SUPPLEMENTAL MATERIAL

Dual labeling biotin switch assay to reduce bias derived from different cysteine subpopulations: A method to maximize S-nitrosylation detection

Heaseung Sophia Chung[1,3], Christopher I. Murray[1,8], Vidya Venkatraman[2,3,6], Erin L. Crowgey[6], Peter P. Rainer[2,9], Robert N. Cole[1,4], Ryan D. Bomgarden[5], John C. Rogers[5], Wayne Balkan[7], Joshua M. Hare[7], David A. Kass[2,3], Jennifer E. Van Eyk[1,2,3,6]

[1]Department of Biological Chemistry, [2]Department of Medicine, Division of Cardiology, [3]The Johns Hopkins NHLBI Proteomics Innovation Center on Heart Failure, [4]Mass Spectrometry and Proteomic Facility, Johns Hopkins University, Baltimore, MD, USA [5]Thermo Fisher Scientific, Rockford, IL, USA [6]Advanced Clinical Biosystems Research Institute, Heart Institute, Cedars-Sinai Medical Center, Los Angeles, CA, USA [7]Department of Medicine, Interdisciplinary Stem Cell Institute, University of Miami Miller School of Medicine, Miami, FL 33136, USA [8]Department of Anesthesiology, Pharmacology and Therapeutics, University of British Columbia, Vancouver, Canada [9]Division of Cardiology, Medical University of Graz, Austria

Corresponding Author:
Dr. Jennifer E. Van Eyk
Advanced Health Science Pavilion Room A9307
127 S. San Vicente Blvd.
Cedars Sinai Medical Center
Los Angeles, CA 90048
E-mail: Jennifer.VanEyk@cshs.org
Fax: 310-423-3522
Phone: 310-384-6674

Detailed Methods

Reagents. IodoTMT^6, cysTMT^6, cysTMT^0 (a non-multiplex version), antibody to TMT, anti-TMT affinity resin and Zeba desalt spin column were from Thermo Fisher Scientific. All other reagents and chemicals including N-ethylmaleimide (NEM), reduced glutathione (GSH), oxidized glutathione (GSSG), S-nitrosoglutathione (GSNO) and triethylammonium bicarbonate buffer (TEAB) pH 8.5 were obtained from Sigma-Aldrich and solution of GSNO was prepared fresh before each experiment. SOD1 recombinant protein was purchased from ProSpec and bovine actin and human beta-actin were from Cytoskeleton.

Cell culture. Human embryonic kidney (HEK) 293 cells were grown in Dulbecco's modification of Eagle's medium with 4.5 g/L glucose, L-glutamine, & sodium pyruvate (Corning Cellgro) at 37°C in a humidified atmosphere containing 5% CO_2. Cells were lysed in PBS pH 7.4, 0.02 mmol/L neocuproine, 0.2 mmol/L EDTA, 150 mmol/L NaCl and 1x protease inhibitor (Roche) containing 1.0% (v/v) triton X-100 using a probe sonicator and centrifuged for 10 min at 2000 X g. The protein concentration of the resulting supernatant was determined by BCA assay kit (Pierce).

Mice. S-nitrosoglutathione reductase (GSNOR) knock-out mice were generated as previously described[1]. Wild-type C57BL/6J mice were purchased from the Jackson Laboratories. 4 to 5-month-old GSNOR knock-out and C57BL/6J mice (male and female) were used in the study performed under same reducing conditions (PEN pH 8.0, 5 mmol/L sodium ascorbate and 0.3 mmol/L cys- or iodoTMT6) for two labels. For the study in optimized conditions for parallel labeling (1 mmol/L sodium ascorbate, 1 mmol/L CuSO4 for cysTMT label, 5 mmol/L sodium ascorbate for iodoTMT), 8-month-old male C57BL/6 were used.

Whole-heart Homogenates Preparation. Animal studies were performed in accordance with National Institutes of Health Guidelines for the Care and Use of Animals and approved by the Institutional Animal Care and Use Committee at Johns Hopkins University. In the dark, left ventricles of C57BL/6 and GSNOR knock-out mouse were excised, immediately washed and homogenized in the homogenization buffer (on ice) containing (in mmol/L) sucrose (300), HEPES-NaOH 8.0 (250), EDTA (1), Neocuproine (0.1), N-ethylmaleimide (NEM) (20) and EDTA-free protease inhibitor tablet 1X (Roche) and centrifuged for 2 min at 1,500 g. All procedures were performed in the dark. Protein concentration was determined using the BCA assay.

Western Blot Analysis. General western blotting procedures were followed as described in a previous study[2] for all TMT labeled samples. Exceptionally, half of each sample for one experiment of simultaneous labeling (Online Figure VIIC) was reduced by DTT prior to gel loading. Separated by SDS-PAGE and then transferred iodoTMT or cysTMT labeled proteins to nitrocellulose membranes were blocked with 5% (w/v) nonfat milk powder in Tris-buffered saline completed with 0.1% (v/v) Tween 20. They were detected using the primary antibody to TMT at room temperature for 1h followed by alkaline phosphatase conjugated secondary antibody to mouse (Jackson Immunoresearch Inc.) or anti-mouse horseradish peroxidase-conjugated secondary antibody (Jackson Immunoresearch Inc.) at room temperature for 30 min. Blots were washed and developed using immun-star substrate (Bio-Rad) or ECL substrate (GE Healthcare).

Cys and IodoTMT^6 Switch Assay in identical conditions with 'no ascorbate' control group. HEK cell lysates (or recombinant proteins) were diluted to 0.8 g/l in PEN buffer (PBS pH

7.4, 1 mmol/L EDTA and 0.1 mmol/L neocuproine). For parallel labeling, 520 μg of HEK cell lysate per condition in PEN buffer was treated with 0.1 mmol/L S-nitrosoglutathione (GSNO) or vehicle as a control) for 20 min at 37 °C (n=4, from separately cultured plates). GSNO was removed using a Zeba desalt spin column (Thermo) equilibrated with PEN pH 7.4 according to the manufacturer's protocol. The solution of remaining free thiols diluted to 0.5 g/l was blocked with 20 mmol/L of NEM in the presence of 2.5% (w/v) SDS and incubated for 20 min at 50°C. Excess NEM was removed by the PEN pH 8.0 equilibrated Zeba desalt spin column. GSNO-treated and control samples were split and half of them (260 μg/each) were diluted to 0.41 g/l in PEN pH 8.0, 5 mmol/L sodium ascorbate and 0.3 mmol/L cysTMT^6 for 2 h at 37 °C. The other half were diluted with equivalent incubation without sodium ascorbate. TMT switch assay with iodoTMT^6 was performed in the same manner as cysTMT^6 labeling.

For TMT switch assay with heart homogenates, non-reduced condition (no-sodium ascorbate) was used as a negative control (450 μg/each).

All steps were protected from light.

SNO-peptides Preparation for Mass Spectrometry (MS) Analysis. 200 μg of each iodoTMT^6 (or cysTMT^6) sample were combined, desalted and digested with trypsin (Promega, 1: 40 of trypsin: protein)[2]. A positive control (all available cysteine residues) sample was processed separately. The digested peptides were incubated with 600 μl of the anti-TMT antibody-affinity resin at room temperature for 2 h. Elutions were performed in two ways: Unlabeled peptides were removed by washing with 5 x 5 ml of TBS and TMT labeled peptides were eluted with 3 X 0.6 ml of 500 mmol/L TEAB pH 8.5 buffer containing 10 mmol/L N,N-diisopropylethylamine. Or elution was performed as described previously with 50% (v/v) acetonitrile /0.4% (v/v) trifluoroacetic acid[2]. Both elution methods were applied alternatively in different replicates to increase TMT peptide identification. Samples were then desalted using solid phase extraction with Oasis HLB (Waters).

Peptide Identification by Liquid Chromatography/Tandem Mass Spectrometry (LC/MS/MS). LC/MS/MS analysis was performed using an LTQ Orbitrap Velos MS (Thermo Fisher Scientific) interfaced with a nanoAcquity UPLC system (Waters). Peptides from the parallel labeling were fractionated by reverse-phase HPLC on a 75 μm x 15 cm PicoFrit column with a 15 μm emitter (PF3360-75-15-N-5, New Objective) packed in-house with Magic C18AQ (5 μm, 120Å, Michrom) using 0–60% acetonitrile/0.1% formic acid gradient over 90 min at 300 nlmin^{-1}. Eluting peptides were sprayed directly into an LTQ Orbitrap Velos at 2.0 kV. Isolation width was set to 1.3 Da and normalized collision energy was set to 38. Precursors were acquired at 30,000 resolution and 15,000 and 7,500 for the fragment ions. High energy collision dissociation was used exclusively with a lock mass of the polysiloxane at 371.101230 for MS1 and MS2.

For peptides from sequential and simultaneous labeling and TMT- labeled samples described in in this Online supplemental, LC/MS/MS analysis was performed using an Easy-nLC 1000 (Thermo Scientific, mobile phase A was 0.1% formic acid in water and mobile phase B was 0.1 % formic acid in acetonitrile) connected to an Orbitrap Elite mass spectrometer (Thermo Scientific) equipped with a nanoelectrospray ion source. Peptides were loaded onto a Dionex Acclaim® PepMap100 trap column (Thermo, 75 μm x 2 cm, C18 3 μm 100Å) and fractionized on a Dionex Acclaim® PepMap RSLC analytical column (Thermo, 50 μm x 15 cm, C18 2 μm 100Å) at a flow rate of 300 nLmin^{-1} using a linear gradient of 2–15% B for 70 minutes, 15–25%for 15 min,25–35% B for 5 minutes, 35–98% B for 2 minutes then holding at 98% for 8 minutes. The nano-source capillary temperature was set to 275 °C and the spray voltage was set to 2.0 kV. MS1 scans were acquired at a resolution of 60,000 FWHM (380–2000 m/z) with

an AGC target of $1x10^6$ ions over a maximum of 250 ms. MS2 spectra were acquired for the top 10 ions from each MS1 scan in the Orbitrap at a resolution 30,000 FWHM with a target setting of $3x10^4$ ions, accumulation time of 200 ms, and an isolation width of 1.2 Da. The normalized collision energy was 33% for 0.1ms activation time and one microscan was acquired for each spectra. Monoisotopic precursor selection was enabled and only MS1 signals exceeding 500 counts triggered the MS2 scans, with +1 and unassigned charge states not being selected for MS2 analysis. Dynamic exclusion was enabled with a repeat count of 1, repeat duration of 30 seconds and exclusion duration of 80 seconds.

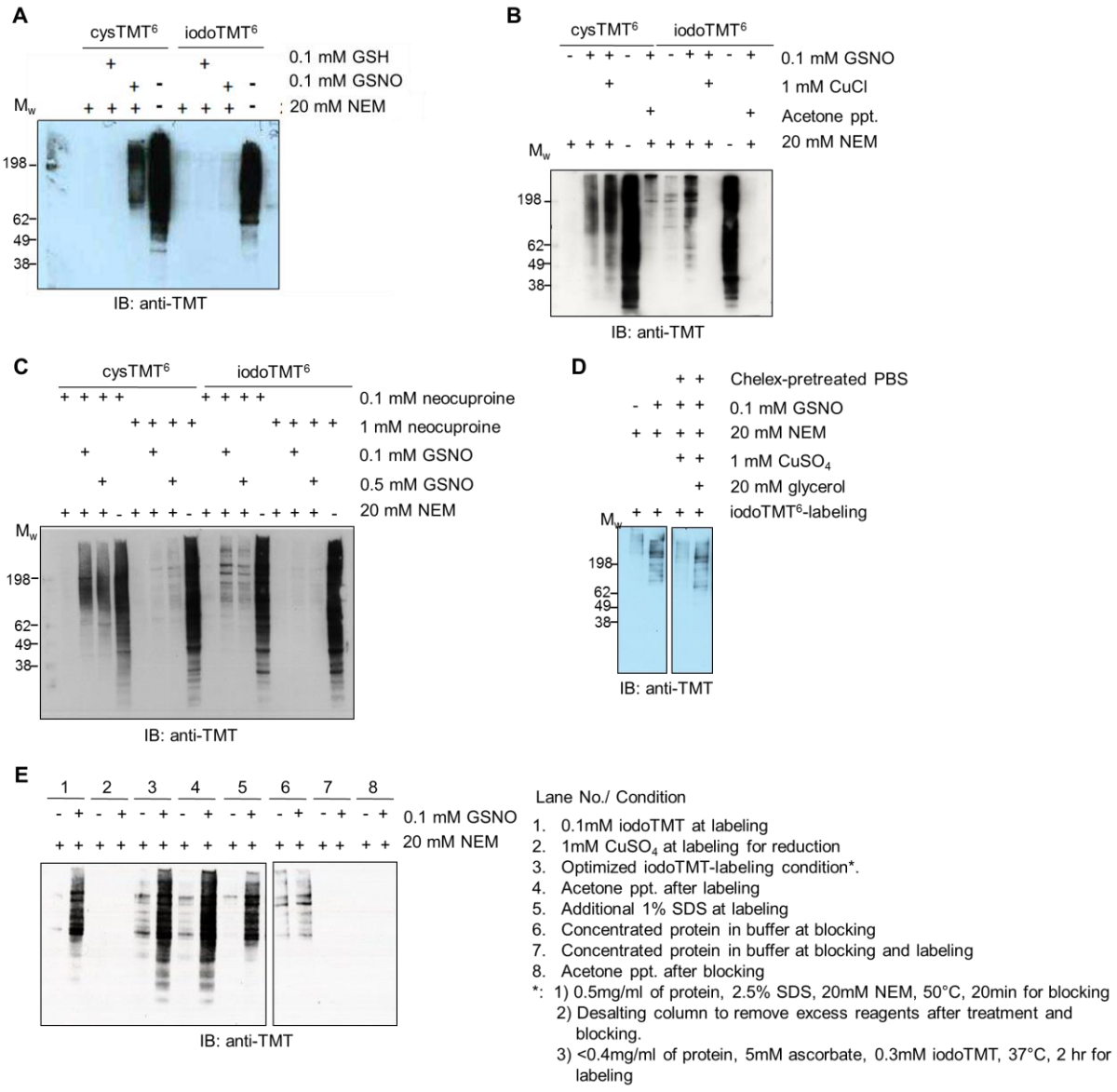
Data Analysis. Raw MS data was converted to mzXML format and searched using OMSSA (version 2.1.9)[3] against the concatenated target/decoy human or mouse Uniprot database. Search parameters included full digestion with trypsin with up to 2 missed cleavages, a parent mass error of 10 ppm and a fragment mass error of 0.03 Da with the variable modifications as NEM, cysTMT[6], cysTMT[0] or iodoTMT[6] for (C) and oxidation (M). For reduced and alkylated samples, IAA (C) was additionally set as variable modification. Software msConvert (version 2.1.2132)[4] from ProteoWizard was used for peaklist generation. Search results were processed using the trans-proteome pipeline (TPP, version v4.4, rev 1)[5] implementing the PeptideProphet[6], iProphet[7] and ProteinProphet[8] algorithms. Peptide and protein identifications were accepted if the peptide confidence was greater than 95.0% as specified by the PeptideProphet[6] algorithm and a protein group probability greater than 95.0% as assigned by the ProteinProphet[8] algorithm. False discovery rates (FDR) were calculated by the ProteinProphet[8]. FDRs were less than 1.0 % using a minimum probability of 0.95 for peptide and protein group as a threshold. After search, TMT-modified peptides were filtered and considered for the SNO-modification. The MS spectra was also searched using the X!Tandem algorithm (version TPP v4.4, rev 1)[9] with the k-score plug-in. X!tandem[9] only accepts one variable modification to a particular amino acid. As a result we chose to set TMT as a fixed modification to utilize in order to compare the OMSSA[3] search results. For sequential and simultaneous labeling, updated versions of msConvert (version 3.0.3858)[4] and TPP (version v4.6, rev 1)[5] were used. All peptide IDs from independent replicates of HEK cells were combined and analyzed. Values from the independent replicates and peptides for an individual cysteine residue were averaged and peptide probability, mass difference (Da), charge and observed peptide mass (Da) were assigned from those of the best identified peptide. A specific site of SNO-modification was accepted only if the value of GSNO-condition was at least two fold (one fold, for sequential and simultaneous labeling) greater than the largest control condition (untreated, GSH, GSSG). For experiments performed without ascorbate (described in Online supplemental), a site of SNO-modification was determined if the TMT intensity in the ascorbate treated sample was greater than controls (HEK cells: no ascorbate/untreated, no ascorbate/GSNO-treated and ascorbate/untreated; Tissue samples: no ascorbate condition). When the fold change was calculated, the values of GSNO-condition (reduced with ascorbate) were > 10-fold greater than the largest control condition on average and in some sites it was >6000-fold greater. However, when background signals were observed in a very few sites, we speculate that this is due to 1) default background noise in MS spectra 2) incomplete distribution of isobaric tag and/or 3) the higher sensitivity of MS over western blot analysis. Quantitative values for the TMT reporter ions were collected with the Libra module of the TPP using a custom condition file. Subcellular locations and functional analysis of proteins were performed based on Gene Ontology

Physiochemical Properties Analysis. 21 amino acid sequences flanking the N- and C-terminus of the SNO-modified cysteine residues were obtained based on Uniprot FASTA sequences. pI prediction for the flanking residues in cys- or iodoTMT specific labeled groups

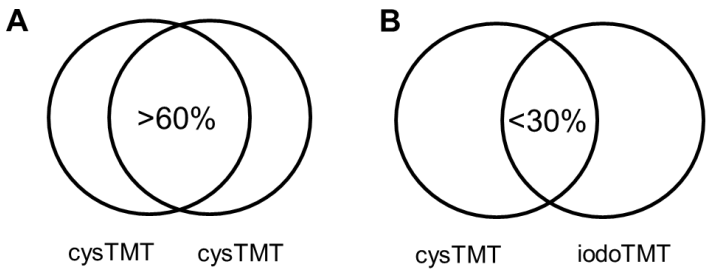
was performed with ExPASy server[10] (http://web.expasy.org/compute_pi/) and the distribution of their pI was represented as color scale from low (red) to high (green) pI and grouped bar graph (Figure 5A) The top 15 of flanking residues whose SNO-cysteine residues were extremely reactive (or had the highest intensity) to either of TMT reagents was selected and the frequencies of the amino acids of 15 flanking residues in each group were computed with WebLogo[11] (http://weblogo.berkeley.edu/logo.cgi). For global proteins' properties analysis, top 20 proteins whose SNO-cysteines were most reactive (or had the highest intensity) and whose all cysteine residues were exclusively labeled with either of TMT were selected. Aliphatic index and molecular weights were computed with ProtParam tool[10] (http://web.expasy.org/protparam/) and represented as grouped color scale (Figure 5C and 5D).

Steps	Conditions	cysTMT	IodoTMT
Buffer preparation	[neocuproine]	0.1mM	0.1mM
	Buffer	HEPES or PBS	PBS
Blocking	pH	7.4	7.4
	Protein concentration	0.5	0.5
	Removal of excess reagent	Acetone precipitation or spin column	Spin column
Reduction and Labeling	Protein concentration	0.4 μg/μl–3 μg/μl	≤0.4 μg/μl
	Solvent for TMT	Acetonitrile	Methanol
	Copper	1 mM	No addition of copper
	[Ascorbate]	1 mM	5 mM
	pH	7.4	8.0
	Temperature	37 ºC	37 ºC
	Duration	2 h-3.5 h	2 h, exactly

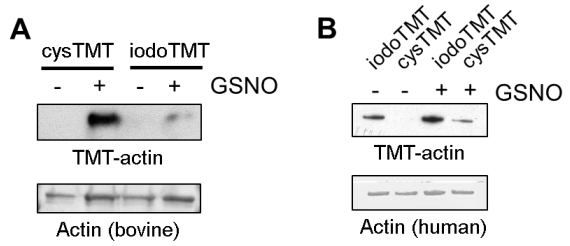
Online Table I. Experimental conditions modified during optimization of iodoTMT labeling. Although IodoTMT[6] has similar mass and structure to cysTMT[6], it could not be simply substituted into the previous TMT-switch protocol. In this study, we establish an optimized protocol for iodoTMT[6] specifically for the switch assay. The multi-step nature of the TMT-switch assay required optimizing several factors to achieve peak efficiency: including concentration of metal, maximum concentration of protein and labeling buffer.



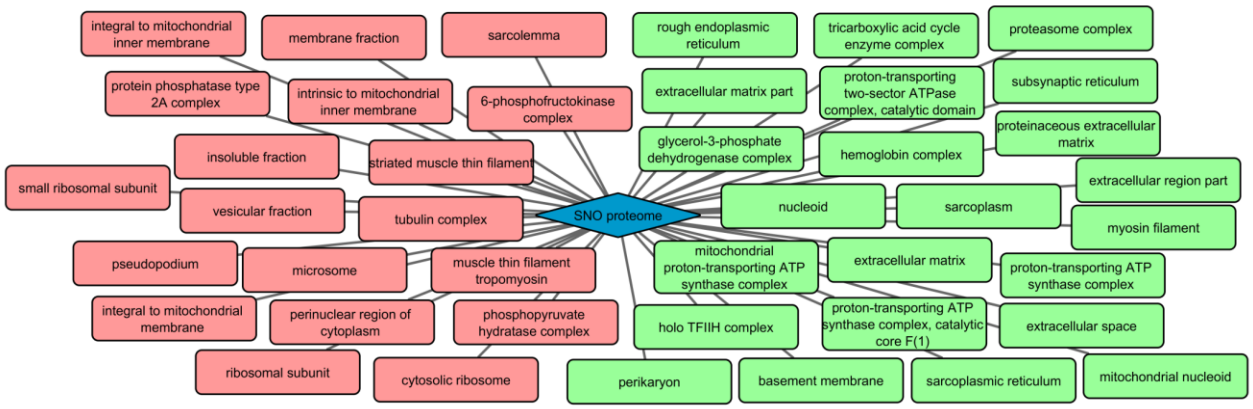
Online Figure I. Troubleshooting for iodoTMT labeling. TMT-switch assay was performed with HEK cell lysates. **A**, TMT-switch assay using cys- and iodoTMT in conditions previously described for cysTMT [2]. Using these conditions, SNO-modified proteins were not labeled with iodoTMT. **B**, Comparison of cys- and iodoTMT in the TMT-switch assay using optimized conditions (PBS at pH 8.0). The addition of 1mM of copper increased labeling for cysTMT but inhibited iodoTMT labeling. **C**, TMT-switch assay in the presence of the metal chelator, neocuproine (0.1 and 1 mM) demonstrating the quantitative effect of metal ions on cys- and iodoTMT labeling. **D**, Decreased iodoTMT labeling by additional 1mM copper was recovered by the addition of 20 mM glycerol, a radical scavenger. **E**, Various conditions to optimize iodoTMT-switch assay were tested: optimal concentration of iodoTMT, ascorbate and SDS, addition of copper, protein: buffer used for blocking and/or labeling and effect of acetone precipitation.



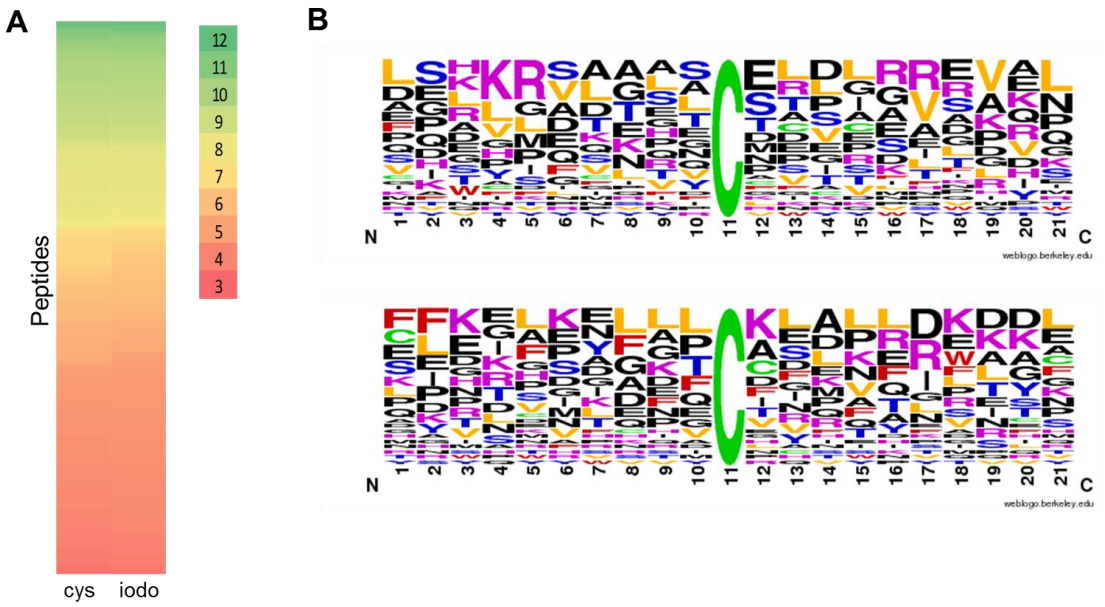
Online Figure II. Comparison of SNO-sites. A, More than 60% of the sites were commonly detected between two replicates of the same cysTMT **B**, Less than 30% of the total SNO-sites from HEK cells were identified by both of the reagents. SNO- sites were labeled with either cysTMT or iodoTMT, under identical conditions.



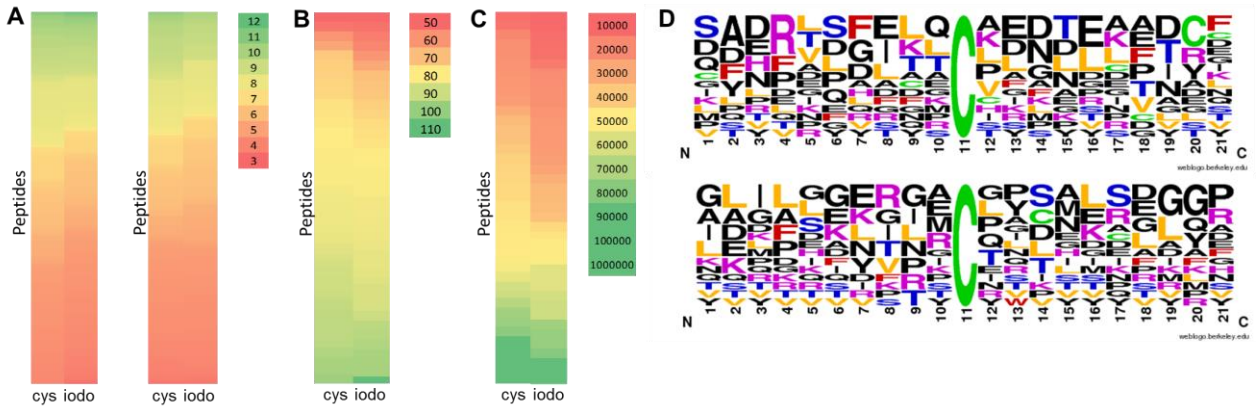
Online Figure III. Western blot analysis of TMT-labeling on recombinant human and bovine actin after cys or iodoTMT-switch assay. A, Western blot analysis of different TMT labeling on recombinant bovine actin after cys- or iodoTMT switch assay using the optimized protocol (1mM copper, 1mM ascorbate for cysTMT labeling and 5mM copper for iodoTMT labeling). **B**, TMT-switch assay with individual recombinant human actin was performed in the same manner for cys- and iodoTMT labeling (right, 5mM ascorbate for both TMT labeling). SNO-modified actin was detected preferentially by iodoTMT.



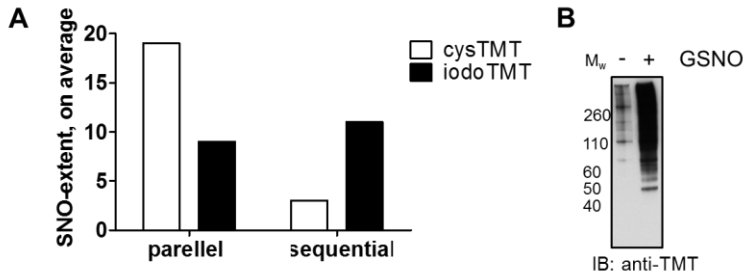
Online Figure IV. Cellular compartment analysis. Gene Ontology-based analysis of SNO-proteome of the two subpopulations in GSNOR knock-out heart: hyper-nitrosylated group (red) and hypo-nitrosylated group (green). Proteins that were clustered to unique categories.



Online Figure V. Physiochemical properties analysis of two subpopulations. SNO sites from cys- and iodoTMT-switch using the optimized conditions were analyzed. **A**, The theoretical pIs of 360 exclusively cysTMT^6-labeled residues and 277 iodoTMT^6-specific residues from HEK cells were analyzed and illustrated using a color scale from low (red) to high (green) pI (left, cysTMT^6; right, iodoTMT^6). **B**, The frequency of amino acids surrounding cysteine residues of two groups were analyzed (top, cysTMT^6; bottom, iodoTMT^6). The pool of fifteen SNO residues from mouse hearts and fifteen SNO residues from HEK cell proteome were used for the analysis. Positively charged lysine/arginine were more frequently observed in cysTMT^6 specific group. Reactive cysteine is highlighted in green at position 11.

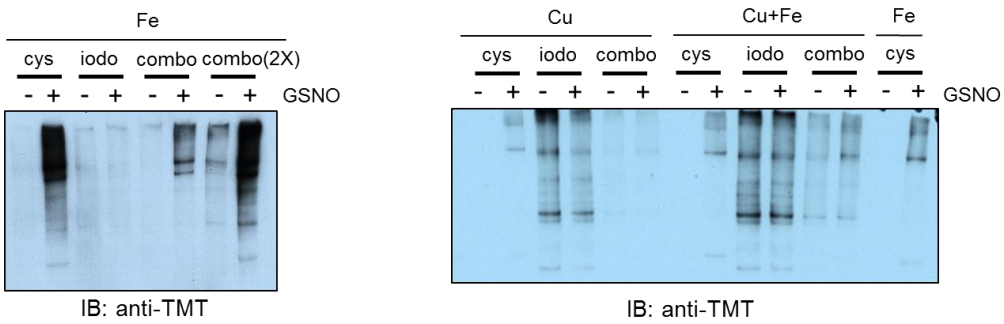


Online Figure VI. Physiochemical properties analysis of two subpopulations. The pool of datasets of SNO-modified sites labeled under optimized conditions and identical conditions were analyzed. **A**, The theoretical pIs of all cysTMT^6-labeled residues and iodoTMT^6-specific residues from HEK cell (left) and WT mouse hearts were illustrated using a color scale from low (red) to high (green) pI (left, cysTMT^6; right, iodoTMT^6). Higher proportion of high pI was observed in iodoTMT^6 specific group. **B**, Aliphatic index for proteins (whose cysteine residues were reactive) were computed based on the amino acid sequence and compared between protein groups which were detected with only one TMT reagent. The color scale represents aliphatic index of proteins from HEK cell (left, cysTMT^6; right, iodoTMT^6). **C**, Molecular weight of the same proteins for (**B**) was analyzed and represented in the same way. IodoTMT^6 group had higher proportion of low molecular weight proteins compared to cysTMT^6 group. **D**, The frequency of amino acids surrounding the cysteine residues of two groups was analyzed (top, cysTMT^6; bottom, iodoTMT^6). Reactive cysteine is highlighted in green at position 11.



Online Figure VII. Analysis of sequential labeling in the TMT-switch assay. A, Comparison of TMT-SNO labeling using the different labeling protocols as calculated by the fold difference of SNO reporter ion intensity over the background reporter ion intensity. In sequential labeling protocols, the average of SNO intensity detected by iodoTMT labeling was only slightly reduced compared to labeling experiments done in parallel while cysTMT SNO intensity was significantly reduced in the sequential protocol compared to the parallel experiments. **B**, Western blot (WB)-analysis of TMT-switch assay indicating non-specific labeling was increased with the sequential labeling protocol.

A



B

Type of metal		No metal			Fe				Cu			Cu+Fe		
Label		cys	iodo	combo	cys	iodo	combo	combo (2X)	cys	iodo	combo	cys	iodo	combo
TMT signal intensity	NO-induced	++	++	+++	++	<+	+	++ ~+++	++	+++	- ~ +	++	+++	++
	Untreated (control)	-	- ~ <+	+	-	<+	-	+	-	++++	- ~ +	-	+++	+ ~ ++
	Effective SNO	++	~++	++	++	-	+	+ ~++	++	-	< +	++	-	<+
	Expected SNO	++	++	++++	++	++	++	++++	++	++	++++	++	++	++++

Online Figure VIII. Analysis of simultaneous cys- and iodoTMT labeling in the TMT-switch assay. A, Simultaneous labeling was evaluated in various conditions including the addition of metals to augment ascorbate reduction[12] best optimized for cysTMT. These conditions were not compatible; labeling intensity was reduced compared to the sum of the optimized single cys- and iodoTMT labeling conditions. Additionally, non-specific labeling increased in simultaneous labeling. 'Combo' experiments were performed using 0.5x the standard concentration for each reagent, while 'combo (2X)' experiments used concentrations for each reagent that matched those of a single label experiment. Both cys- and iodoTMT were immunoblotted with the anti-TMT antibody. Metal concentrations were 1mmol/L $CuSO_4$, 0.1 μmol/L $FeSO_4$ or combination of 10 μmol/L $CuSO_4$ and 0.1 μmol/L $FeSO_4$. **B**, Summary of WB analysis for various labeling conditions anti-TMT signal from WB-analysis was indicated as +; 'combo' denotes simultaneous labeling by cys- and iodoTMT. Effective labeling was determined by comparison of the untreated control signal with the NO-induced.

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Online Figure IX. Libra condition file. When the isobaric tags are released from the manufacturer, each isobaric tag is not 100% pure. Based on the information provided by the manufacturer, correction factors were included in the libra condition file when searching for TMT reporter ion intensities (A: cysTMT labeled samples, B: iodoTMT labeled samples).

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