aqueous acetonitrile containing 0.1% TFA. The optimized sequence, FLNCCPGCCMEP (H₂N- Phe-Leu-Asn-Cys-Cys-Pro-Gly-Met-Glu-Pro-CONH₂) is preferable.
- iv.** Prepare a 100 mM aqueous solution of MOPS neutralized with NaOH to pH 7.2.

- v. To 2.5 ml of the MOPS buffer in a fluorescence cuvet, add 25 μ L of 1M MES followed by 2.5 μ L of 10mM EDT to give final concentrations of 10mM and 10 μ M respectively.
- vi. With a fluorimeter, measure the emission intensity of the solution at 530 nm with excitation at 508 nm (for FIAsh) or emission at 608 nm with excitation at 597 nm (for ReAsH). This is best done continuously, for example every 1 or 5 s.
- vii. Add 2.5 μ L of 1 mM FIAsh-EDT₂ or ReAsH-EDT₂ to give a final concentration of 1 μ M. Mix well and continue to measure the fluorescence at the appropriate wavelength for 5–10 min. If there is a noticeable decrease in fluorescence during this period, this indicates there has been some loss of EDT from the biarsenical.
- viii. Add 10 μ L of 10 mM tetracysteine peptide to the cuvet and mix well. The fluorescence will increase rapidly but will take up to 1 hour to plateau as the complex forms.
- ix. Measure the percent fluorescence of the FIAsh-EDT₂ or ReAsH-EDT₂ compared to that of the complex with peptide after subtracting the baseline fluorescence (before addition of the biarsenical).

Troubleshooting

1. Heavy emulsions when washing toluene extract with water:

Heavy emulsions are usually formed but these can be broken by adding ~10 ml of saturated NaCl solution to the funnel. Do not reshake the funnel. Separate the two layers as much as possible and repeat the washing with water step twice, each time breaking the emulsion as described. Note that this step produces heavy precipitation of salts that are best carried through the extraction rather than removed before by filtration. Dry the combined toluene layers with anhydrous Na₂SO₄.
2. FIAsh-EDT₂ or ReAsH-EDT₂ that have lost EDT. Excess fresh EDT can be added to DMSO solutions of FIAsh and ReAsH that have lost EDT but reaction is incomplete and repurification is necessary (addition of EDT occurs quickest at pH~7 so mixing the DMSO solution with an aqueous buffer might be advantageous). However, repeating the synthesis is probably the best option.
3. No solid on washing evaporated FIAsh-EDT₂ or ReAsH-EDT₂ with ethanol. This may result from incomplete removal of toluene from the pooled fractions, so re-evaporate (ethanol and toluene form an azeotrope aiding the removal of residual toluene) and re-add ethanol. If still no solid is formed either before or after adding a little fresh cold ethanol; chill in ice and scratch glass with spatula. If no solid after a few hours, re-evaporate and dissolve residue in DMSO. Measure concentration of biarsenical by absorbance (see expected results)
4. TLC analysis of chromatography fractions and purified products gives baseline material in addition to that with expected mobility. This reflects breakdown of the products under the TLC conditions rather than impure fractions. For example, simply spotting pure FIAsh-EDT₂ or ReAsH-EDT₂ on silica TLC plates leads to formation of the corresponding arsenoxides probably by hydrolysis of EDT and subsequent oxidation by air. Excess EDT prevents this so the problem only occurs during the chromatography step that removes free EDT. To assess the presence of any arsenoxides in a sample of FIAsh-EDT₂ or ReAsH-EDT₂; dissolve in DMSO and

analyze by reverse-phase HPLC (see expected results for conditions; any arsenoxides will be eluted almost immediately).

5. Inability to get large fluorescence increases when FIAsh-EDT₂ and ReAsH-EDT₂ are mixed with tetracysteine peptides. See section (2. **Quality control of the products by measuring fluorescence before and after addition of tetracysteine tagged peptide**) for optimal conditions for this reaction. It is important to have 10 μ M EDT present in the assay as millimolar concentrations of monothiol such as 2-mercaptoethanesulfonate (MES) or 2-mercaptoethanol (BME) slowly react with the biarsenicals to form fluorescent products and thereby decrease the final enhancement on adding peptide. MES is preferred as millimolar BME can compete with the peptide for the biarsenicals, probably because the hydroxyl group can weakly bind to the arsenic in addition to the thiol. A slow decrease in fluorescence on adding the sample of biarsenical to this solution is indicative of a loss of EDT in the sample. Simply waiting until the EDT complex is reformed (fluorescence stops decreasing) before the addition of tetracysteine peptide should give the usual 1000–2000 fold increase in fluorescence upon peptide addition. If the fluorescence enhancement is smaller than this, fluorescent impurities or breakdown products (e.g. monoarsenicals or fluorescein or resorufin) are probably present and the sample should be repurified. Analytical HPLC (see analytical data) will also confirm their presence.
6. Stability and color changes on storage of FIAsh-EDT₂ and ReAsH-EDT₂ solutions in DMSO. Although stock solutions of the biarsenicals in EDT are stable at -20°C for many years (measured by HPLC), color changes of these (FIAsh and ReAsH solutions becoming redder and bluer respectively) are usually seen over time and even vary from aliquot to aliquot of the same solution. This probably reflects uptake of traces of moisture into the solutions resulting in more of the quinonoid form of FIAsh (rather than the lactone) and deprotonation of ReAsH. These changes have no effect on protein or cell-labeling experiments.

Anticipated Results

Analytical data—Fluorescein 4', 5'-bis(mercuric trifluoroacetate): ¹H-NMR: (D₆-DMSO) δ 6.48 (d, 2H, H-2'), 6.63 (d, 2H, H-1'), 7.23 (d, 1H, H-7, $J = 7.5$ Hz), 7.72 (m, 1H, H-6), 7.80 (m, 1H, H-5), 7.98 (d, 1H, H-4, $J = 7.5$ Hz), 9.98 (s, 2H, OH). ¹³C-NMR (d₆-DMSO): 110.81, 112.91, 116.50, 118.14, 120.35, 124.78, 125.44, 127.15, 130.18, 130.79, 136.24, 154.84, 159.12, 159.61, 160.09, 160.58, 162.41, 169.31. Storage : Stable at room temperature indefinitely but protect from light.

FIAsh-EDT₂: ¹H- NMR (CDCl₃) δ 2.3 (s, OH), 3.57 (m, -SCH₂CH₂S-), 6.60 (d, H-2'), 6.69 (d, H-1'), 7.19 (d, H-7), 7.66 (m, H-5, 6), 8.03 (d, H-4). ¹³C-NMR (d₆-DMSO): δ 42.50, 42.43, 83.95, 110.36, 113.30, 116.75, 124.82, 125.44, 126.91, 130.58, 130.90, 136.36, 152.54, 153.19, 161.77, 169.18. HPLC ES-MS (4.6 $\times$ 250mm Phenomenex Luna 2 C₁₈, 1ml/min of 10–90 % ACN-H₂O with 0.05% TFA linear gradient in 20 min then 90% for an additional 10min); retention time, 23.9 min +ve mode: (M + 1) 664.8. Calcd for C₂₄H₁₈As₂O₅S₄, 664.85. HR-ES 664.8536 (100%), 665.8565 (30%), 668.8508 (24%). Calcd [M+H]⁺ 664.8542 (100%), 665.8571 (30%), 666.8522 (24%). UV-Vis (10mM K.MOPS pH 7.2): λ_{max} 508 nm, λ_{max} 63500 M⁻¹ cm⁻¹; (0.1M NaOH): λ_{max} 496 nm, $\epsilon = 69500$ M⁻¹ cm⁻¹. Storage: Keep at -20°C . No deterioration detected in 10 years.

Resorufin 4, 5-bis(mercuric trifluoroacetate): ¹H-NMR: (D₆-DMSO) δ 6.97 (d, 2H, $J = 8.8$ Hz), 7.04, (s, 1H, OH), 7.26 (d, 2H, $J = 8.7$ Hz). Storage : Stable at room temperature indefinitely but protect from light.

ReAsH-EDT₂: ¹H-NMR (CDCl₃): δ 3.49 (m, 8H, S-CH₂), 6.22 (d, 2H, *J* = 8.3 Hz), 6.30 (d, 2H, *J* = 8 Hz). ¹³C-NMR (d₆-DMSO): δ 42.32, 110.94, 115.34, 117.71, 125.92, 144.02, 153.33. HPLC ES-MS (4.6× 250mm Phenomenex Luna 2 C₁₈, 1ml/min of 10–90 % ACN-H₂O with 0.05% TFA linear gradient in 20 min then 90% for an additional 10min); retention time, 21.4 min +ve mode: (M + 1) 545.8. Calcd for C₁₆H₁₃NO₃As₂S₄ 545.8. HR-ES 545.8283 (100%), 546.8313 (18%), 547.8253 (17%). Calcd [M+H]⁺ 545.8283 (100%), 546.8310 (20%), 547.8253 (19%). UV-Vis (0.1M NaOH): λ_{max} 579 nm, ε = 63000 M⁻¹ cm⁻¹. Storage: Keep at –20 °C. No deterioration detected in 8 years.

Quality control of the products by measuring fluorescence before and after addition of tetracysteine tagged peptide

i. Absorbance—ReAsH-EDT₂ has low solubility in aqueous solutions at pH 7 and FIAsh-EDT₂ can partially form the colorless lactone form. Therefore, FIAsh-EDT₂ and ReAsH-EDT₂ solutions are best quantified by reading the absorbance of a suitable dilution in 0.1N NaOH in which they are more soluble and any lactone forms are minimal. The measurement should be made immediately after addition to the base as the absorbance decreases with time. Note that under these conditions, EDT is lost from the biarsenicals and the product absorbs at slightly shorter wavelengths than the EDT complex.

ii. Fluorescence enhancement of FIAsh and ReAsH upon binding to a tetracysteine peptide—Typical preparations of FIAsh-EDT₂ and ReAsH-EDT₂ from this method have 0.05–0.1 % of the fluorescence of the tetracysteine complex. However, samples with higher values (up to 0.5%) were still suitable for cell staining. Typical time courses for the reaction of the biarsenicals with excess tetracysteine peptide are shown in Figure 3. Absorbance and emission spectra of the FIAsh and ReAsH complexes with a tetracysteine peptide are shown in Figure 4 and Figure 5 respectively.

REFERENCES

1. Tsien RY. The green fluorescent protein. *Annu. Rev Biochem* 1998;67:509–544. [PubMed: 9759496]
2. Giepmans BN, Adams SR, Ellisman MH, Tsien RY. The fluorescent toolbox for assessing protein location and function. *Science* 2006;312:217–224. [PubMed: 16614209]
3. Griffin BA, Adams SR, Tsien RY. Specific covalent labeling of recombinant protein molecules inside live cells. *Science* 1998;281:269–272. [PubMed: 9657724]
4. Adams SR, et al. New biarsenical ligands and tetracysteine motifs for protein labeling in vitro and in vivo: synthesis and biological applications. *J. Am. Chem. Soc* 2002;124:6063–6076. [PubMed: 12022841]
5. Martin BR, Giepmans BN, Adams SR, Tsien RY. Mammalian cell-based optimization of the biarsenical-binding tetracysteine motif for improved fluorescence and affinity. *Nat. Biotechnol* 2005;23:1308–1314. [PubMed: 16155565]
6. Gaietta G, et al. Multicolor and electron microscopic imaging of connexin trafficking. *Science* 2002;296:503–507. [PubMed: 11964472]
7. Martin BR, Deerinc TJ, Ellisman MH, Taylor SS, Tsien RY. Isoform-specific PKA dynamics revealed by dye-triggered aggregation and DAKAP1α-mediated localization in living cells. *Chem Biol* 2007;14:1031–1042. [PubMed: 17884635]
8. Poskanzer KE, Marek KW, Sweeney ST, Davis GW. Synaptotagmin I is necessary for compensatory synaptic vesicle endocytosis in vivo. *Nature* 2003;426:559–563. [PubMed: 14634669]
9. Tour O, Meijer RM, Zacharias DA, Adams SR, Tsien RY. Genetically targeted chromophore-assisted light inactivation. *Nat. Biotechnol* 2003;21:1505–1508. [PubMed: 14625562]
10. Gaietta GM, et al. Golgi twins in late mitosis revealed by genetically encoded tags for live cell imaging and correlated electron microscopy. *Proc. Natl. Acad. Sci. U. S. A* 2006;103:17777–17782. [PubMed: 17101980]

11. Tour O, et al. Calcium Green FAsH as a genetically targeted small-molecule calcium indicator. *Nat. Chem. Biol* 2007;3:423–431. [PubMed: 17572670]
12. Hoffmann C, et al. A FAsH-based FRET approach to determine G protein-coupled receptor activation in living cells. *Nat. Methods* 2005;2:71–76.
13. Keppler A, et al. A general method for the covalent labeling of fusion proteins with small molecules in vivo. *Nat. Biotechnol* 2003;21:86–89. [PubMed: 12469133]
14. Chen I, Howarth M, Lin W, Ting AY. Site-specific labeling of cell surface proteins with biophysical probes using biotin ligase. *Nat. Methods* 2005;2:99–104. [PubMed: 15782206]
15. Fernández-Suárez M. Redirecting lipoic acid ligase for cell surface protein labeling with small-molecule probes. *Nat Biotechnol* 2007;25:1483–1487. [PubMed: 18059260]
16. Griffin BA, Adams SR, Jones J, Tsien RY. Fluorescent labeling of recombinant proteins in living cells with FAsH. *Methods Enzymol* 2000;327:565–578. [PubMed: 11045009]
17. Cao H, Chen B, Squier TC, Mayer MU. CrAsH: a biarsenical multi-use affinity probe with low non-specific fluorescence. *Chem. Commun* 2006;24:2601–2603.
18. Spagnuolo CC, Vermeij RJ, Jares-Erijman EA. Improved photostable FRET-competent biarsenical-tetracysteine probes based on fluorinated fluoresceins. *J. Am. Chem. Soc* 2006;128:12040–12041. [PubMed: 16967933]
19. Bhunia AK, Miller SC. Labeling tetracysteine-tagged proteins with a SplAsH of color: a modular approach to bis-arsenical fluorophores. *Chembiochem* 2007;8:1642–1645. [PubMed: 17694522]
20. Cao H, et al. A red cy3-based biarsenical fluorescent probe targeted to a complementary binding peptide. *J. Am. Chem. Soc* 2007;129:8672–8673. [PubMed: 17585763]

Figure 1. Synthesis of FIAsh-EDT₂ and ReAsH-EDT₂; (a) HgO, TFA (b) AsCl₃, Pd(OAc)₂, DIEA, NMP (c) EDT, aqueous acetone.

Figure 2.

Structures of some other useful biarsenicals reagents and intermediates. CrAsH-EDT₂ (refs 4· 11· 17), fluorogenic biarsenicals and intermediate for CaG FIAsH and immobilized biarsenicals; sFIAsH-EDT₂ (ref 4) membrane-impermeable fluorogenic biarsenicals; F2-FIAsH (ref 18), photostable fluorogenic biarsenicals; CHoXAsH-EDT₂ (refs 4· 17), aggregator for BA-GFP; SpLAsH (ref, colorless and nonfluorescent biarsenicals for targeting other dyes e.g. Alexa594; CaG FIAsH (ref 11), targetable low-affinity Ca²⁺ indicator; AsCy3 (ref 20), fluorogenic biarsenical based on cyanine dye.

Figure 3. Typical time courses for the reaction of FIAsh-EDT₂ (green trace) and ReAsH-EDT₂ (red trace) with the tetracysteine peptide, FLNCCPGCCMEP (added at 700 s).

Figure 4. Absorbance (red trace) and fluorescence emission (blue trace) of FIAsh complex with FLNCCPGCCMEP.

Figure 5. Absorbance (red trace) and fluorescence emission (blue trace) of ReAsH complex with FLNCCPGCCMEP.