SUPPLEMENTAL INFORMATION

Specific biarsenical labeling of cell surface proteins allows fluorescent- and biotin-tagging of APP and prion proteins

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Supplemental Figure 1. Optimization of protocols for FlAsH labeling of cell surface TC-PrP.

(A) Confocal microscopy images of N2a cells expressing PrP(230TC*) and FlAsH-labeled using protocols without DTT. For the cells in the upper and middle panels, cells on glass bottom dishes were preincubated in 5 mM MES and 0.5 mM TCEP in MEM, labeled with 1 μM FlAsH in MEM for 1 hour at 37 ˚C, postincubated in 0.5 mM EDT in MEM for 30 minutes at 37 ˚C and then incubated in Disperse Blue in HBSS. For the cells in bottom panels, the labeling procedure was basically the same as the above two samples except that the ingredients of the preincubation medium were 5 mM MES, 0.5 mM TCEP and 0.5 mM EDT in MEM. For all the samples, FlAsH was preincubated with 12.5 mM of EDT for 10 minutes, diluted in 100 μl of HBSS and then added to the preincubation medium. The cells were then observed by confocal microscopy. Scale bars = 15 μm. MES+TCEP, cells preincubated in medium containing MES and TCEP. MES+TCEP+EDT, cells preincubated in medium containing MES, TCEP and EDT. Wt-PrP, N2a cells transfected to express wild-type PrP. TC-PrP, N2a cells transfected to express PrP(230TC*). DIC, differential interference contrast.

(B) Fluorescent gel analysis of FlAsH-PrP(230TC*) labeled with similar protocols as used in (A) with the indicated modifications. Note that none of the samples were deglycosylated. Cells stably-transfected with PrP(230TC*) were preincubated in 5 mM MES, 0.5 mM TCEP, 0.25 mM EDT in MEM for 40 minutes at 37˚C, labeled with 1.5 μM FlAsH in MEM for 1 hour either at 4˚C or 37˚C, post-incubated in 0.5 mM EDT in MEM for 30 minutes either at 4˚C or at 37˚C and finally incubated in Disperse Blue in HBSS for 15 minutes at room temperature (RT). The cells were rinsed twice with phosphate-buffered saline and harvested with TX100/DOC lysis buffer.

The protocol for TX114 lysate-derived samples (right panel) was basically the same as described above but with some modifications: preincubation with 5 mM MES and 0.5 mM TCEP in MEM; labeling with 1 μM FlAsH in MEM at RT for 45 minutes; postincubation in 0.5 mM EDT in MEM with 50% FBS for 30 minutes at RT. The lysate from one well of a 6-well plate was used for the sample per lane. TX114 lysates were phase-separated at 37 ˚C for 10 minutes and the aqueous phase was discarded. Then, after addition of 0.1% TX114 wash buffer, the lysate was phase-separated again and the aqueous phase was discarded again, proteins in the detergent phase were extracted by methanol/ chloroform precipitation. The extracted proteins were dissolved in sample buffer and finally boiled for 5 minutes. In whole cell lysate samples prepared with TX100/DOC lysis buffer to create a total cell protein extract, fluorescence suggestive of FlAsH-PrP was not identified even when the background was somewhat improved by labeling at 4^{o}C. After concentration of FlAsH-PrP (and other GPI-anchored proteins) from the detergent phase of a TX114 lysate, some fluorescence specific to TC-PrP-expressing cells was seen (indicated by the bracket). WCL, whole cell lysate samples prepared with TX100/DOC lysis buffer. TX114, samples prepared from detergent phase of TX114 lysates after phase-separation. Temp, temperatures at which the cells were labeled. TC+ or -, TC-PrP-transfected cells or non-transfected cells, respectively.

(C) Fluorescent gel analysis of cells labeled with one early protocol using DTT. "MES+TCEP" samples (lanes 1,4) were labeled with the same protocol as for "TX114-lysate sample" in (B) except that OptiMEM with 1% FBS was used for the preincubation and labeling media and OptiMEM with 50% FBS was used for the post-incubation media. For samples with "DTT" and "DTT+TCEP" (lanes 2-3 and 5-6) cells were preincubated in 10 mM DTT and 0.5 mM TCEP in OptiMEM with 1% FBS for 5 minutes at 4 ˚C, labeled with 1 μM FlAsH in OptiMEM with 1% FBS for 30

minutes (first 10 minutes at 4 ˚C then at RT to reduce cytotoxicity of DTT and low temperature), postincubated in 0.5 mM EDT in OptiMEM with 50% FBS (30 minutes, RT), and finally incubated in Disperse Blue in HBSS (10 minutes, RT). Cells confluent on 6-well plates were harvested immediately after labeling with 250 μl of TX114 lysis buffer and 12 μl of lysate was taken to prepare a sample without phase-separation. Therefore, 21-fold fewer cell equivalents/lane were loaded here as compared to (B), illustrating the increased efficiency of labeling in addition to the enhanced specificity. The bracket indicates the multiple glycoforms of FlAsH-labeled PrP(230TC*). Non-Tf, non-transfected cells. TC-PrP, TC-PrP transfected cells. MES, 2-mercaptoethanesulfonate. TCEP, tris-(2-carboxyethyl)phosphine. DTT, dithiothreitol.

Supplemental Figure 2. Washing PTA-precipitated pellets with 2% N-lauroylsarcosine buffer decreases FlAsH-PrPsen in PTA-precipitated fraction.

Although PTA precipitation can precipitate FlAsH-PrPres very efficiently, a considerable amount of FlAsH-PrPsen is also precipitated when TC-PrP expression levels were high such as in the PrP(230TC) cells. N2a(90TC) cells on 12-well plates were IDEAL-labeled with FlAsH and harvested immediately after labeling. The lysates were subjected to PTA precipitation and the pellets were sonicated in 100 μl of buffer (TX100/DOC lysis buffer with 0.25% phosphotungstate and 10 mM magnesium chloride) with the designated concentrations of N-lauroylsarcosine. Then they were centrifuged and samples were prepared from the final pellets. Sarc, concentrations of N-lauroylsarcosine in wash buffers. FlAsH-PrPsen was efficiently reduced by ~60-70% by sonicating the PTA pellet in 2% N-lauroylsarcosine-containing buffer and centrifuging again.

Supplemental Figure 3. The 21 kDa truncated PrPres band is produced by cysteine proteases.

The fluorescent gel compares FlAsH-PrP in PTA-precipitated fractions from 22L(90TC) cells incubated for 7 hours with or without cysteine protease inhibitor after IDEAL-labeling. (-), cells incubated without inhibitors. E64, cells treated with 40 μg/ml E64. Leu, cells treated with 20 μg/ml leupeptin. Arrow indicates full-length PrP. Arrowhead indicates 21 kDa truncated PrPres.

Supplemental Figure 4. Chymotrypsin preserves the 21 kDa PrPres band and efficiently digests FlAsH-PrPsen.

(A) The 21 kDa PrPres band is efficiently precipitated with PTA and partially PK-resistant. 22L(90TC) cells and N2a(90TC) cells were IDEAL-labeled with FlAsH and harvested 16 hours after labeling with TX114 or TX100/DOC lysis buffers. TX114, detergent phases of TX114-lysates. PTA, pellets of TX100/DOC lysates after PTA precipitation. PK+ or -, samples with or without PK digestion before PTA precipitation, respectively. Note that the scrapie infection-specific 21 kDa PrPres band is efficiently precipitated by PTA (lane 4 vs. 5, arrowhead). Although some resistance to PK digestion is detected, the intensity of the 21 kDa PrPres band is greatly diminished by PK digestion (lane 5 vs. 6).

(B) Full-length FlAsH-PrPsen is completely digested to undetectable levels at relatively low chymotrypsin concentrations both in cell lysate and PTA-precipitated samples. N2a(90TC) cells on a 12-well plate were IDEAL-labeled with FlAsH and harvested immediately after labeling with TX100/DOC lysis buffer without protease inhibitors. The lysates were pooled, dispensed at 100 μl/tube, and digested with the designated concentrations of chymotrypsin for 30 minutes at 37 ˚C. The digestion was stopped with Pefabloc, 10 μl of cell lysate was set aside for the "Lysate" sample, and the rest of the lysate was used for PTA-precipitated samples. Lysate, cell lysates. PTA, PTA-precipitated fractions. Chy, concentrations of chymotrypsin. The arrow indicates position of full-length FlAsH-PrP(90TC).

(C) Comparison between Chy-res and PK-res over time after FlAsH-labeling demonstrates enhanced detection of the 21 kDa PrPres band. 22L(230TC) cells on 12-well plates were IDEAL-labeled with FlAsH and harvested at designated periods of time with TX100/DOC lysis buffer without protease inhibitors. The lysates were digested either with 15 μg/ml of chymotrypsin or 15 μg/ml of PK at 37˚C for 30 minutes and the digestion was stopped with 2 mM Pefabloc. Then the lysates were subjected to PTA precipitation (without wash), PNGase F digestion, and fluorescent gel analysis. PTA, PTA-precipitated fractions. C, chymotrypsin-digested lysates. P, PK-digested lysates. The arrowhead indicates the 21 kDa PrPres band and the arrow indicates digestion products presumably derived from both FlAsH-PrPsen and FlAsH-PrPres. The graph shows average band intensity ± SEM (n=4).

Supplemental Figure 5. Specific cell surface labeling of PrP(230TC) with AF568-FlAsH. Stably-transfected N2a cells expressing PrP(230TC) were IDEAL-labeled with AF568-FlAsH prior to the treatments described below. (A) After removal of labeling medium, the cells were washed with ice cold HBSS and fixed immediately with pre-chilled 4% paraformaldehyde/5% sucrose (in PBS) for 30 min at RT. (B) After removal of labeling medium, the cells were placed in OptiMEM-I with 1% FBS for 5 minutes then OptiMEM-I with 10% FBS and maintained live. Samples were imaged by confocal microscopy. Panels represent selected individual confocal Z-slices and numbers indicate distance (μm) above the adherent surface of the cells. Images shown in (B) were captured about 22 minutes after initial exposure of cells to AF568-FlAsH and show the presence of intracellular, perinuclear PrP(230TC) (arrow) that is not detected in cells that were kept cold and fixed immediately (A). Scale bar = 10 μm.

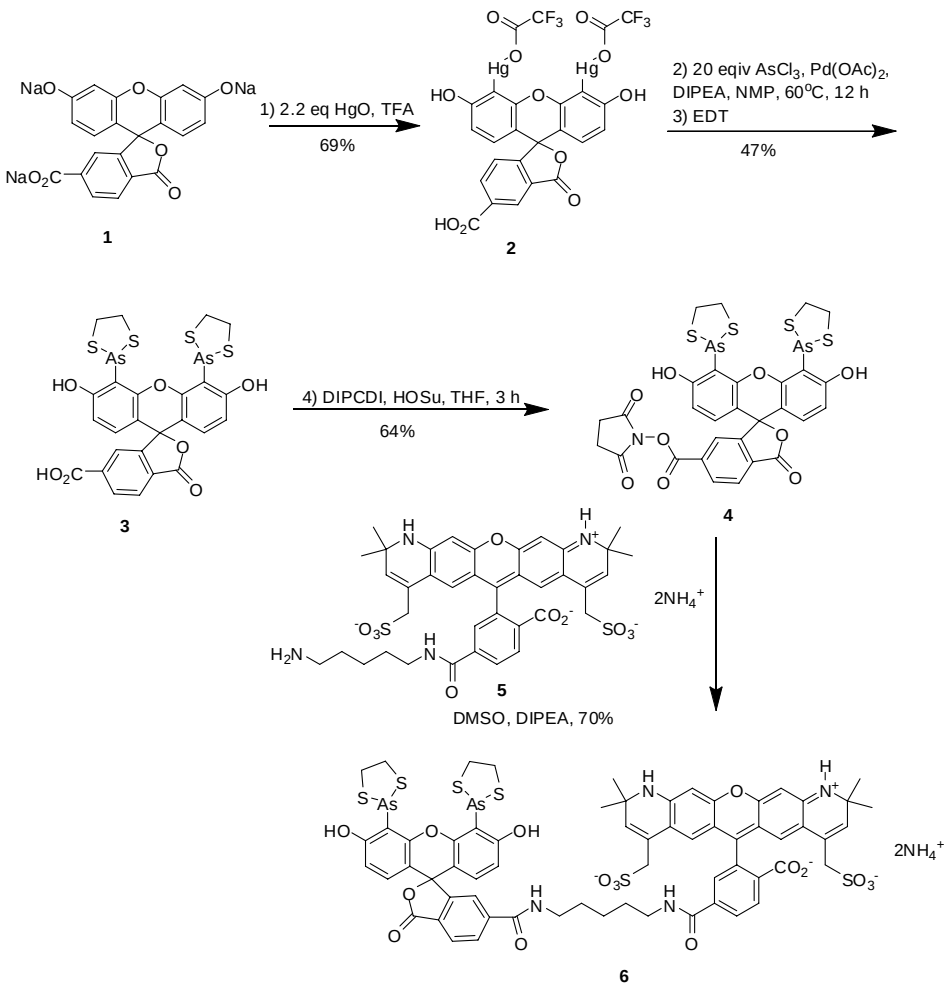
Supplemental Video 1. Endocytic trafficking of AF568-FlAsH-labeled PrP(230TC). Stably-transfected cells expressing PrP(230TC) were IDEAL-labeled with AF568-FlAsH and many (n=18) independent fields of view were subsequently monitored hourly by live cell confocal microscopy over the next 6 hours. Imaging was initiated 30 minutes after the start of IDEAL-labeling to correspond with the biochemical analysis described in Fig. 3D. A representative example of the events observed is shown. Images correspond to 3D volume renderings of the entire cell volume at each time point. A single confocal Z-slice from each time point in this series is shown in Fig. 5E. The movie shows the progressive intracellular accumulation of labeled PrP(230TC) in perinuclear endocytic vesicles with a concomitant decrease in cell surface PrP(230TC).

SUPPLEMENTAL METHODS

Synthesis of Alexa Fluor 568-FlAsH (AF568-FlAsH)

Chemistry

The synthesis of the 6-carboxy FlAsH-Alexa Fluor® 568 cadaverine conjugate (AF568-FlAsH) is outlined in Scheme 1. According to original published literature(Adams et al., 2002), mercuration of 6-carboxyfluorescein trisodium salt **1** with mercuric acetate produced a mono-, di-mercuration mixture (1:3). This proved troublesome in the next transmetalation step. Without purification of the complex, our yields of the desired arsenical product were <10% and involved tedious chromatography. This methodology was modified(Griffin et al., 1998) using an alternate substrate, dimercuric triflate **2,** which was readily prepared from the exclusive dimercuration of **1** using mercuric oxide and trifluoroacetic acid (TFA) in a yield of 69%. Transformation of dimercurate **2** to its diarsenical species was performed in the presence of arsenic trichloride and palladium acetate at 60^{o}C. Immediate treatment of **2** with excess 1,2-ethanedithiol (EDT) led to a 47% yield of intermediate **3**. Using 1,3-di-isopropylcarbodiimide (DIPCDI) and *N*-hydroxysuccinimide (NHS), the free carboxylic acid **3** was converted to the activated succinimidyl ester **4** with a 64% yield. The activated acid **4** was conjugated with Alexa Fluor® 568 6-carboxyl cadaverine to give the final imaging agent **6** in a 70% yield.



Scheme 1. Synthesis of 6-carboxy FlAsH-cadaveryl-Alexa Fluor® 568 conjugate

Experimental

General. Silica gel 60 F254 TLC plates were from Merck. Flash chromatography was done on an Analogix Intelliflash Workstation with a SuperFlash Sepra Si 50 SF column. Mass spectral analyses were done on an Agilent 1200 mass spectrometer and ^{1}H-NMR obtained on a Varian 400 MHz. Most starting materials are commercially available from Aldrich, while the Alexa Fluor® dyes are available from Invitrogen Corporation.

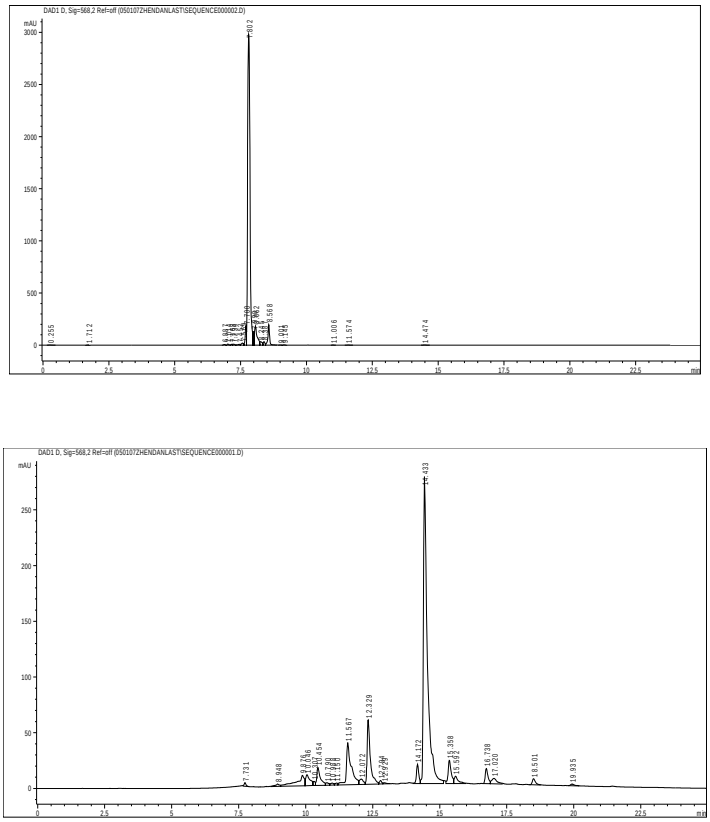
6-Carboxyfluorescein-4',5'-bis(mercuric trifluoroacetate) (2). 6-Carboxyfluorescein trisodium salt **1** (848 mg, 1.92 mmol) was added to a solution of mercuric oxide (917 mg, 4.22 mmol) in trifluoroacetic acid (10 mL). After stirring for 24 hours, the yellow mixture was evaporated and diluted with water (50 mL). The precipitate was collected by filtration and dried *in vacuo* over phosphorus pentoxide (P_2O_5) to constant weight, to give **2** as a red powder. Yield, 1.32 g (69%). Mp: >300°C; 1H NMR (D_2O+Na_2CO_3, 400 MHz) 8.01 (dd, 1 H, J = 1.6 Hz, J = 8.0 Hz), 7.79 (d, 1 H, J = 8.0 Hz), 7.68 (s, 1H), 7.13 (d, 2H, J = 9.2 Hz), 6.64 (d, 2H, J = 9.2 Hz).

4',5'-Bis(1,2,3-dithioarsolan-2-yl)-fluorescein-6-carboxylic acid (3). Compound **2** (502 mg, 0.50 mmol) was suspended in anhydrous *N*-methyl-2-pyrrolidone (NMP) (6 mL) under an argon atmosphere. To this suspension was added arsenic (III) trichloride (0.85 mL, 10 mmol), palladium (II) acetate (40 mg) and *N,N'*-diisopropylethylamine (DIPEA) (0.70 mL, 4.0 mmol). The orange suspension quickly changed to a clear yellow solution. The resulting solution was stirred at 60°C for 13 hours as it turned into a dark red suspension. This suspension was poured into a mixture of aqueous potassium phosphate buffer (125 mL, 0.2 M, pH = 7) and acetone (125 mL) and treated with ethanedithiol (EDT) (2.5 mL). After stirring for 30 minutes, chloroform ($CHCl_3$) (125 mL) and acetic acid (AcOH) (6 mL) were added to the heavy brown mixture and the suspension was stirred for an additional 1 hour before separation. The aqueous layer was extracted with $CHCl_3$ (2 X 125 mL). The combined organic layer was washed with 5% AcOH (100 mL) and dried over anhydrous sodium sulfate (Na_2SO_4). Evaporation of the solvents gave an orange residue which was purified by flash chromatography using a gradient of 0.5% AcOH in toluene to 0.5% AcOH in toluene:ethyl acetate (6:1) to obtain product **3** as a pale pink solid. Yield, 168 mg (47%). [R_f 0.36 in 0.5% AcOH toluene:ethyl acetate (1:1)]. Mp: 184-185 °C. ^{1}H NMR (d^6-dimethylsulfoxide (DMSO), 400 MHz) δ 13.60 (br, 1H), 10.64 (s, 2H), 8.22 (dd, 1H, J = 1.0 Hz, J = 8.0 Hz), 8.20 (d, 1H, J = 8.0 Hz), 7.64 (s, 1H), 6.59 (d, 2H, J = 8.6 Hz), 6.54 (d, 2H, J = 8.6 Hz), 3.35-3.26 (m, 8H); MS (m/z): 708.9 $(M-H)^-$.

4',5'-Bis(1,2,3-dithioarsolan-2-yl)-fluorescein-6-carboxylic acid, succinimidyl ester (4). Compound 3 (60 mg, 84 μmol) was dissolved in anhydrous tetrahydrofuran (THF) (8 mL) under an argon atmosphere. To the solution was added DIPCDI (34 μl, 100 μmol) and NHS (24 mg, 100 μmol). After 3 hours, the mixture was evaporated and the residue was purified by flash chromatography using a gradient of 0.5% AcOH in toluene:ethyl acetate (10:1 to 4:1) to give product **4** as an orange solid. Yield, 43 mg (64%). R_f 0.53 in 0.5% AcOH in toluene:ethyl acetate (1:1). ^{1}H NMR ($CDCl_3$, 400 MHz) δ 9.98 (s, 2H), 8.38 (d, 1H, J = 8.0 Hz), 8.14 (d, 1H, J = 8.0 Hz), 7.95 (s, 1H), 6.59 (d, 2H, J = 8.8 Hz), 6.55 (d, 2H, J = 8.6 Hz), 3.65-3.53 (m, 8H), 2.90 (s, 4H); MS (m/z): 805.9 $(M+H)^+$.

Coupling to Alexa Fluor® 568 6-carboxycadaverine. Compound **5** (4.3 mg, 5.3 μmol) was dissolved in DMSO (250 μL). To the solution was added Alexa Fluor® 568 6-carboxycadaverine (6.0mg, 7.4 μmol) and DIPEA (1.4 μL, 8.0 μmol). The resulting solution was shaken for 24 hours at room temperature and monitored by TLC and HPLC until completion. The solution was purified *via* preparative TLC (developed in CH_2Cl_2/MeOH (2:1) system) to give product **6** as a red solid. Yield, 5.6 mg (70%). R_f 0.47 in CH_2Cl_2:MeOH (1:1).

HPLC Analysis of the Final Product. Analysis of the final product was performed using an Agilent 1200 Series High Performance Liquid Chromatography System. A = 0.05% TFA in water and B = 0.05% TFA in acetonitrile. An Agilent XDB-C_{18}, 3.5 μm, 3.0 x 150 mm column was used with a flow of 1.0 mL/min. A linear gradient from 5-85% acetonitrile was used with the Alexa Fluor® 568 cadaverine starting material eluting at 7.802 minutes and the product **6** eluting at 14.433 minutes, λ = 568 nm. Silica gel 60 F254 TLC plates were from Merck. Final HPLC traces are illustrated in Supplemental Figure 6. The overall yield was 14.5%.

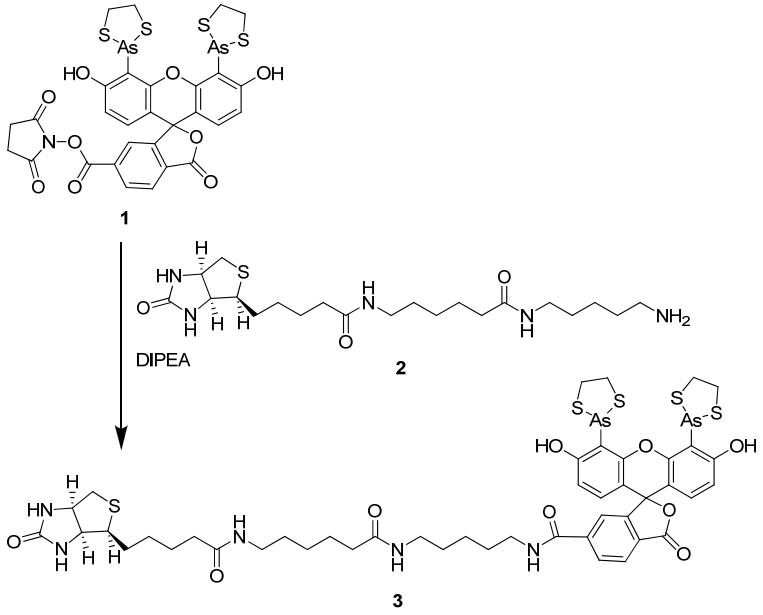


Supplemental Figure 6. HPLC elution traces of Alexa Fluor® 568 6-carboxycadaverine starting material (top) and the final product, the 6-carboxy FlAsH-cadaveryl Alexa Fluor® 568 (AF568-FlAsH) conjugate (bottom) measured at an absorbance of 568 nm. X-axis is time (minutes); y-axis is UV absorbance (arbitrary units) of eluting compound.

Synthesis of Bio-FlAsH

Chemistry

The preparation of the Bio-FlAsH conjugate was based on standard conjugation chemistries, as shown in Scheme 2, using the 6-hydroxysuccinimidyl ester of the FlAsH dye. Diisopropylethylamine (DIPEA) was used as base and a long-chain derivative of biotin (**2**) was used to generate Bio-FlAsH.



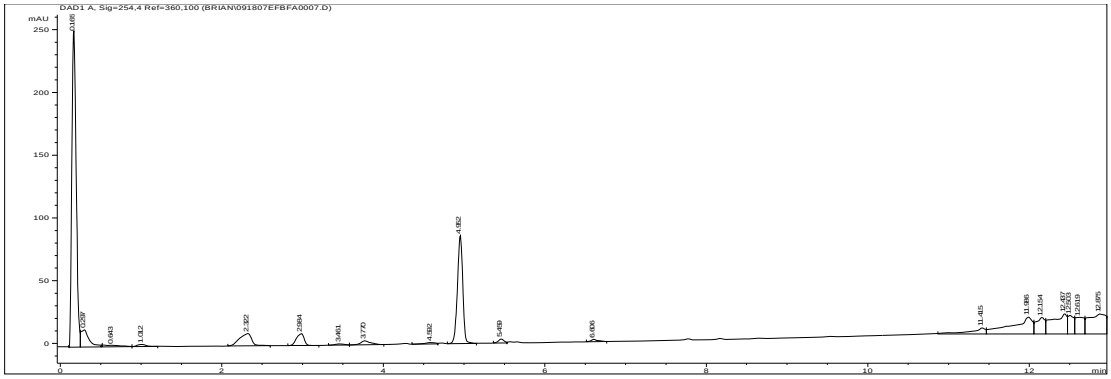
Scheme 2. Coupling of N-hydroxysuccinimidyl-FlAsH to amino derivative of biotin.

Experimental

General. Biotin-cadaverine was purchased from Invitrogen. Analytical Silica gel 60 F254 TLC plates were from Merck and Preparative Silica gel GF 2000μm 20x20cm plates from Analtech. Flash chromatography was done on an Analogix Intelliflash Workstation with a SuperFlash Sepra Si 50 SF column. HPLC analyses were performed using an Agilent 1200 Series High Performance Liquid Chromatography System. Mass spectral analyses were done on an Agilent 1200 mass spectrometer.

Coupling of FlAsH succinimidyl ester to biotin-cadaverine. Compound (**1**) (4.8 mg, 6.0 μmol) was dissolved in DMSO (150 μL). To this solution was added biotin-cadaverine (**2**) (3.3 mg, 6.0 μmol) and DIPEA (2.0 μL, 11 μmol). The resulting solution was shaken for 24 hours at room temperature. The solution was purified *via* preparative TLC (developed in CH_2Cl_2/MeOH, 5:1) to give product (**3**) as a pink solid. Yield, 1.6 mg (24%). MS (m/z): 1132.3 $[M+1]^+$. For HPLC analysis an Agilent XDB-C_{18}, 5.0 μm, 4.6x150 mm column was used with a flow

rate of 1.0 mL/min. Solvent A was 0.05% TFA in water and solvent B was 0.05% TFA in acetonitrile. A linear gradient from 25-75% acetonitrile was used with (**3**) eluting at 5.0 minutes, λ = 254 nm (Supplemental Figure 7).



Supplemental Figure 7. HPLC elution traces of Bio-FlAsH conjugate measured at an absorbance of 254 nm. X-axis is time (minutes); y-axis is UV absorbance (arbitrary units) of eluting compound.

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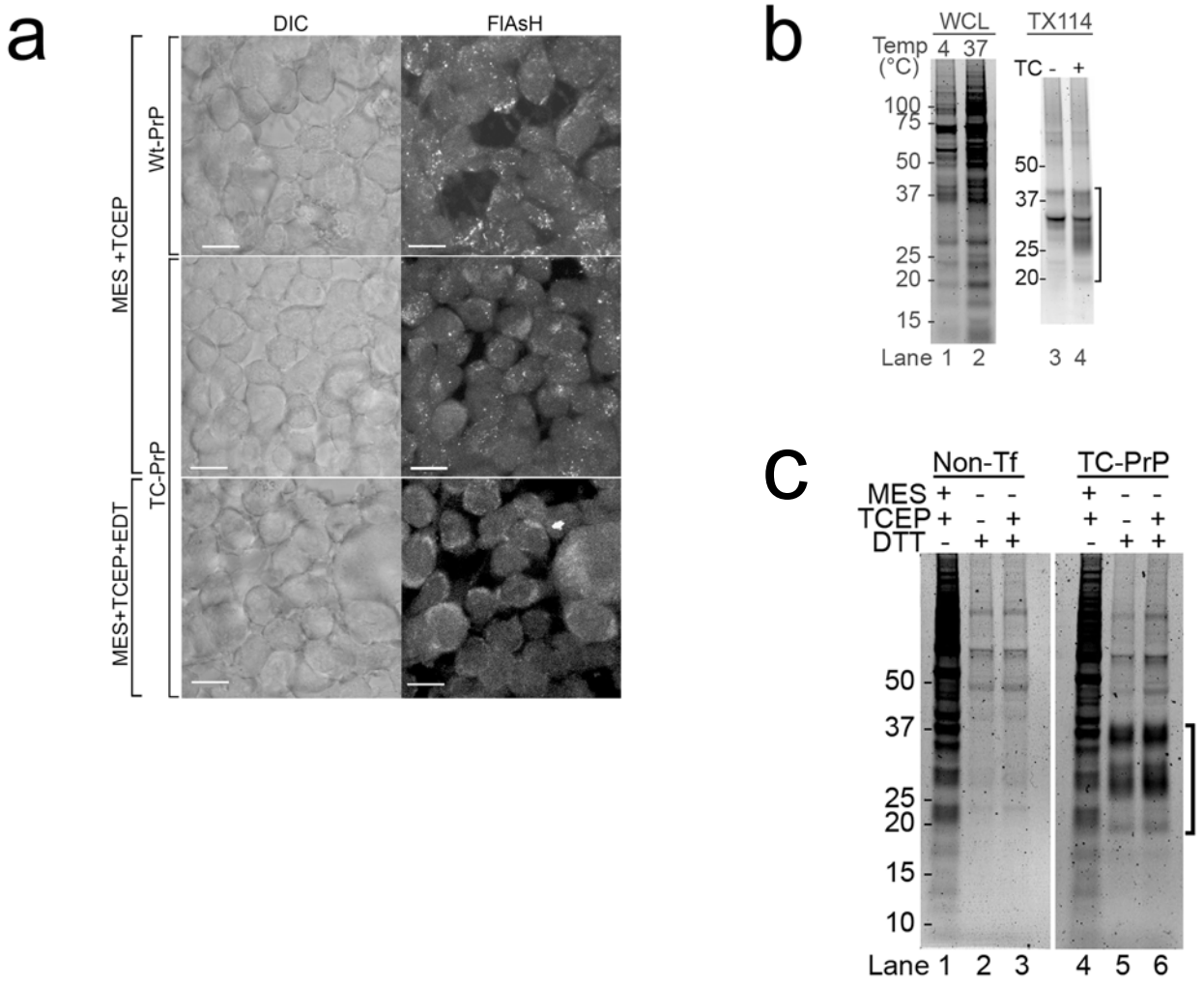
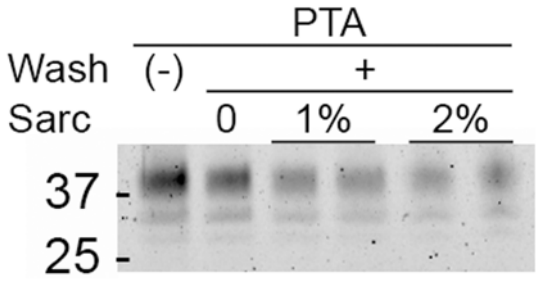
Griffin,B.A., Adams,S.R., and Tsien,R.Y. (1998). Specific covalent labeling of recombinant protein molecules inside live cells. Science *281*, 269-272.

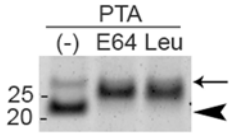
Supplemental Figure 1



Supplemental Figure 2

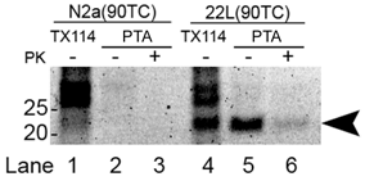


Supplemental Figure 3



Supplemental Figure 4

a



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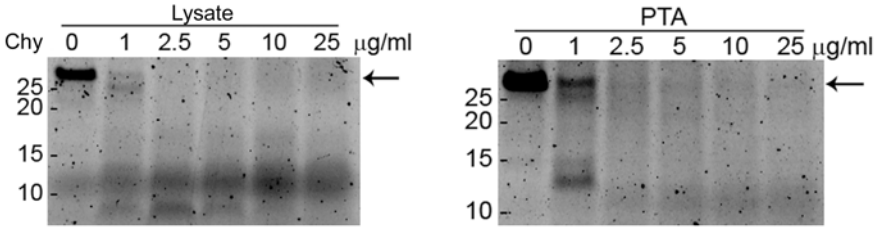

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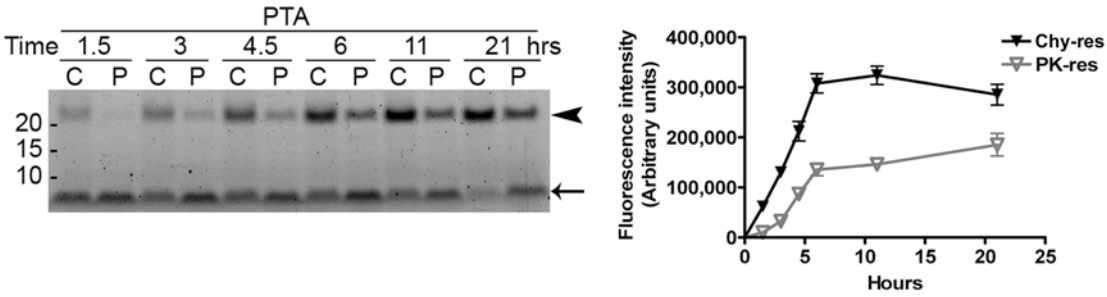

Supplemental Figure 5