Supporting Information

Cysteine Sulfoxidation Increases the Photostability of Red Fluorescent Proteins

Haiyan Ren[†], Bing Yang[†], Cheng Ma[‡], Ying Hu[§], Peng George Wang[‡], Lei Wang[†]*

[†] Department of Pharmaceutical Chemistry and the Cardiovascular Research Institute, University of California San Francisco, San Francisco, CA 94158, USA

[‡] Center for Diagnostics & Therapeutics and Department of Chemistry, Georgia State University, Atlanta, Georgia 30303, USA

[§] Nomis Center for Immunobiology and Microbial Pathogenesis, The Salk Institute for Biological Studies, La Jolla, CA 92037, USA

* Correspondence to: Lei.Wang2@ucsf.edu

Supporting Methods

Molecular cloning and mutagenesis

All plasmids were cloned in DH10β *E. coli* strain and confirmed by DNA sequencing. PfuTurbo (Stratagene, La Jolla, CA) was used for QuikChange reactions and Phusion (Finnzymes, Finland) for gene amplification reactions. Primers were synthesized by ValueGene (San Diego, CA) and are listed in Supporting Information. Restriction enzymes were purchased from New England Biolabs (Ipswich, MA). For protein expression in *E. coli*, wild-type mKate2 gene was amplified from pLei-tRNAopt-mKate2 [1] and cloned into the pBAD plasmid using *Spe* I and *Hind* III to afford pBAD-mKate2. A His6 tag was appended to the C-terminus for protein purification. mStable was similarly cloned into pBAD to afford pBAD-mStable for expression in *E. coli*. pBAD-mKate2(S143D) mutant was generated from pBAD-mKate2 through QuikChange. For expression in mammalian cells, mKate2 and mStable were respectively cloned into pmCardinal-Actin-C-18 or pmTagRFP-T-H2B-6 plasmids[2] for tagging human actin and human histone H2B, respectively. *Age* I and *Bgl* II were used to replace the mCardinal gene in plasmid pmCardinal-Actin-C-18, and *Age* I and *Not* I were used to replace the TagRFP gene in plasmid pmTagRFP-T-H2B-6. For expression in the mitochondrion, the genes were cloned into plasmid pKillerRed-dMito (Evrogen, Moscow, Russia) using enzyme sites *Age* I and *Not* I to replace the KillerRed gene.

Protein expression and purification

E. coli BL21 cells harboring the expression plasmid (pBAD-mKate2, pBAD-mKate2(S143D) or pBAD-mStable) were cultured at 37 °C in 2xYT medium containing ampicillin. Protein expression was induced in dark at 25 °C with arabinose (final concentration 0.2%) when the OD_{600} reached 0.4-0.5. After 16 hr, cells were collected and lysed in 30 mL lysis buffer (50 mM TrisHCl, pH 8.0, 500 mM NaCl, 20 mM imidazole, 1% (v/v) Tween 20, 10% (v/v) glycerol and 0.5 mg/mL lysozyme) with protease inhibitors. Lysed cells were centrifuged for 50 min at 180,000 g after sonication. Supernatant was loaded onto a Ni-NTA resin column (Qiagen), and the column was washed 3 times with washing buffer (lysis buffer without Tween 20 and lysozyme). Protein was then eluted with 1 mL of elution buffer (washing buffer containing 250 mM imidazole, pH 8.0). Imidazole was removed by dialysis. Protein induction and all purification steps were performed in dark.

Gel filtration chromatography

The oligomeric state of mStable was analyzed using a HiLoad 16/60 Superdex 200 gel filtration column in 25 mM HEPES, pH 7.0 with 150 mM NaCl (Pharmacia Biosystems) in FPLC at 4 °C. mStable fluorescent protein was injected into the column with a flow rate of 0.5 mL/min and absorbance was monitored at 280 nm. Gel filtration standard proteins were also loaded under the same conditions to calibrate the column. The oligomeric state of the mStable was determined based on the calibration plot and the monomeric mKate2 elution time. A single peak corresponding to monomeric mStable was detected. The eluted mStable protein was used for measuring its absorption and fluorescent properties.

Cell culture

HEK293T and HeLa cells were cultured at 37 °C and 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS), penicillin (100 units/mL) and streptomycin (100 mg/mL). Cells were passaged 3 times a week and seeded at a density of 75,000 cells/cm^2 and transfected the following day with 1 μg DNA (35 mm dishes) using Lipofectamine 2000 according to the manufacturer's protocol. Cells were imaged 24-36 hr after transfection.

Absorption and fluorescence spectroscopy

The absorption was measured on UVIKON XS Spectrophotometer in a 100 μL quartz microcuvette with black side walls (Hellma 105-251-QS, Hellma Ltd). Measurements were carried out in buffer solutions containing 50 mM Tris (pH = 8). Fluorescence spectra were measured on a Fluorolog-3 (Horiba Jobin Yvon) in the same cuvette. The emission spectra were recorded at the excitation wavelength of 600 nm, and the excitation spectra were recorded at the emission of 640 nm. Purified mStable kept in dark was measured to obtain fluorescence spectra before photoactivation. To photoactivate mStable for acquiring its fluorescence spectra after photoactivation, purified mStable was illuminated with white light (400-700 nm, 25 W/cm^2) for 7.5 min (30 s on, 30 s off).

Extinction coefficient and quantum yield measurement

The absorbance of protein sample was measured on UV-vis at its peak absorption wavelength. Protein concentration was determined by comparison of band intensities on Coomassie brilliant blue-stained SDS-PAGE using BSA of known concentrations as standards. The molar extinction coefficient ε was calculated using $\varepsilon = A/cl$, where A is the absorbance, c is the molar concentration, and l is the path length. The quantum yields of mStable and mCardinal were

determined by comparison of the absorbance and integrated fluorescence emission intensity relative to the WT mKate2 using the following equation: $QY_{sample} = (Emi_{sample}/Emi_{mKate2}) \times (Abs_{mKate2}/Abs_{sample}) \times QY_{mKate2}$, in which QY represents quantum yield, Emi represents integrated emission intensity, and Abs represents absorbance. To measure the QY of mStable after photoactivation, we photoactivated the mStable protein in a quartz cuvette for 30 s, measured its fluorescence emission, and determined the QY. This procedure was repeated at each time point, and the QY was plotted against the number of treatments. The QY of mStable increased with photoactivation time and reached the maximum QY of 0.17 (Figure S2).

Single molecule measurement

Glass bottom dishes (GBDs) with 35-mm No. 0 glass (MatTek P35G-0-20-C) were used for protein measurements. Prior to protein coating, GBDs were incubated with 1 M NaOH for 10 min, rinsed with MilliQ water for 1 min, and dried with filtered air. An aliquot of protein dilute (5-20 nM) was evenly coated onto the glass surface for 5 min. After brief air-drying, the GBDs with damp glass surface were promptly used for measurements. Single-molecule measurements were taken using an in-house total-internal reflection microscope (TIRF) setup. A 561 nm diode-pump solid-state laser (Cobolt Jive) was used as excitation. Typical excitation power was measured to be 22 mW before the back aperture of the objective lens. A Nikon Apo TIRF 60×, 1.49 oil immersion objective was used for imaging. Image acquisition was performed by an Andor iXon3 EMCCD camera. The exposure time was set to 40 ms and frame rate to 12 fps. A collection of time-lapsed image frames capturing the bleaching of single proteins was obtained for analyses. A script was written to identify individual bleaching events and calculate their lifetimes and brightness levels. In brief, the algorithm detects bright spots in an image frame at a particular time instance, and traces 50-100 frames before and after this time point. The integrated brightness level of the same pixel and its immediate neighboring pixels is plotted versus time. Human analyses were performed to select single-molecule events from these trajectories and to discard protein aggregates with multiple stages of bleaching. The lifetime was measured from the duration of the fluorescence emission and the brightness was registered as the averaged intensity of fluorescence emission with background removal.

Photobleaching measurements

Arc-lamp photobleaching of purified protein in aqueous microdroplets in PBS (pH = 7.4) were performed exactly as previously described[3]. Droplets of proteins were formed under mineral oil in a chamber, which is put on a wide field fluorescence microscope for imaging. Droplets with

size comparable to mammalian cells were selected for continuous illumination (580/20 nm), and their images (653/95 nm) were recorded at different time points to generate the bleaching curve.

Laser photobleaching was performed on a Zeiss light scanning microscope (LSM 700). Purified protein was deposited onto a nickel-nitrilotriacetic acid coated glass slide (MicroSurface Inc.) and incubated for 1 h at room temperature. The glass slide was washed 3 times using the protein buffer, covered with a glass cover slide and sealed with nail polish. Bleaching was achieved in conjunction with the acquisition of a series of images over time. Samples were bleached with full laser power (10 mW, 555 nm). The bleaching tool in the Zeiss ZEN2012 imaging software was used to characterize the fluorescence intensity decrease. For Rose Bengal treatment of mPlum and mPlum(S146C), purified proteins were incubated with 10 mM of Rose Bengal and sensitized with 540/40 nm of light for 5 min. The proteins were then separated from Rose Bengal using the PD10 column (GE Healthcare Life Sciences) and subjected to photobleaching measurement.

Live-cell photobleaching was performed on live HeLa or HEK293T cells using a laser-scanning confocal microscope (Zeiss LSM 700). Bleaching was achieved in conjunction with the acquisition of a series of images over time (excitation 555 nm, emission 600-700 nm). Full laser power (10 mW, 555 nm) was used to bleach the cells expressing mStable. To match the fluorescence emission intensity of other samples expressing mKate2 or mCardinal, the laser power was decreased so that the mean fluorescence intensity of the initial images were the same as that of mStable after photoactivation. Because mKate2 and mCardinal are brighter than mStable, the laser power used was ~60% for mKate2 and ~80% for mCardinal on average. The bleaching tool in the Zeiss ZEN2012 imaging software was used to characterize series images acquired over time to generate the bleaching curve. For singlet oxygen trapping experiment, HEK293T cells expressing H2B-mStable were incubated with 10 mM NaN_3 for 30 min. These cells were then photobleached using 75% laser power (7.5 mW, 555 nm) side-by-side with H2B-mStable expressing HEK293T cells that were not treated with NaN_3.

Structured-illumination microscopy

The Zeiss Elyra PS.1 Super-Resolution Microscope with an Andor iXon3 888 EMCCD camera (1024 x 1024, 9 fps) was used for actin imaging. Images were taken with a 100× oil-immersion objective, 10% laser power (20 mW, 561 nm) and 200 ms exposure time. The structure illumination tool in the Zeiss ZEN2011 imaging software was used to reconstruct the final SIM images. Each SIM image was reconstructed from 100 raw images. The final SIM images for mStable and mCardinal were shown on the same intensity scale.

CD spectra measurement

CD spectra were recorded on a JASCO spectrophotometer (J-810) at 25 °C. A quartz cell with 0.1 cm path length was used for range of 200–260 nm and 1 cm path length was used over the range of 500-650 nm. Protein concentrations for both samples were about 1×10^{-4} M for range 200–260 nm and 2 mM for measurement over 500-650 nm range in phosphate buffer (pH 7.4). Obtained CD spectra for range 200–260 nm were the average of six accumulations made at 100 nm/min. For range 500-650 nm, the average of eight accumulations made at 50 nm/min was used.

Supporting Table 1. Primer sequences

Name	Sequence
mKate2 S143C P1	5′-GGGTACAGGGTCTCGGTGCAGGCCTCCCAGCCGAGTG-3′
mKate2 S143C P2	5′-ACCGAGACCCTGTACCCCACTCGGCTGGGAGGCCTGC-3′
mKate2 S143D P1	5′-GGGTACAGGGTCTCGGTGTCGGCCTCCCAGCCGAGTG-3′
mKate2 S143D P2	5′-CACTCGGCTGGGAGGCCGACACCGAGACCCTGTACCC-3′
mKate2 SpeI P1	5′-CTCAACTAGTGTGAGCGAGCTGATTAAGG-3′
mKate2 Hind III P2	5′-CTCAAAGCTTTTAGTGATGGTGATGGTGATGAG-3′
H-mKate2 AgeI P1	5′-CTCAACCGGTCGCCACCATGGTGAGCGAGCTGATTAAGG-3′
H-mKate2 NotI P2	5′-CTCAGCGGCCGCTTATCTGTGCCCCAGTTTGCTAG-3′
A-mKate2 BglII P2	5′-CTCAAGATCTGAGTCCGGATCTGTGCCCCAGTTTGCTAG-3′
(Actin and H2B use the same P1)	

mStable DNA sequence:

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ATGGTGAGCGAGCTGATTAAGGAGAACATGCACATGAAGCTGTACATGGAGGGCACCGTGAACA
ACCACCACTTCAAGTGCACATCCGAGGGCGAAGGCAAGCCCTACGAGGGCACCCAGACCATGAG
AATCAAGGCGGTCGAGGGCGGCCCTCTCCCCTTCGCCTTCGACATCCTGGCTACCAGCTTCATG
TACGGCAGCAAAACCTTCATCAACCACACCCAGGGCATCCCCGACTTCTTTAAGCAGTCCTTCC
CCGAGGGCTTCACATGGGAGAGAGTCACCACATACGAAGACGGGGGCGTGCTGACCGCTACCCA
GGACACCAGCCTCCAGGACGGCTGCCTCATCTACAACGTCAAGATCAGAGGGGTGAACTTCCCA
TCCAACGGCCCTGTGATGCAGAAGAAAACACTCGGCTGGGAGGCCTGCACCGAGACCCTGTACC
CCGCTGACGGCGGCCTGGAAGGCAGAGCCGACATGGCCCTGAAGCTCGTGGGCGGGGGCCACCT
GATCTGCAACTTGAAGACCACATACAGATCCAAGAAACCCGCTAAGAACCTCAAGATGCCCGGC
GTCTACTATGTGGACAGAAGACTGGAAAGAATCAAGGAGGCCGACAAAGAGACCTACGTCGAGC
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A
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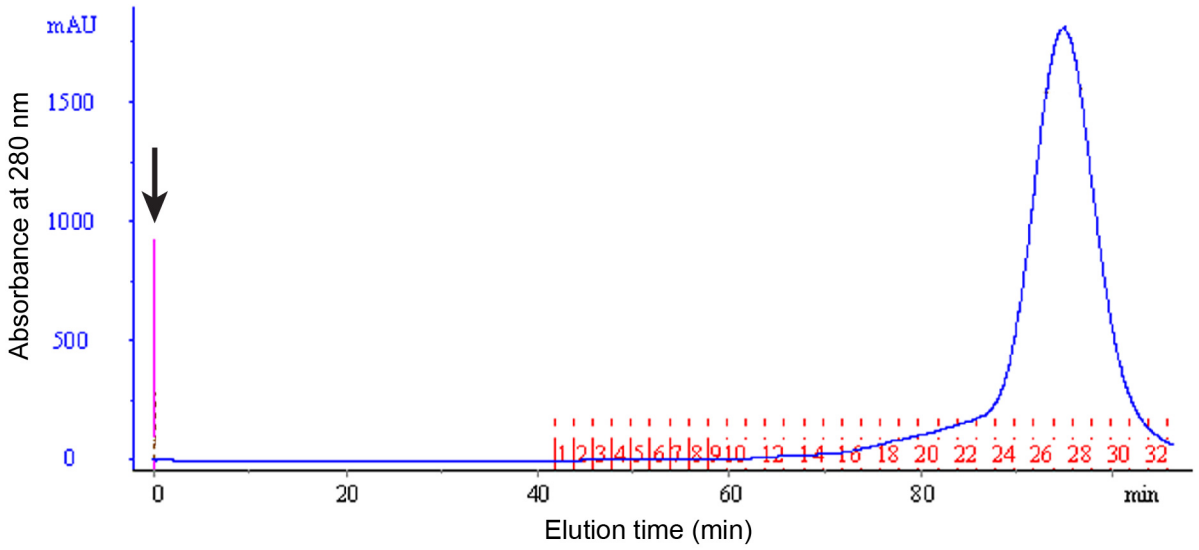


Figure S1. Gel filtration chromatography of mStable. Arrow indicates injection. The blue trace was the UV absorbance at 280 nm. Only a single peak corresponding to the monomer was detected.

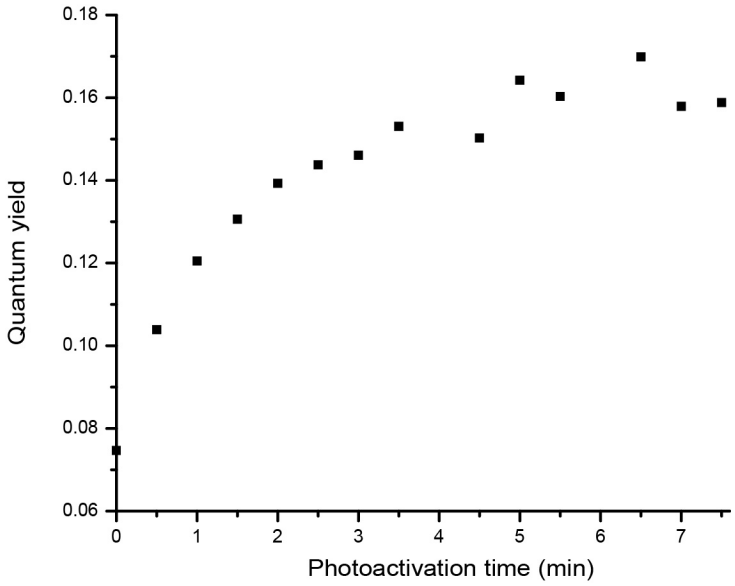
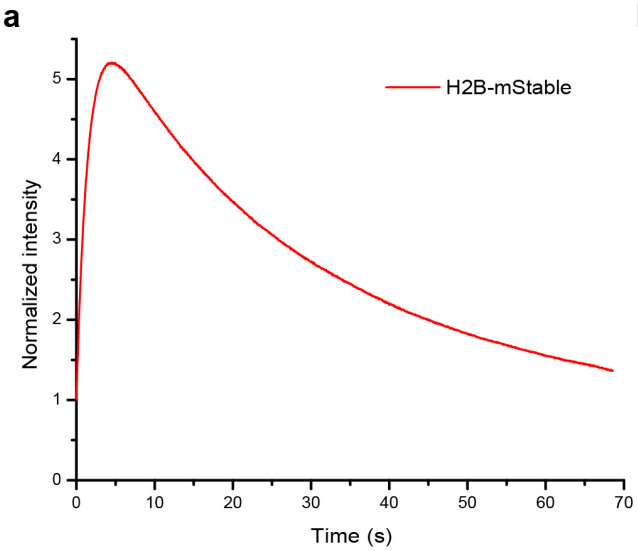
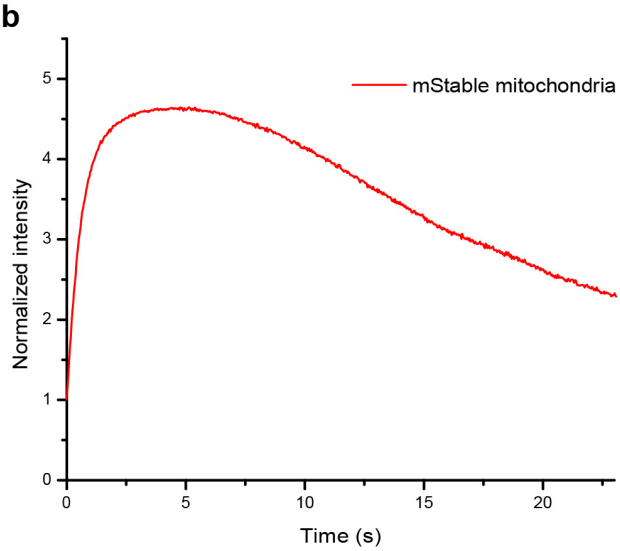
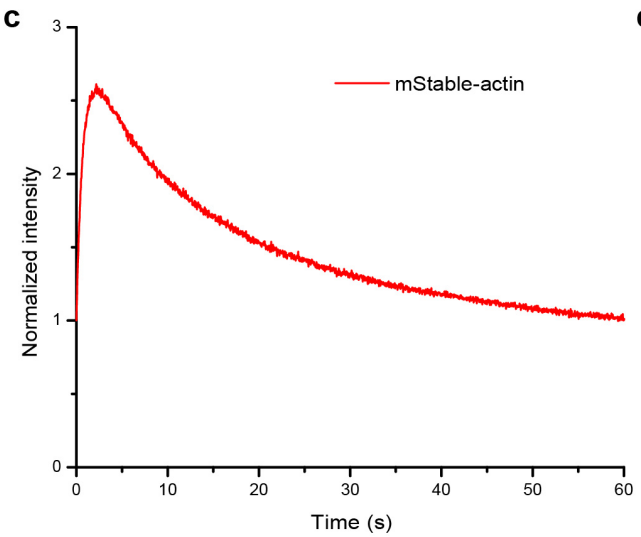


Figure S2. The quantum yield of mStable after photoactivation. Quantum yield was measured along with photoactivation until the maximum was reached.





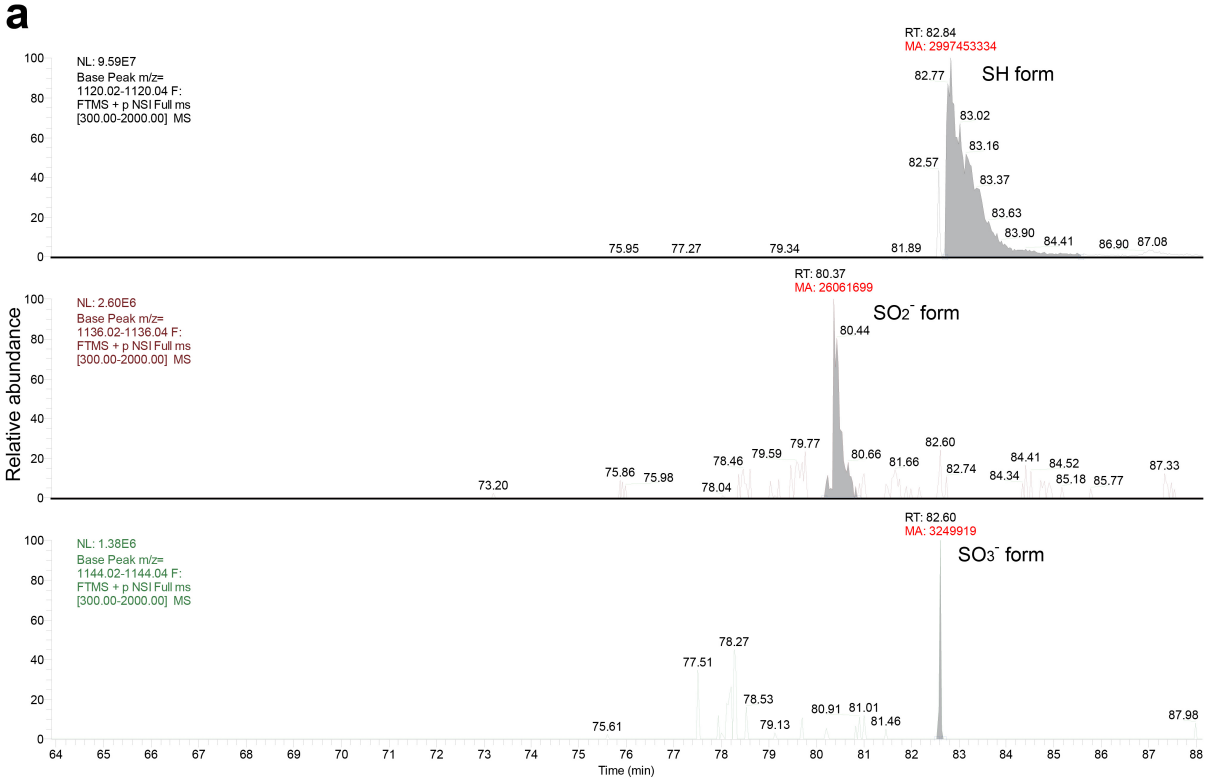


d

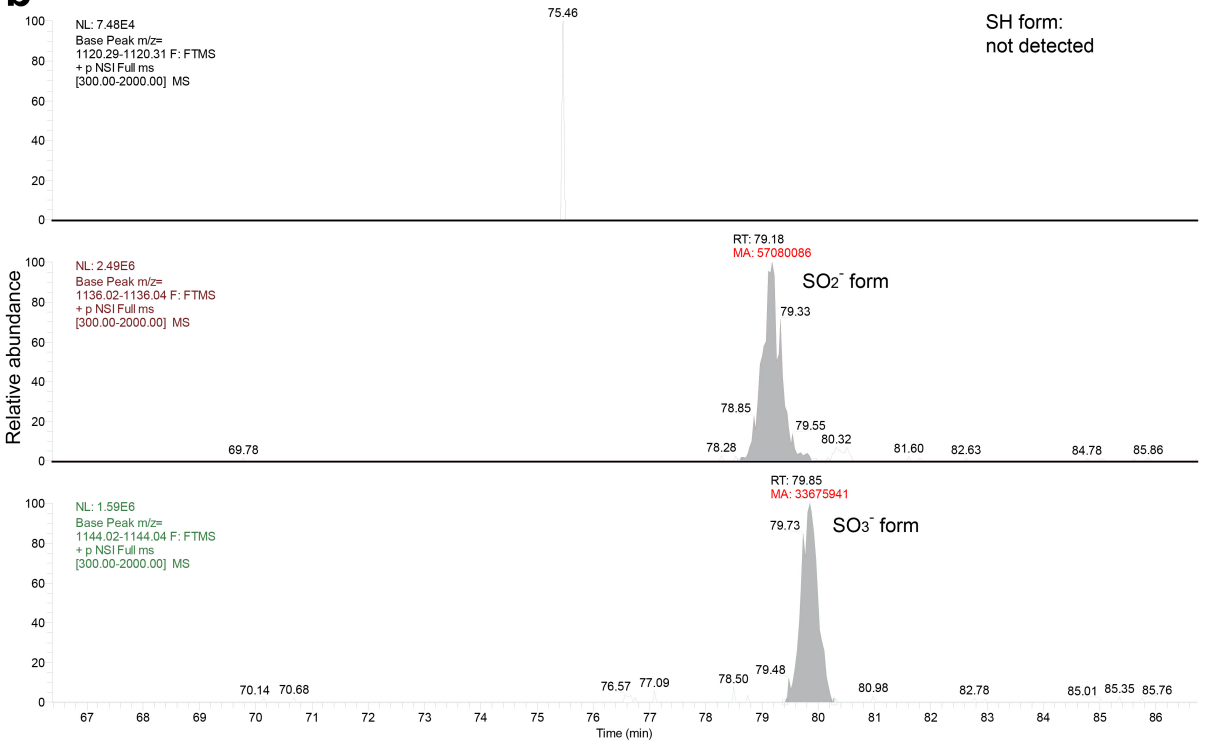
Subcellular location	Photoactivation time (s)	Photoactivation fold
Nucleus	4.4 ± 0.2	5.2 ± 0.2
Mitochondria	4.0 ± 0.2	4.6 ± 0.2
Cytosol	3.6 ± 0.2	2.6 ± 0.1

Figure S3. Photoactivation of mStable expressed in different cellular locations of mammalian cells. (**a**) In nucleus and fused to the C-terminus of human histone H2B. (**b**) In mitochondria targeted with mitochondrial localization signals. (**c**) In cytosol and fused to the N-terminus of actin. The curves were measured using procedures described for photobleaching on the laser-scanning confocal microscope Zeiss LSM 700. Each curve is the mean of three independent experiments. (**d**) Summary of measured photoactivation time (time taken to reach the peak intensity) and photoactivation fold (peak intensity/initial intensity). Data are mean ± s.e., n = 3. We note that the photoactivation fold is correlated with the redox status of the subcellular location. When expressed in a more reduced environment, mStable would have Cys143 pre-oxidized to a lesser extent and thus show a higher fold of photoactivation.

a



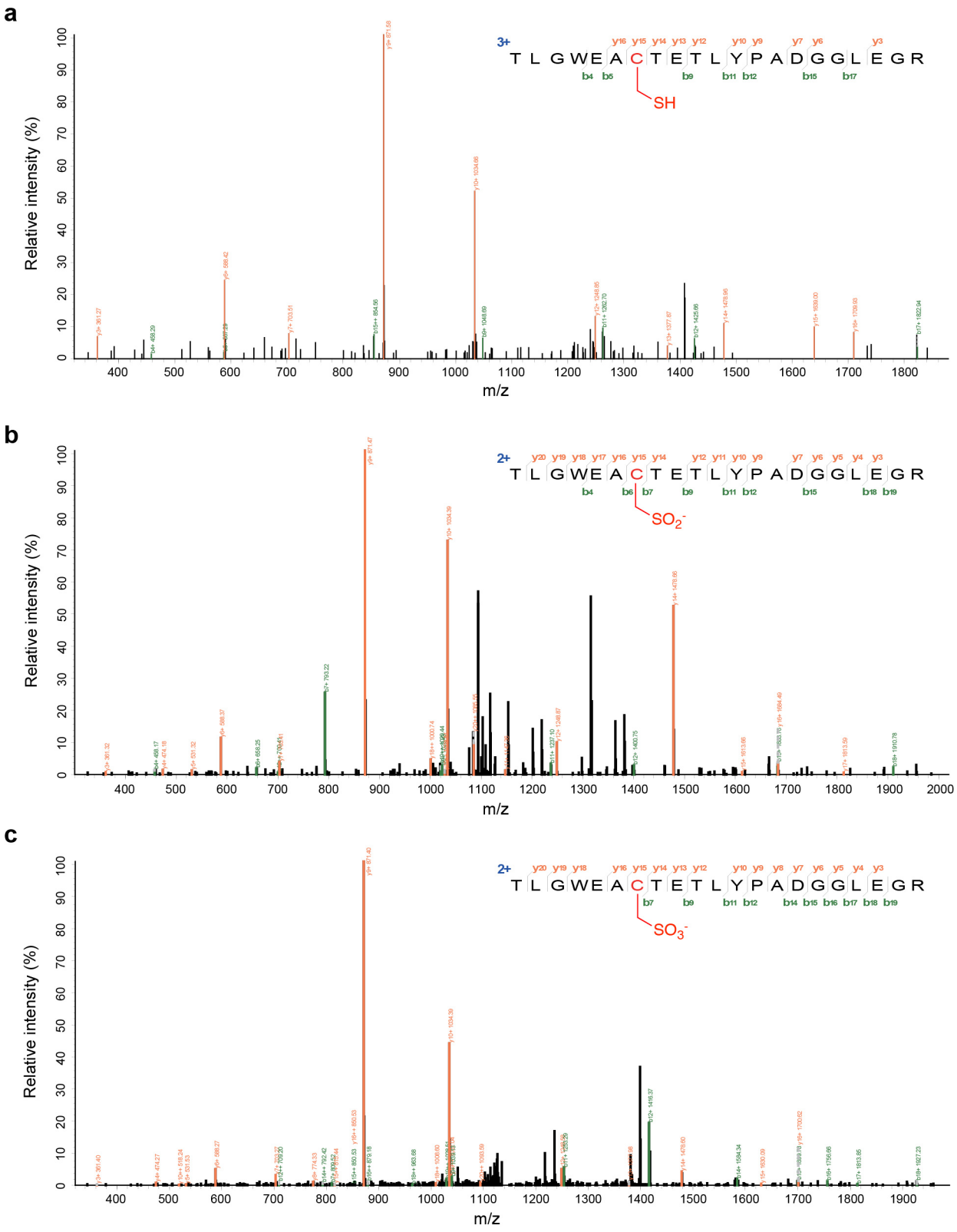
b



c

mStable	SH form	SO_2^- form	SO_3^- form
before photoactivation	99.21%	0.70%	0.09%
after photoactivation	not detected	62.89%	37.11%

Figure S4. Extracted ion chromatograms of LC-MS showing the oxidation of Cys143 after mStable photoactivation. (**a**) Extracted ion chromatograms of trypsin-digested peptides of mStable before photoactivation. Peptide peaks corresponding to the SH form, SO_2^- form, and SO_3^- form of Cys143 are labeled, respectively. The integrated area of each peak is indicated in red color. Disulfide bond formation of Cys143 with Cys in other peptides was measured to have total peak area of 0.676×10^9. (**b**) Extracted ion chromatograms of trypsin-digested peptides of mStable after photoactivation. Peptide containing the thiol form of Cys143 was not detectable, and disulfide bond formation of Cys143 with Cys in other peptides was not detected either; oxidized SO_2^- form and SO_3^- form of Cys143 are labeled and integrated peak area colored in red. (**c**) Estimation of the percentage of SH form, SO_2^- form, and SO_3^- form of Cys143 in mStable before and after photoactivation based on the corresponding peak areas. Note: the high-resolution m/z measured by FTMS for these peaks are shown in Figure 3a and 3b, and these peptide fragments were subsequently sequenced using MS/MS to confirm their amino acid identity and the Cys143 oxidation status in Figure S5.


a
3+
T L G W E A C T E T L Y P A D G G L E G R
SH
Relative intensity (%)
m/z
b
2+
T L G W E A C T E T L Y P A D G G L E G R
SO2-
Relative intensity (%)
m/z
c
2+
T L G W E A C T E T L Y P A D G G L E G R
SO3-
Relative intensity (%)
m/z

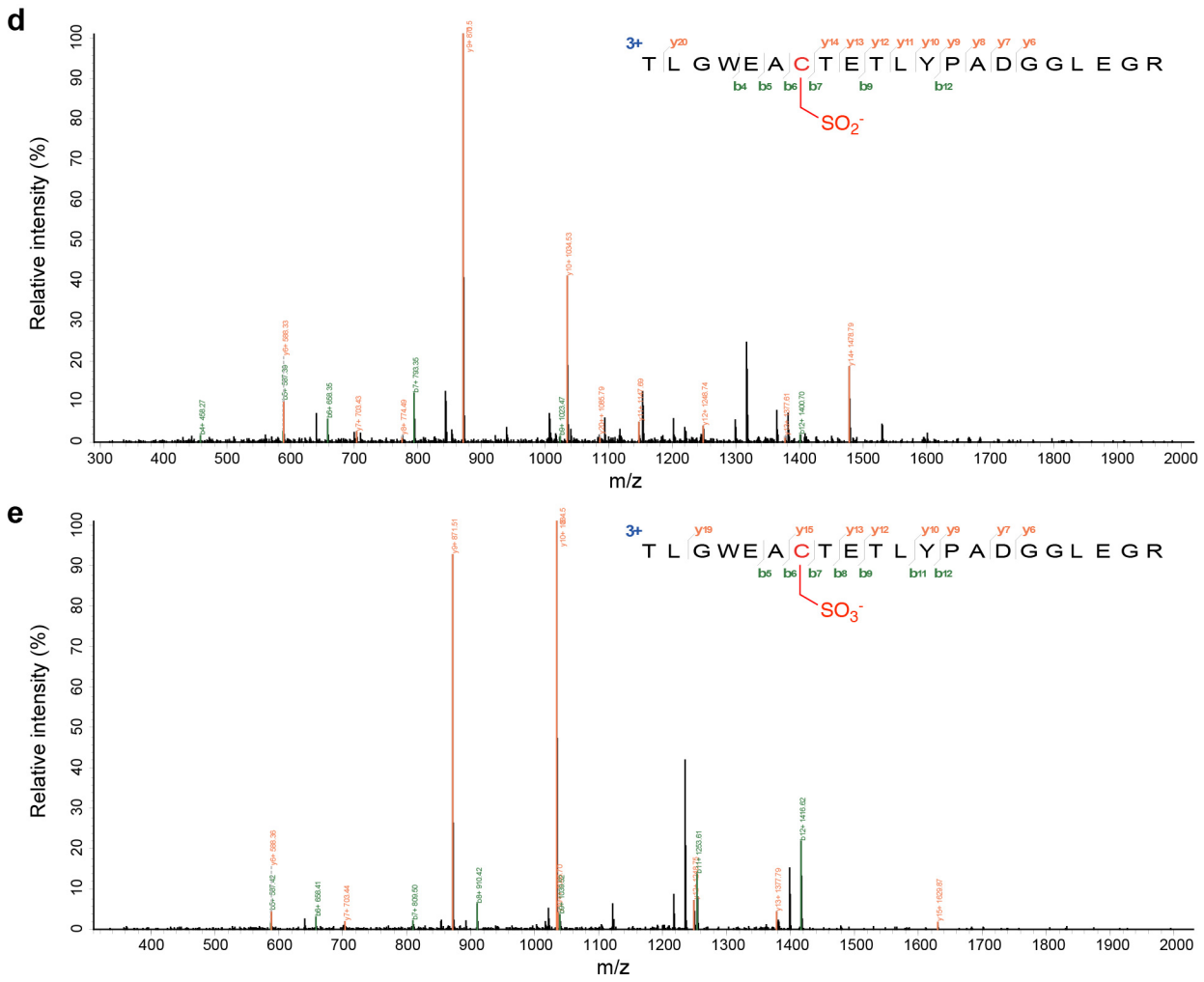


Figure S5. MS/MS analysis of Cys143 oxidation in mStable before and after photoactivation. (**a-c**) mStable before photoactivation confirming Cys143 in the thiol form (**a**), SO_2^- form (**b**), and SO_3^- form (**c**). (**d-e**) mStable after photoactivation confirming Cys143 in the SO_2^- form (**d**) and SO_3^- form (**e**).

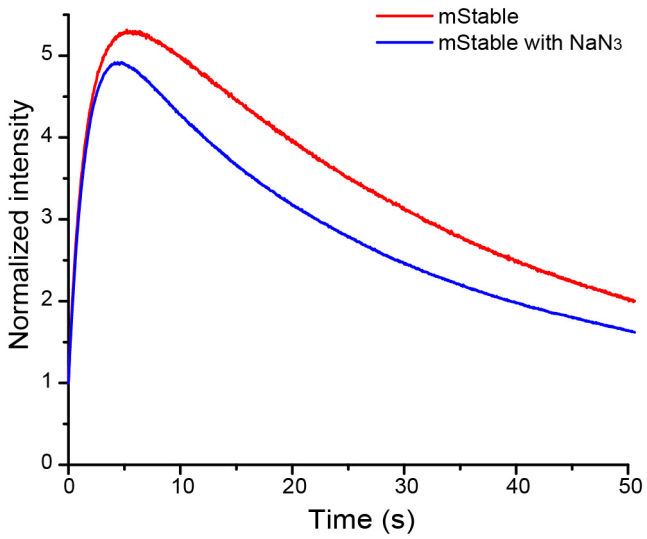


Figure S6. The presence of sodium azide, a specific quencher of singlet oxygen, decreased the photostability of mStable. Curves show the photobleaching kinetics of mStable fused to human histone H2B expressed in HEK293T cells in the presence and absence of sodium azide. $t_{1/2}$: 32.1 s for mStable; 25.2 s for mStable with NaN_3. Note that 75% laser power was used in these photobleaching experiments while experiments in Figure 2c used 100% laser power. Each curve is the mean of three independent experiments. In the presence of sodium azide, the photostability of mStable decreased by 21%; correspondingly, the extent of photoactivation also decreased 7.5%. These observations are consistent with the ability of sodium azide to quench singlet oxygen, thus reducing the extent of oxidation of Cys143. The decrease of mStable photostability by sodium azide was not 100%, possibly because the singlet oxygen was generated by the chromophore inside the β-barrel and Cys143 is directly next to the chromophore with its side chain pointing toward the chromophore inside the β-barrel (Fig. 3c). Therefore, it is difficult for sodium azide to completely quench the single oxygen before the singlet oxygen reaches the Cys143, as sodium azide needs to enter the β-barrel to exert its quenching effect. As a control, we also performed the experiment using a different singlet oxygen quencher glutathione. Glutathione showed no effect on the photostability of mStable; glutathione is a large molecule, which cannot enter the β-barrel of mStable to quench singlet oxygen generated inside the β-barrel.

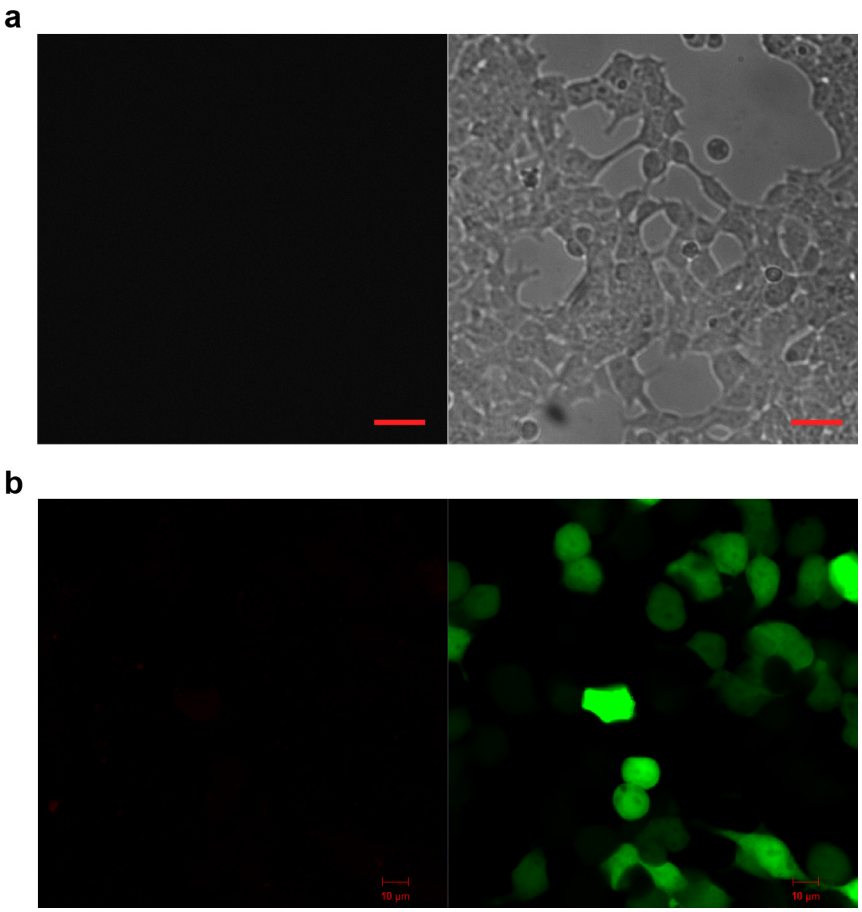


Figure S7. mStable(C143D) mutant was nonfluorescent in HEK293T cells. (**a**) Left panel: Expression of mStable(C143D) in HEK293T cells showed no green fluorescence; excitation: 488 nm, emission: 492-590 nm. Right panel: bright field image of the cells. Scale bar: 50 μm. (**b**) Left panel: Expression of mStable(C143D) in HEK293T cells showed no red fluorescence; excitation: 555 nm, emission: 600-700 nm. Right panel: GFP was cotransfected with mStable(C143D) in HEK293T cells to indicate successful transfection; excitation: 488 nm, emission: 492-590 nm. Scale bar: 10 μm.

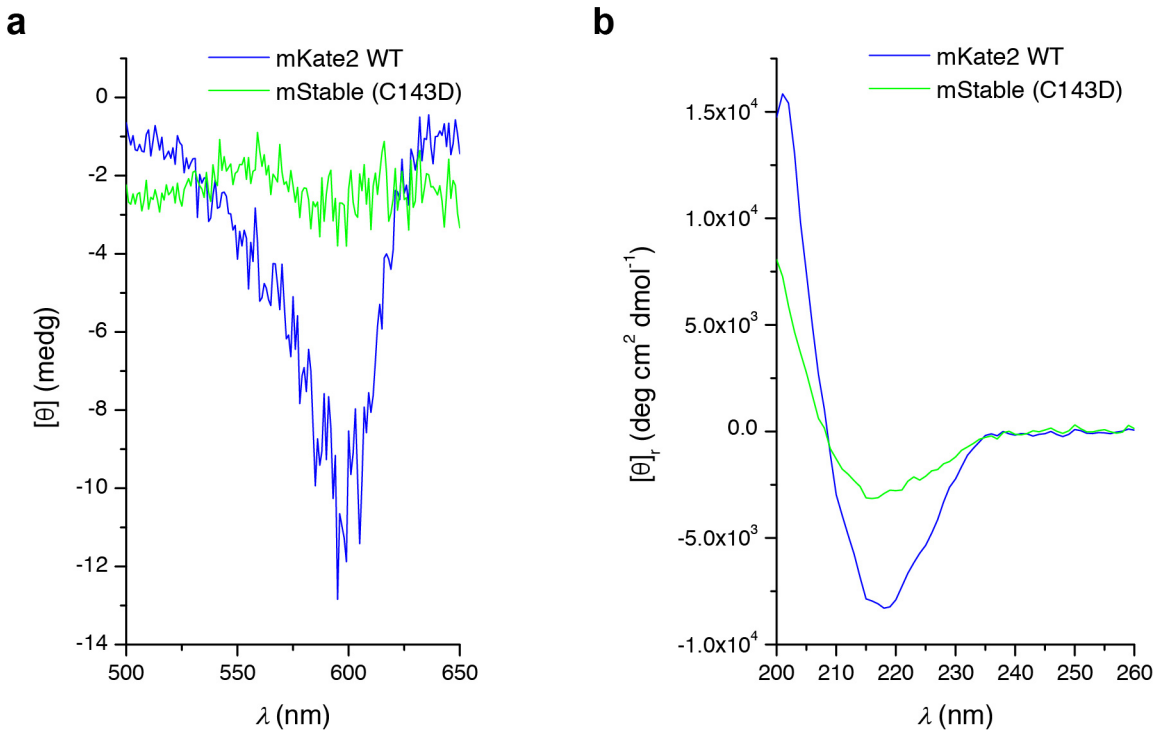
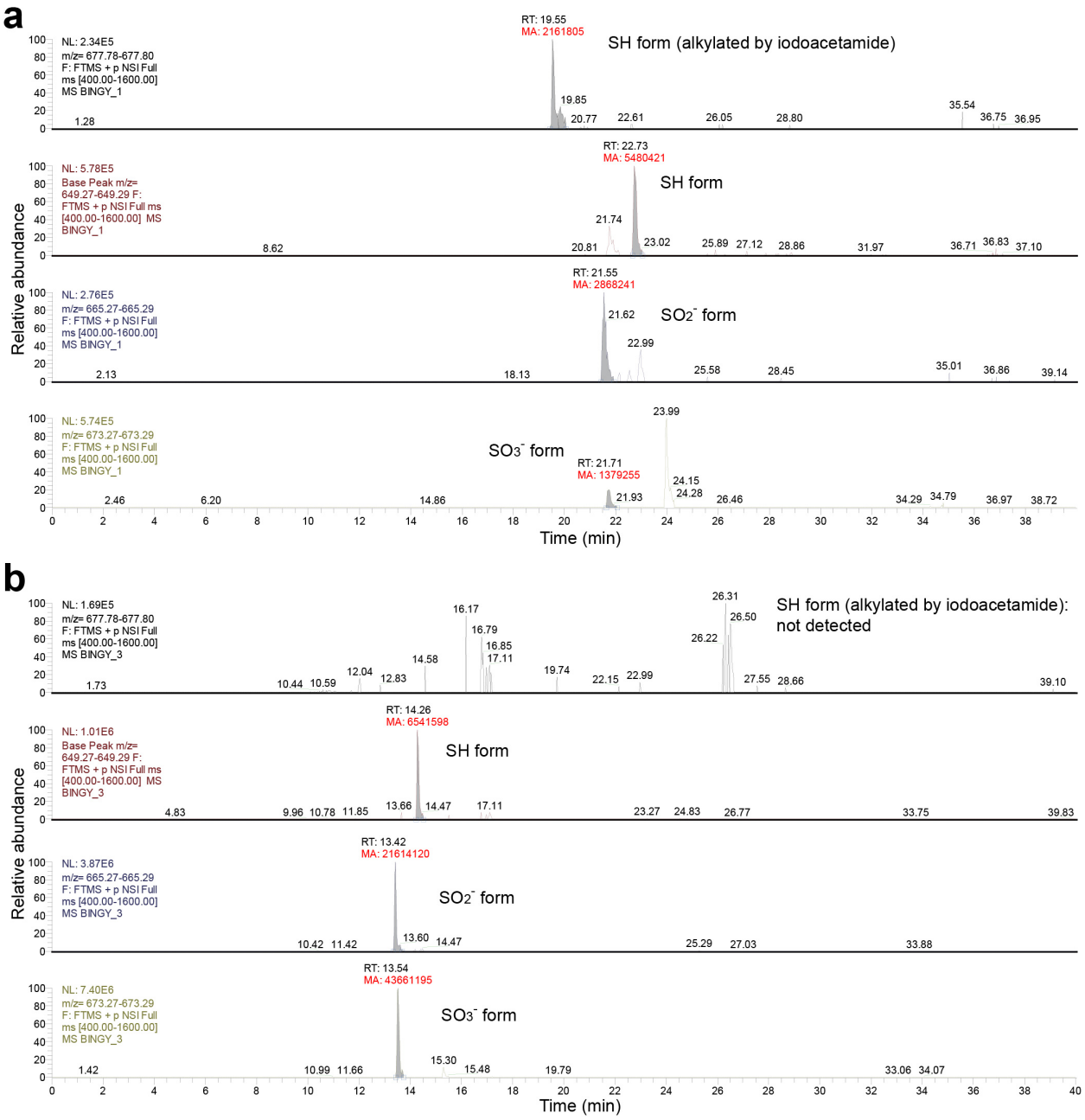


Figure S8. Circular dichroism (CD) spectra of WT mKate2 and mStable(C143D). (**a**). Ellipticity of WT mKate2 and mStable(C143D) in the spectral region of the chromophore indicates that the red chromophore of mStable(C143D) mutant did not form efficiently. (**b**) Molar ellipticity of WT mKate2 and mStable(C143D) in the UV region indicate that the β-barrel structure was disrupted in the mStable(C143D) mutant.

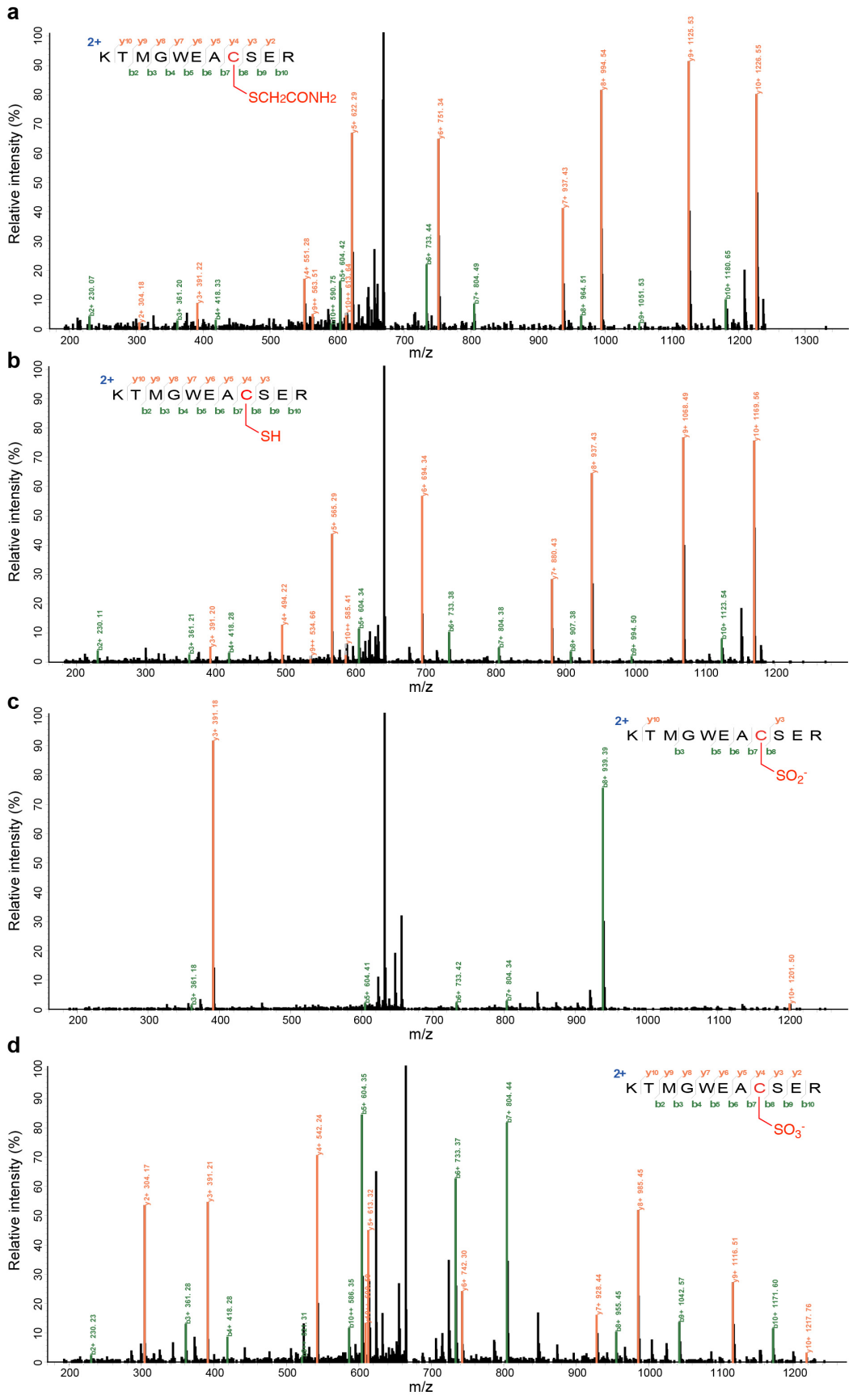


c

mPlum	SH form	SO_2^- form	SO_3^- form
without rose bengal	64.3%	24.1%	11.6%
with rose bengal	9.1%	30.1%	60.8%

Figure S9. Extracted ion chromatograms of LC-MS showing that rose bengal photosensitization increased the extent of Cys146 oxidation of mPlum(S146C). (**a**) Extracted ion chromatograms of trypsin-digested peptides of mPlum(S146C) without rose bengal photosensitization. Peptide peaks corresponding to the SH form, SO_2^- form, and SO_3^-

form of Cys143 are labeled, respectively. Note some SH form was alkylated by iodoacetamide during MS sample preparation. The integrated area of each peak is indicated in red color. (**b**) Extracted ion chromatograms of trypsin-digested peptides of mPlum(S146C) after rose bengal photosensitization. Peptide peaks corresponding to the SH form, oxidized SO_2^- form and SO_3^- form of Cys146 are labeled and integrated peak area colored in red. (**c**) Estimation of the percentage of SH form, SO_2^- form, and SO_3^- form of Cys146 in mPlum(S146C) before and after rose bengal photosensitization based on the corresponding peak areas. Note: the peptide fragments in the peaks were subsequently sequenced using MS/MS to confirm their amino acid identity and the Cys146 oxidation status in Figure S10.


a
2+
K T M G W E A C S E R
SCH2CONH2
Relative intensity (%)
m/z
b
2+
K T M G W E A C S E R
SH
Relative intensity (%)
m/z
c
2+
K T M G W E A C S E R
SO2-
Relative intensity (%)
m/z
d
2+
K T M G W E A C S E R
SO3-
Relative intensity (%)
m/z

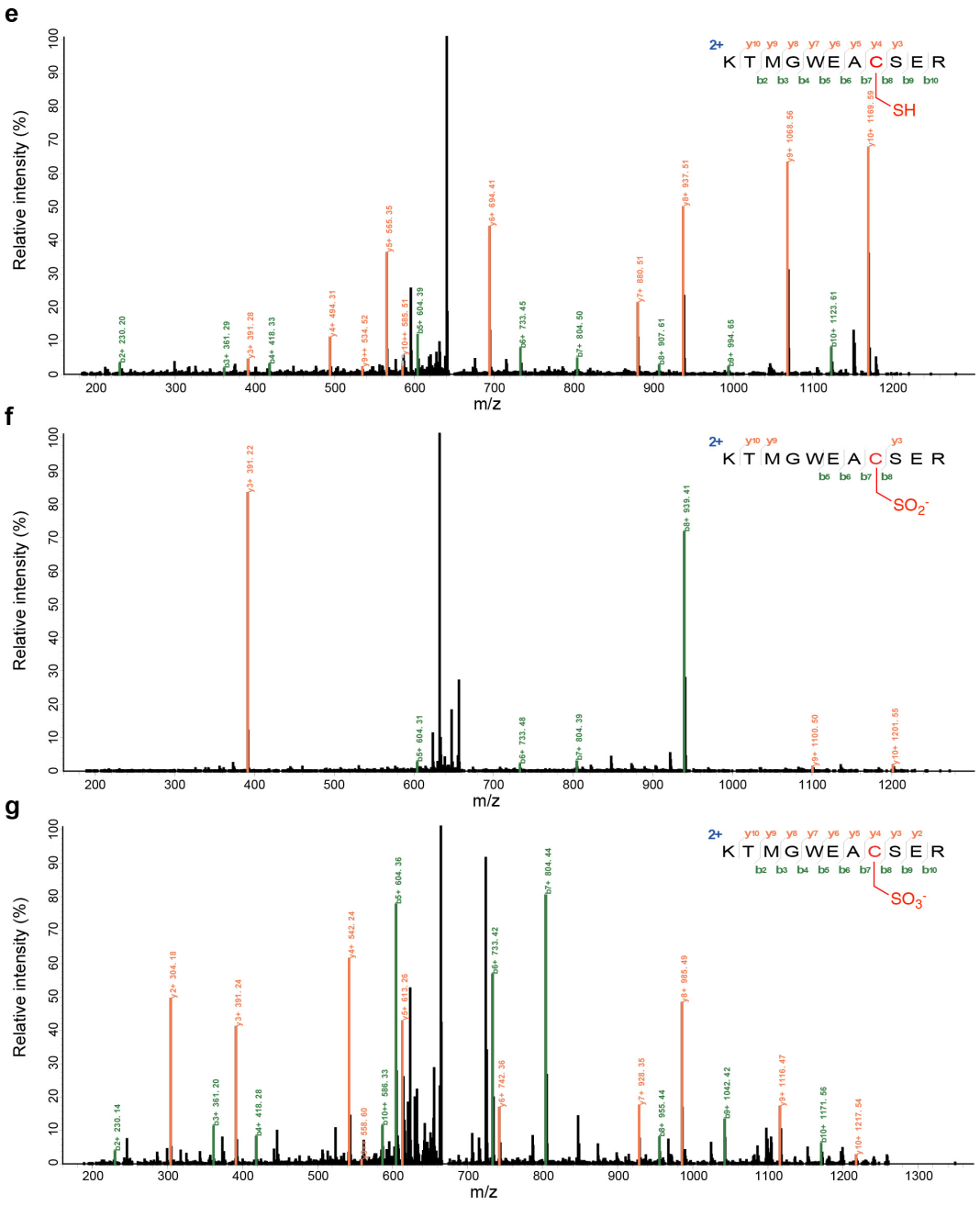


Figure S10. MS/MS analysis of Cys146 oxidation in mPlum(S146C) without and with rose bengal photosensitization. (**a-d**) mPlum(S146C) without rose bengal photosensitization confirming Cys146 in the thiol form alkylated by iodoacetamide (**a**), free thiol form (**b**), SO_2^- form (**c**), and SO_3^- form (**d**). (**e-g**) mPlum(S146C) after rose bengal photosensitization confirming Cys146 in the thiol form (**e**), SO_2^- form (**f**), and SO_3^- form (**g**).

Supporting references:

1. Xiang, Z., Ren, H., Hu, Y. S., Coin, I., Wei, J., Cang, H., and Wang, L. (2013) Adding an unnatural covalent bond to proteins through proximity-enhanced bioreactivity, *Nat. Methods 10*, 885-888.
2. Chu, J., Haynes, R. D., Corbel, S. Y., Li, P., Gonzalez-Gonzalez, E., Burg, J. S., Ataie, N. J., Lam, A. J., Cranfill, P. J., Baird, M. A., Davidson, M. W., Ng, H. L., Garcia, K. C., Contag, C. H., Shen, K., Blau, H. M., and Lin, M. Z. (2014) Non-invasive intravital imaging of cellular differentiation with a bright red-excitable fluorescent protein, *Nat Methods 11*, 572-578.
3. Shaner, N. C., Steinbach, P. A., and Tsien, R. Y. (2005) A guide to choosing fluorescent proteins, *Nat Methods 2*, 905-909.