Published in final edited form as:

Nat Protoc. 2013 November ; 8(11): . doi:10.1038/nprot.2013.128.

Quantification of free cysteines in membrane and soluble proteins using a fluorescent dye and thermal unfolding

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Abstract

Cysteine is an extremely useful site for selective attachment of labels to proteins for many applications, including the study of protein structure in solution by electron paramagnetic resonance (EPR), fluorescence spectroscopy and medical imaging. The demand for quantitative data for these applications means that it is important to determine the extent of the cysteine labeling. The efficiency of labeling is sensitive to the 3D context of cysteine within the protein. Where the label or modification is not directly measurable by optical or magnetic spectroscopy, for example, in cysteine modification to dehydroalanine, assessing labeling efficiency is difficult. We describe a simple assay for determining the efficiency of modification of cysteine residues, which is based on an approach previously used to determine membrane protein stability. The assay involves a reaction between the thermally unfolded protein and a thiol-specific coumarin fluorophore that is only fluorescent upon conjugation with thiols. Monitoring fluorescence during thermal denaturation of the protein in the presence of the dye identifies the temperature at which the maximum fluorescence occurs; this temperature differs among proteins. Comparison of the fluorescence intensity at the identified temperature between modified, unmodified (positive control) and cysteine-less protein (negative control) allows for the quantification of free cysteine. We have quantified both site-directed spin labeling and dehydroalanine formation. The method relies on a commonly available fluorescence 96-well plate reader, which rapidly screens numerous samples within 1.5 h and uses <100 µg of material. The approach is robust for both soluble and detergent-solubilized membrane proteins.

INTRODUCTION

The chemical versatility of the thiol moiety of cysteine lends itself to a range of chemical transformations of proteins. When coupled to site-directed mutagenesis^{1,2}, it allows site-specific labels or modifications to be introduced, in turn allowing the study of proteins in exquisite detail^{3,4}. The reactivity of cysteine to modification within a protein is subject to a number of variables such as temperature, buffer composition and structural context. Several methods have been developed that measure the efficacy of the transformation, including Ellman's assay and the maleimide-PEGylation assay⁵⁻⁷. With further modification, these protocols have now been extended to buried cysteines^{8,9}.

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AUTHOR CONTRIBUTIONS: E.B., C.P. and G.H. performed experiments. E.B., C.P., G.H. and J.H.N. analyzed the data. E.B., C.P. and J.H.N. wrote the paper.

COMPETING FINANCIAL INTERESTS: The authors declare no competing financial interests.

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Our approach uses a cysteine-reactive dye and heat-denatured protein to accurately quantify the amount of cysteine remaining in proteins after either spin labeling or dehydroalanine formation (Fig. 1a). This is an extension of a classic thermal fluorescence assay in which protein stability and ligand binding can be tested via heat-induced protein unfolding. In this assay, the unfolding temperature can be monitored by measuring the fluorescence of newly exposed tryptophan residues or fluorescent dyes that bind to either newly exposed protein hydrophobic regions or cysteine residues^{10–14} (Fig. 1b,c).

We have used the fluorescent dye DCIA (7-diethylamino-3-(4-(iodoacetyl)-amino)phenyl)-4-methylcoumarin), which contains a coumarin fluorophore and conjugates specifically with free cysteines in the protein¹⁵. The coumarin fluorescence only occurs after conjugation with thiols as the fluorescence is otherwise quenched¹⁵ (Fig. 1a). This assay uses only very small amounts of labeled protein combining both low protein concentration and sample volume. The small volumes allow trial-scale reactions of valuable membrane proteins to be carried out to develop experimental conditions. By denaturing the protein in the presence of the fluorescent dye, reaction rates are observed in real time directly from the fluorescence measurement (Fig. 1b,c). The fluorescence measurement at a particular temperature is directly quantitative and multiple measurements can be performed simultaneously, thereby providing controls and error estimates. An experimental run, including data evaluation, takes up to 3 h 30 min. The procedure can be seen as an updated Ellman approach using fluorescence rather than visible spectroscopy.

Application of cysteine modifications to the study of proteins

Site-specific introduction of cysteine not only can function as a chemical handle for the introduction of localized spectroscopic probes¹ and synthetic modifications⁴, enabling the use of biophysical techniques such as EPR spectroscopy³, but also can simplify the analysis of the diversity of possible functional outputs in biochemical assays of naturally occurring post-translational modifications¹⁶. In our research in which we characterize protein structure using EPR, the most commonly used label is MTSSL (S-(2,2,5,5-tetramethyl-2,5-dihydro-1H-pyrrol-3-yl) methylmethanesulfonylthioate), which contains a nitroxide radical and is attached via a disulfide bond to the cysteine. Measurement of the environment of the spin by continuous-wave (CW) EPR has been used to report on the structure of proteins³. Pulsed electron-electron double resonance (PELDOR) spectroscopy has proven to be powerful for the measurement of accurate distance measurements within highly symmetric labeled macromolecules or protein complexes^{7,17,18}. Its extension to membrane proteins (such as ion channels) has allowed testing of gating models and has been predicted to become an essential tool in membrane protein structural biology¹⁷. The quality of PELDOR data for multimeric ($n > 3$) proteins is particularly sensitive to the uniformity of spin labeling (the efficiency with which free cysteine is converted to labeled cysteine), although the precise relationship of signal to noise is complex^{7,17,18}. Knowledge of the labeling efficiency is important for subsequent data analysis including determination of its oligomerization state¹⁹. Even for simpler (two-spin) systems, poor labeling efficiency (<60%) requires compensating higher protein concentrations (which may be hard to achieve) to ensure sufficient signal-to-noise ratios²⁰. As a rule of thumb, an average spin-labeling efficiency of 60–70% is recommended²¹. For the integral membrane heptameric channel MscS, lower efficiencies of labeling led to uninformative data^{17,22}. Other experimental and sample variables can also undermine data quality; excluding incomplete labeling has allowed focused effort on these other experimental parameters.

The application of this thermofluor method satisfies the increasing need in the field of EPR to assess the extent of spin-labeling efficiency, in particular on membrane proteins, allowing many spin-labeled cysteine mutants to be tested. Moreover, the rapidity of the assay allows feedback and optimization of the labeling reaction while it is in progress. We used this

approach to adjust our labeling protocols for individual sites before embarking on time-consuming EPR experiments.

Protein modifications at cysteine via dehydroalanine

The chemical conversion of cysteine to dehydroalanine and the subsequent chemical addition of a nucleophile have been exploited to create stable protein modifications analogous to natural post-translational modifications^{4,16,23}. This approach has been useful for the study of an array of modifications that are otherwise dynamic in nature and therefore difficult to isolate from *in vivo* preparations⁴. These modifications control cellular processes such as DNA repair and replication, protein conformational change and transcription¹⁶.

Dehydroalanine can be formed using a dibromide reagent, α,β -di-bromoadipoyl(bis)amide, which performs a bis-alkylation elimination on cysteine to yield dehydroalanine²³. In our hands, the formation of dehydroalanine is slow (hours and, in some cases, days), and accurate and rapid monitoring to determine the extent of the reaction is essential, as protein with unreacted cysteines would give a different biochemical response, confounding downstream analysis; equally prolonged incubation also led to protein precipitation. We used the thermofluor method to determine the extent of conversion of cysteine to dehydroalanine.

Current methods for quantifying cysteine modification

The use of Ellman's reagent (5,5'-dithiobis-(2-nitrobenzoic acid)) was one of the first chemical methods for quantifying free thiol groups in proteins. The reagent, once conjugated to a free thiol moiety, gives rise to absorbance at 412 nm, which can be converted to cysteine concentration⁵. Although the technique is accurate for reactive cysteines, it does require substantial amounts of protein (e.g., >100 μ M, according to instructions from Thermo Scientific Pierce Protein Biology Products at <http://www.piercenet.com/instructions/2160311.pdf>), a major drawback for hard-to-purify proteins such as membrane proteins. Modifications to the reagent and the use of chemically denatured protein have been used to reduce (but not eliminate) its second important limitation: the bulky substituent may not react (or react only very slowly) with some cysteines, thus yielding an underestimate of their quantity^{8,9}.

The addition of maleimide-PEGylation relies on detecting modified protein on SDS-PAGE gels. The large PEG group slows the electrophoretic mobility of the protein. The PEGylated protein gives rise to a distinct band at a higher molecular weight on the gel⁶. The reactivity of the reagent with different cysteines is variable, and thus the same problems observed with Ellman's reagent can occur. The quantification of the extent of labeling relies on the digitization of bands on gels.

Liquid chromatography electrospray ionization mass spectrometry (LCT-ESIMS) relies on the ionization ability of the protein, which is directly linked to its detection efficiency. MS on either tryptic fragments or whole proteins is very powerful with respect to identifying the nature of the modification, and it requires very small amounts of sample²⁴. However, MS is not quantitative unless stable isotope labeling is used, and often some tryptic peptides are not detected²⁴. Further, LCT-ESI-MS analysis is challenging when detergent is present, as is very often the case for membrane proteins. Detergent ionizes readily and can mask the protein signal.

For EPR-active labels, double integration of the CW-EPR spectrum with a subsequent comparison with a known standard is an alternative method of quantifying spin-label modification in proteins^{25,26}. The accuracy is very sensitive to a number of experimental parameters such as the Q value (the ratio of energy stored in the resonator to the energy

dissipated by the resonator), sample volume, sample positioning, tubing diameter, and cavity and temperature, which all must be identical between multiple experimental measurements. This makes it both technically demanding and time-consuming.

Our approach overcomes many of the limitations imposed by other methods, such as requiring $<25 \mu\text{M}$ of protein. In addition, unfolding the protein by heat denaturation allows access to buried cysteines overcoming limitations of both the Ellman's and PEGylation assays. Unlike MS, the method quantifies the remaining free cysteine (thus yielding a reaction efficiency). Quantification is achieved by measuring the fluorescence obtained from the modified protein, the unmodified protein with 100% free thiol (positive control) and, if available, a cysteine-less protein (negative control), often the wild-type protein. Moreover, multiple measurements and replicate measurements can be monitored within the same assay plate.

Experimental design

Fluorescence measurement—Samples are loaded in a 96-well plate, and placed in the fluorimeter. The temperature is increased at a ramp rate of 1°C min^{-1} . For slowly reacting proteins, for which a plateau in the fluorescence maximum is not observed, a slower temperature ramp of $0.5^\circ\text{C min}^{-1}$ can be used. After each 1°C increment, the temperature is maintained while the samples are excited at a wavelength of 350 nm and emission is recorded at 440 nm. This is outside the region of the protein's absorption spectrum in order to avoid any background-noise signal from the protein itself. As the fluorescence emission signal intensity for each sample is recorded, it provides a fingerprint of fluorescence emission intensity versus temperature (Figs. 1c and 2).

The protocol was developed on multiple single-cysteine mutants of MscS (a heptameric membrane protein) and a single-cysteine mutant of soluble protein UbcH5a (a soluble monomeric E2 enzyme). To determine the efficacy of cysteine modification (Fig. 3a and Supplementary Table 1), we tested the modified cysteine mutant alongside unmodified cysteine-containing protein (positive control) and a cysteine-less variant (negative control). For MscS, we used a wild-type protein as the negative control, and for UbcH5a, we used a C85K mutant as the negative control. All samples were tested in duplicate or triplicate. Fluorescence measurements for quantification were recorded at the temperature at which the maximum fluorescence intensity was observed for the unmodified cysteine protein (positive control) incubated with the dye; this is denoted by a vertical black line in Figure 2. To our surprise, there were large variations in this measurement temperature between cysteine mutants (Fig. 2), possibly reflecting difference in chemical reactivity. We obtained full melting curves ($25\text{--}100^\circ\text{C}$) once for each cysteine mutant to monitor for artifacts. Two cysteine mutants completely reacted at 25°C , and no thermal unfolding appeared to be required.

To interpret the data, we assume that the difference between the fluorescence intensity of the positive (unmodified thiol) and negative control (no thiol) at the chosen temperature corresponds to 100% of free cysteine. The percentage of free cysteine remaining in the spin-labeled or dehydroalanine samples (that is, the spin-labeling or dehydroalanine efficiency) can be determined by a simple ratio (having subtracted the negative control; see equation in PROCEDURE Step 11; Fig. 3a).

Control experiments—The positive control (the corresponding unmodified cysteine) has to be used for each different mutant, as the coumarin fluorophore is itself sensitive to hydrophobicity and thus to the precise location of the label. This is an important factor particularly for cysteine mutants located in the transmembrane region of a membrane protein. Similarly, the same concentration of detergent in the modified sample must be used

in the control sample, as different detergent concentrations have an effect on the fluorescence intensity.

Second, cysteine mutagenesis can change protein stability and therefore produce very different melting curves for different mutants. It was our experience that the precise shape and initial fluorescence values of the curve varied from day to day, even for identical samples. We were unable to eliminate this variability, and we reasoned that it could arise from dye stocks, optics or plate imperfections. As quantification is based on the difference in fluorescence intensity, our method requires that the control samples are run at the same time on the same plate, thus removing the need for comparison between absolute values from different plates. The percentage of cysteine modifications derived from measurements did not change between plates.

We loaded samples into a fluorimeter in a 96-well plate, with control samples and test samples in the same row. When samples are run in triplicate, the contents of a typical assay plate followed that shown in Table 1. The negative control (cysteine-less protein) is particularly important the first time a new protein is tested. It reports fluorescence of the dye arising from nonspecific protein interactions. A large fluorescence output would undermine the accuracy of our method. We did not observe this in our experiments but cannot eliminate it as a possibility in other samples. It was these negative control samples that confirmed that the variation between plates was not simply sample dependence.

Validation of the method—We validated the fluorescence measurement of cysteine conversion using a second quantification method that is suitable for the protein and chemical transformation of cysteine. We carried out double integration of CW-EPR spectra for spin-labeled MscS mutants as an orthogonal quantification method to compare with fluorescence. We used MS to validate the fluorescence method for the conversion of cysteine to dehydroalanine. Materials and procedures for these methods are supplied in the Supplementary Methods.

We recorded CW-EPR spectra using identical CW-EPR parameters and sample volume (Supplementary Fig. 1). Spectra were baseline corrected and subsequently doubly integrated, thus yielding the number of spins for each sample with known MscS concentration. Consequently, the higher this number was, the higher the efficiency. The number of spins for each sample was then compared with 4-amino TEMPO standards of known concentration, which acted as the positive control (Fig. 3a and Supplementary Table 1). Errors were estimated on the basis of the s.d. of triplicate measurements, performed with identical EPR parameters.

We judged the chemical conversion of cysteine to dehydroalanine in UbcH5a by fluorescence to be ~81% complete. Whole-protein LCT-MS detected dehydroalanine and identified an intermediate product of the reaction, which corresponded to a covalent adduct of cysteine and the dibromide reagent (Fig. 3b). As this adduct, as in dehydroalanine, is unreactive with DCIA, the thermal fluorescence method cannot distinguish between them. In our experience with different batches of the dehydroalanine reaction, MS consistently underestimated cysteine content with the fluorescence assay.

Limitations—The assay can reliably quantify the amount of free cysteine in a sample in which cysteine has undergone modification and thus is no longer a reactive nucleophile. It does not distinguish between the different types of modified cysteine; for example, in the dehydroalanine experiment, only MS revealed the presence of the alkylated cysteine intermediate. Where one relies on multiple sequential reactions and the rate-determining step does not correspond to the initial removal of cysteine nucleophilicity, greater care is

needed in data interpretation. In the case of dehydroalanine formation, we found that prolonging the reaction with the dibromide reagent by 4 h with additional monitoring by MS was sufficient to deal with this.

MATERIALS

REAGENTS

- DMSO (Sigma-Aldrich)
- DCIA (7-diethylamino-3-((4-(iodoacetyl)-amino)phenyl)-4-methylcoumarin; Invitrogen)
- , -di-bromo-adipoyl(bis)amide (dibromide)
- NaCl (Sigma-Aldrich)
- TCEP (tris-(2-carboxyethyl)phosphine; Thermo Scientific)
- n-Dodecyl- β -D-maltopyranoside (DDM, a detergent; Anatrace)
- Sodium phosphate (Fluka)
- MscS
- UbcH5a
- Milli-Q water

EQUIPMENT

- Mx3000P 96-well plates, non-skirted (Agilent Technologies)
- Optically clear adhesive seals (Bio-Rad)
- NanoDrop UV spectrophotometer (Thermo Scientific)
- Stratagene Mx3005P fluorimeter

REAGENT SETUP

DCIA—Prepare a 10 mg ml⁻¹ DCIA solution in 100% DMSO stocks.

This solution can be stored at -20 °C for 24–48 h.

Proteins—To accomplish this protocol, you need the protein that is to be analyzed. You will mostly be interested in the protein that has been labeled at cysteine residues, but you will need to analyze unreacted control proteins (some of which may be mutants). Protein buffer components should be considered carefully. Avoid reducing agents that form a covalent adduct with cysteine. Avoid nucleophilic reagents that can react with DCIA yielding a false-positive fluorescence reading. Reducing agents cannot be incorporated into the buffer of MTSSL-labeled proteins, as this will cleave the label from cysteine. Reagents involved in the modification of cysteine with spin labeling or dehydroalanine must be removed from the sample before the assay, as they will compete with the fluorescent dye to react with cysteine.

In our work, we used single-cysteine mutants, labeled cysteine mutants with MTSSL, and wild-type MscS. These were solubilized in DDM as described previously²⁷. A single-cysteine-containing UbcH5a construct was provided by A. Plechanovova²⁸ (University of Dundee) and was purified as described earlier²⁸. Dehydroalanine was prepared according to a method described by Chalker *et al.*²³. MscS and UbcH5a with a cysteine concentration of

about 100–200 μM were prepared in their respective buffers (MscS: 0.05% (wt/vol) DDM, 50 mM sodium phosphate (pH 7.4) and 150 mM NaCl. UbcH5a: 0.05% (wt/vol) DDM, 50 mM Tris (pH 8.0), 150 mM NaCl and 0.5 mM TCEP). The protein concentration was measured using a NanoDrop UV spectrophotometer.

EQUIPMENT SETUP

StratageneMx3005P—The Stratagene instrument is setup with MxPro software. Turn on the instrument 20 min before the experiment to allow the UV lamp to warm up.

Stratagene software—The fluorescence profile for each sample is obtained by using MxPro software. Choose the SYBR Green (with dissociation curve) experiment. Collect fluorescence data with Alexa Fluor dye filters (excitation: 350 nm; emission: 440 nm). Set up a thermal profile with 30 s at 25 °C, followed by 75 cycles of 1 or 2 min with a 1 °C increment per cycle between 25 °C and 99 °C; end with 1 min at 25 °C. Three emission intensity values are recorded at the end of each 1 °C increment. Average data are recorded using Excel (Microsoft Office).

PROCEDURE

Thermofluorescence assay TIMING 2–3 h

1. Dilute DCIA 100-fold in the corresponding protein buffer (MscS: 0.05% (wt/vol) DDM, 50 mM sodium phosphate (pH 7.4), and 150 mM NaCl. UbcH5a: 0.05% (wt/vol) DDM, 50 mM Tris (pH 8.0), 150 mM NaCl and 0.5 mM TCEP).

CRITICAL STEP Cover DCIA solution in aluminum foil to prevent photobleaching.

TROUBLESHOOTING

2. Add buffer to the 96-well plate before adding protein and DCIA. The final volume in each well will be 130 μl once protein and DCIA are added (for some controls, neither or only one is added).
3. Add each protein to a final concentration of 25 μM to separate wells in the 96-well plate. Place the buffer and a negative and positive control on each row.

TROUBLESHOOTING

4. Add DCIA in a molar ratio of twofold excess of the proteins, and then cover the 96-well plate with an adhesive seal. Allow the plate to stand for 10 min at 4 °C to equilibrate the mixture.

CRITICAL STEP Cover the 96-well plate in aluminum foil to prevent photobleaching of DCIA.

5. Transfer the 96-well plate to the Stratagene Mx3005P fluorimeter and set up the instrument.
6. Subject the samples to thermal denaturation by heating them with increasing temperatures from 25 °C to 100 °C at a ramp rate of 1°C per 1 or 2 min.

TROUBLESHOOTING

7. Use an excitation wavelength of 350 nm and record the signal intensity of the light emitted at 440 nm for each well containing either buffer or protein sample. Use these data to generate thermofluor profiles of emission signal intensity at 440 nm versus temperature for each one of the samples. We do not use data from

temperatures above the point at which the fluorescence signal decreases, as this is an indication of protein precipitation and/or photobleaching.

TROUBLESHOOTING

Data analysis TIMING 30 min

8. Check the graph of observed fluorescence versus temperature for the modified and unmodified cysteine-containing proteins (positive control) and the cysteine-less protein (negative control) for artifacts.

TROUBLESHOOTING

9. Use a buffer control for each sample to account for any background-signal contribution and to check for any unexpected DCIA reaction with any buffer constituents or serious machine malfunction. Subtract the fluorescence signal from this control from the fluorescence signal of protein samples.
10. Correct the fluorescence of modified and unmodified protein by subtracting the fluorescence observed for the cysteine-less protein (usually wild-type protein and negative control).
11. The experiment measures three fluorescence values at the chosen temperature F_{mod} (the sample), F_{neg} control (usually wild-type, no cysteine) and F_{unmod} (unmodified cysteine). Calculate the labeling efficiency for each modified cysteine mutant as a ratio according to the equations below.

$$F_{\text{mod-corrected}} = F_{\text{mod}} - F_{\text{neg-control}}$$

$$F_{\text{unmod-corrected}} = F_{\text{unmod}} - F_{\text{neg-control}}$$

$$\text{Efficiency (\%)} = (1 - (F_{\text{mod-corrected}}) / (F_{\text{unmod-corrected}})) \times 100$$

12. Estimate the error by averaging the efficiency for three measurements on the same plate and by using the s.d. The controls are run in the same row for each sample.

TROUBLESHOOTING

Troubleshooting advice can be found in Table 2.

TIMING

Steps 1–7, thermofluorescence assay: 2–3 h

Steps 8–12, data analysis: 30 min

ANTICIPATED RESULTS

When both controls and modified cysteines are measured in the same assay plate at the same time, the ratio of these values allows the efficiency of spin labeling or dehydroalanine formation to be estimated. As fluorescence is temperature sensitive, the fluorescence values for samples and controls need to be compared at the same temperature. We chose the temperature to correspond to the highest fluorescence intensity observed for the positive control for each individual mutant. We did so on the basis of the assumption that this is the temperature at which all cysteines have reacted with the dye and at which thermal denaturation or decomposition, which decreases fluorescence, is minimized. We emphasize that the optimal temperature varies for each protein (even between mutants of the same protein) (Fig. 2). If necessary, less protein can be used in the assay as suggested in Table 2.

The results of a fluorescence experiment using this protocol are shown in Supplementary Figure 2.

In the UbcH5a positive control, without detergent the protein precipitated quickly during the assay resulting in a low fluorescence signal, whereas addition of the detergent gave better data. The L124C, mutant of MscS, was most poorly labeled with MTSSL, as estimated by both the thermofluor method and CW-EPR (Fig. 3 and Supplementary Table 1). The crystal structure shows that this mutant is inaccessible. All other cysteine mutants of MscS showed a labeling efficiency of ~90% by the thermofluor method (Fig. 3 and Supplementary Table 1). MS data for dehydroalanine formation in UbcH5a showed the presence of monoalkylated, bisalkylated cysteine as well as dehydroalanine, and no appreciable signal for free thiol was observed (Fig. 3b). Thermofluor data indicated that 19% of free cysteines remained (Fig. 3a and Supplementary Table 1), a reminder that MS is not a reliable method of quantification. To ensure that there was no systematic error in the estimation of cysteine modification in UbcH5a by fluorescence, UbcH5a was also spin labeled with MTSSL, with the cysteine modifications being measured with both fluorescence and CW-EPR. Both measurements indicated complete reaction of UbcH5a with MTSSL, with the fluorescence intensity of the spin-labeled protein close to that observed in the negative control in the fluorescence assay.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We acknowledge the Biomedical Sciences Research Complex Mass Spectrometry and Proteomics Facility for mass spectroscopy analysis and assistance with writing the manuscript; B. Bode and R. Ward for discussing the manuscript; A. Plechanovova[†] at the University of Dundee for providing the plasmid for UbcH5a; and J. Chalker at the University of Oxford for providing the dibromide reagent. The UK Biotechnology and Biological Sciences Research Council (BB/H017917/1), the Wellcome Trust (WT081862) and EaStCHEM (E.B. studentship) funded this work.

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Figure 1. Schematic diagram of the thermofluor assay

(a) Reaction of DCIA with cysteine within a protein, showing fluorescence emission at 440 nm upon conjugation with cysteine and excitation at 350 nm.

(b) Diagram of protein unfolding upon heat denaturation from 25 to 100 °C during the thermofluor assay. MscS is shown in blue. DCIA and the position of the cysteine mutation are denoted by green and red spheres, respectively.

(c) Diagram of a typical thermofluor profile, plotting temperature against fluorescence emission and a sigmoidal melting curve in red. After unfolding the proteins with heat, buried unreacted cysteines are revealed and react with DCIA yielding a DCIA-protein conjugate, which fluoresces and is detected at 440 nm.

Figure 2. Thermofluor profile

(a–f) Thermofluor profiles for each spin-labeled MscS mutant, denoted by R1 in a–e, and dehydroalanine containing UbCH5a, denoted by DHA in f. Each profile shows the negative controls in blue, the positive controls in red and the spin-labeled or dehydroalanine-modified mutants in green. Repeat measurements are denoted with (1) a smooth line, (2) a dashed line and (3) a dotted line. The temperature at which the fluorescence intensity was used is indicated for each mutant with a black line. The intersections of the black line with the blue, red and green lines correspond to F_{neg} control, F_{unmod} and F_{mod} , respectively. WT, wild type.

A

B

Figure 3. Comparison of thermal fluorescence with CW-EPR-derived efficiencies of cysteine modification and MS

(a) Bar chart comparing percentage spin-labeling efficiencies measured by fluorescence (blue) and CW-EPR (green) for each labeled MscS mutant. Dehydroalanine formation efficiency by fluorescence is shown in yellow. Percentage efficiency of cysteine turnover by fluorescence is calculated as described in the PROCEDURE and the average value is reported in this graph. The error bars show means $\pm$ s.d.

(b) Chemical structures and calculated masses of cysteine, dehydroalanine and the alkylated cysteine side product of the dehydroalanine reaction at position 85 in UbcH5a. Mass spectra of UbcH5a (containing the cysteine mutant) and dehydroalanine, analyzed by ESI-MS on a Micromass LCT mass spectrometer.

Table 1
Typical assay plate.

	Buffer Control			Negative Control			Positive Control			Modified Cystein		
Mutant 1	1	2	3	1	2	3	1	2	3	1	2	3
Mutant 2	1	2	3	1	2	3	1	2	3	1	2	3
Mutant 3	1	2	3	1	2	3	1	2	3	1	2	3
Mutant 4	1	2	3	1	2	3	1	2	3	1	2	3
Mutant 5	1	2	3	1	2	3	1	2	3	1	2	3
Mutant 6	1	2	3	1	2	3	1	2	3	1	2	3
Mutant 7	1	2	3	1	2	3	1	2	3	1	2	3
Mutant 8	1	2	3	1	2	3	1	2	3	1	2	3

Table 2
Troubleshooting table.

Step	Problem	Possible reason	Solution
1	Insolubility of dye in buffer	Protein buffers are incompatible with dye	Addition of DMSO up to a final concentration of 5–10% (vol/vol) in the assay may be required to keep dye soluble
3	Small quantity of protein to test	Low-yielding membrane protein; smaller-scale reactions	Use a lower concentration of protein (we used 10 μ M in 40 μ l as shown in Supplementary Fig. 2); adjust controls accordingly
6	Slow reaction with DCIA	Buried cysteine mutants within the transmembrane region of MscS	Extend the cycle time to 2 min to allow sufficient time for DCIA to react with cysteines
7	Low fluorescence signal	Protein precipitation; thermostable protein	Addition of 0.05% DDM (wt/vol) detergent to aid in unfolding of protein and eliminate protein precipitation
8	Decrease in fluorescence signal at high temperatures	Protein aggregation ¹² and photobleaching	Interpret data at an appropriate temperature before protein aggregation has occurred. In most cases, the thermofluorescence profile reaches a characteristic plateau after the highest fluorescence signal has been observed