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Global, *in situ*, site-specific analysis of protein S-sulphenylation

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Abstract

Protein S-sulphenylation is the reversible oxidative modification of cysteine thiol groups to form cysteine S-sulphenic acids. Mapping the specific sites of protein S-sulphenylation onto complex proteomes is crucial to understanding the molecular mechanisms controlling redox signaling and regulation. This protocol describes global, *in situ*, site-specific analysis of protein S-sulphenylation using sulphenic acid-specific chemical probes and mass spectrometry-based proteomics. The major steps in this protocol are (1) optimization of conditions for selective labeling of cysteine S-sulphenic acids in intact cells with the commercially available dimedone-based probe, DYn-2, (2) tagging the modified cysteines with a functionalized biotin reagent containing a cleavable linker via Cu^I-catalyzed azide-alkyne cycloaddition reaction, (3) enrichment of the biotin-tagged tryptic peptides with streptavidin, (4) liquid chromatography-tandem mass spectrometry (LC-MS/MS)-based shotgun proteomics and (5) computational data analysis. We also outline strategies for quantitative analysis of this modification in cells responding to redox perturbations and discuss special issues pertaining to experimental design of thiol redox studies. Our chemoproteomic platform should be broadly applicable to the investigation of other bioorthogonal-chemically engineered posttranslational modifications. The entire analysis protocol takes about 1 week to complete.

Introduction

The oxidation of protein cysteinyl thiols to S-sulphenic acids (-SOH), also called protein S-sulphenylation, is a post-translational modification (PTM) that is important for redox regulation and signaling^{1, 2}. This intrinsically unstable modification can be formed either by

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The authors declare no competing financial interests.

two-electron oxidation of thiols or by hydrolysis of stable cysteine modifications^{3, 4}. The products of protein S-sulphenylation are intermediates that undergo further redox reactions that yield more stable oxidation products. For example, S-sulphenylcysteines can be over-oxidized to sulphinic acids (-SO₂H) or sulphonic acids (SO₃H) or react with nearby cysteinyl thiols to form disulfides, all of which influence protein structure, activity, interaction, and localization^{5–10}. Nonetheless, it has also been suggested that protein S-sulphenic acid redox state can be stabilized by the microenvironment on proteins^{11–14}. The chemistry and biology of protein S-sulphenylation has been summarized in several comprehensive reviews^{4, 15}.

Identification of the specific sites of protein S-sulphenylation on a protein is crucial to understanding the molecular mechanisms of redox control. In the past, formations of cysteine S-sulphenic acids on proteins were detected indirectly, through measurement of cysteine reactivities toward hydrogen peroxide (H₂O₂). Accordingly, proteomic techniques for large-scale redox profiling of cysteine oxidative modifications employed indirect analyses based on differential labeling of protein thiols^{16–25}. These techniques rely on high concentrations of thiol alkylating reagents, which may react with protein S-sulphenic acids as well^{26, 27}. Moreover, these analysis methods also require specific reducing reagents for distinct cysteinyl redox modifications, and there isn't an available reagent for protein S-sulphenylation yet. These redox proteomic techniques are, therefore, not suitable for proteomic profiling of protein S-sulphenylation, even though they have been successfully applied to study cysteine oxidation stoichiometry and distinct oxidation such as S-glutathionylation or S-nitrosylation.

Coincident with the progresses of redox proteomics is the rapid growth of reagents for direct probing and detection of distinct redox modifications. Reagents for direct labeling of S-sulphenic acid groups on proteins take advantage of its distinct chemical reactivity, which exhibits both nucleophilic and electrophilic character^{4, 15, 28–30}. Cyclic 1,3-diketone carbon nucleophiles, such as 5,5-dimethyl-1,3-cyclohexadione (Dimedone) have proven useful for efficient and selective labeling of protein S-sulphenylation²⁸. Thus, most previously reported methods for detecting S-sulphenylated proteins are built upon this chemistry. For example, fluorophore- or biotin-functionalized dimedone analogs have been developed and successfully used for detecting protein S-sulphenylation in cell lysates or isolated tissues^{29, 30}. However, biotinylated and fluorophore-labeled probes have two major limitations: (1) poor cell permeability; and (2) steric encumbrance that limits access to buried sulphenic-acid groups on folded proteins.

To overcome this problem, we developed a cell-permeable alkyne-tagged dimedone analog, DYn-2, which enables *in situ* labeling of S-sulphenylated proteins (Fig. 1a)³¹. The DYn-2 labeled proteins can then be bio-orthogonally conjugated to a fluorophore or biotin via Cu^I-catalyzed azide-alkyne cycloaddition reaction (CuAAC or click chemistry) and visualized in living cells or gels/blots (Fig. 1b, See Ref.³² for detailed experimental considerations). This probe has proven useful for interrogating the roles of protein S-sulphenylation in redox adaption process and growth factor signaling in living cells^{31, 33}. However, proteome-wide and site-specific analysis of DYn-2 labeled protein S-sulphenylations in cells remains a

major challenge. To address this challenge, we developed a highly efficient site-centric chemoproteomic platform³⁴.

In this protocol, we describe the step-by-step procedures and troubleshooting guidelines for global, *in situ*, site-specific analysis of protein S-sulphenylation using our site-centric chemoproteomic workflow. By using this protocol, we were able to identify hundreds of distinct endogenous protein S-sulphenylation sites from single samples³⁴. Here we also outline strategies for quantitative analysis of this modification in cells under redox perturbations. In addition, we discuss adaptations of our chemoproteomic workflow for investigation of other bioorthogonal-chemically engineered posttranslational modifications.

Key elements of site-centric chemoproteomics

In principle, alkyne tagged proteins or peptides can be conjugated with azide-tagged biotin reagents via CuAAC, enriched by streptavidin, and subjected to proteomics analysis (Fig. 1b). However, because of the strong binding interaction, biotinylated proteins are usually eluted from streptavidin only by harsh conditions (detergents, heat, organic solvents), which result in co-elution of nonspecifically bound proteins or peptides. Moreover, the biotin moiety may compromise the ionization of modified peptides and affect their MS/MS fragmentation, thereby interfering with site localization.

To circumvent these limitations, we and others have developed azide-tagged biotin reagents with various cleavable linkers^{35–41}. Most of these reagents have been used mainly in protein-centric chemoproteomics studies to generate protein-level identifications of putative modification targets^{42–47}. Application of site-specific profiling has been restricted to characterization of reactive thiol proteomes in cell or tissue lysates with alkyne tagged alkylating reagents^{48–50}. In these studies (Fig. 2a), alkyne tagged protein is typically conjugated with an azido biotin reagent containing a cleavable linker and then captured by streptavidin beads. After tryptic digestion, the modified peptides are eluted selectively and analyzed by LC-MS/MS. However, the efficiency for enriching modified peptides may be compromised mainly by incomplete alkyne-azide conjugation under aqueous conditions, especially when tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine (TBTA) is used as the reaction ligand (See ref.60, 61). In addition, potential chemical instability of some cleavable linkers could lead to significant loss of modified protein or peptides under digestion condition.

To address these issues and to improve the efficiency of enrichment for labeled peptides, we introduced a complementary workflow (Fig. 2b), which is described in the procedure of this protocol. Alkyne-tagged proteins are first digested into tryptic peptides. After reaction with azidobiotin containing a photocleavable linker, modified peptides are captured with streptavidin and eluted by photolysis for LC-MS/MS analysis. With this workflow, the percentage of S-sulphenylated peptides in all identified peptides is around 20%³⁴. For comparison, a prior proteomic analysis of S-sulphenylated peptides tagged with dimedone without affinity enrichment, only 0.2% of the final identifications are S-sulphenylated peptides⁵¹. This indicates that the sensitivity of our approach for detecting S-sulphenylated peptides is 100-fold higher than that of a method without enrichment.

Experimental design

Overview—The protocol has five major steps (1) selective labeling of cysteine S-sulphenic acids in intact cells with the commercially available dimedone-based probe, DYn-2, (2) tagging the modified cysteines with a functionalized biotin reagent containing a photocleavable linker via click chemistry, (3) enrichment and photorelease of biotin-tagged tryptic peptides with streptavidin, (4) liquid chromatography-tandem mass spectrometry (LC-MS/MS)-based shotgun proteomics and (5) computational data analyses. For the best results, the following aspects should be considered.

***In situ* labeling of protein S-sulphenylation**—The procedure described was the one optimized for analyzing S-sulphenylation targets from RKO cells, which are rapidly growing colon carcinoma cells. An important consideration is that the input material needed for such analysis is reasonably large ($2\sim3\times10^8$ cells), as for most of PTM studies^{52–54}. Thus, rapidly growing cell models can quickly afford sufficient amounts of protein for method testing. Labeling conditions such as probe concentration and incubation time should be optimized for each new cell line. In addition, to maximize the labeling efficiency, we typically release cells from culture plates and label endogenous protein S-sulphenylation in cell suspension. For this reason, the viability of released cells should be routinely monitored, the density of the cell suspension should typically be lower than 5×10^6 cells per ml, and cells in suspension should be gently mixed periodically to avoid cell clumping prior to the labeling process. It is noteworthy that the final result can depend on redox characteristics of different cell types and whether or how a stimulus is used.

Cell lysis, reduction and alkylation—To reduce the possibility of artifactual, sample preparation-related modification and to minimize sample loss, we combine cell lysis and reduction into a single step. We then alkylate all reduced free cysteine residues in the proteome with iodoacetamide to avoid protein aggregation during digestion. The excess chemicals can be simply removed by protein precipitation.

Trypsin digestion—The complete and specific proteolytic cleavage of protein samples into peptides is crucial for the success of every site-centric PTM experiment. Thus, in this protocol, we perform double tryptic digestion to facilitate the most reproducible and complete digestions possible. However, by using other orthogonal or multiple digestion approaches^{55–57}, one may obtain more comprehensive protein cleavage and further increase the site identifications.

CuAAC (Click chemistry)—To date, tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine (TBTA) is the most utilized Cu-binding ligand for CuAAC acceleration, mainly because of its commercial availability. Most previously described CuAAC protocols using the TBTA ligand are associated with slow kinetics in aqueous solution^{58, 59}. However, it has also been suggested that the potency of this ligand can be dependent on the nature of solvent and the ligand: Cu ratio⁶⁰. To improve the efficiency of click derivatization of DYn-2-labeled peptides, we provide an optimized CuAAC protocol which has three features: (1) TBTA and the other reagents are dissolved thoroughly in the reaction solvent containing ~30% acetonitrile in mild acid, (2) the volume of reaction mixture is kept as small as possible once

all the contents are dissolved, (3) a high catalyst concentration is used. In principle, other cleavable azido biotin reagents could also be applicable to this CuAAC protocol, except for reagents with an acid-cleavable linker. To further increase yields of the biotinylation reaction, one may also explore the use of new copper ligands with higher catalytic efficiency than TBTA.

Strong cation exchange (SCX)—Since relatively high concentrations of reactants are used in our CuAAC protocol, the major challenge we faced is removal of excess click reagents, especially biotin related chemicals, from the peptide mixture before the streptavidin capture step. We adopted strong cation exchange (SCX), which has proven useful to separate proteins or peptides from unreacted biotin reagents⁵³. Typically, peptides from CuAAC reaction mixtures are positively charged in acidified SCX loading buffer (pH 3.0, see Reagent Setup), retained on SCX columns, and separated from the neutrally charged free biotins. To reduce the sample complexity for the downstream LC-MS/MS analyses, the peptides can be released by a series of buffers with varying salt concentration. We have also found that SCX fractionation before streptavidin enrichment can significantly increase the number of S-sulphenylated peptides identified.

Streptavidin capture and enrichment—After the SCX cleanup step, we use streptavidin sepharose beads to capture biotinylated peptides. Of the commercially available streptavidin beads, only those with magnetic forms are not recommended, as they are not compatible with the subsequent photorelease step. After the 2h affinity capture reaction, non-biotinylated peptides and other nonspecifically bound substances are removed by stringent washing steps. All the solutions used for streptavidin capture and enrichment are adjusted to mildly acidic pH (pH 4.5) to minimize potential hydrolysis of the photocleavable benzoin ester⁶¹. Direct light exposure should also be minimized at this stage.

Photorelease—To ensure the efficiency of the photorelease, the washed streptavidin beads should be resuspended in at least 5 volumes of clear buffer solution, transferred into thin walled borosilicate glass tubes and irradiated with 365 nm UV light at a distance of 1–3 cm. Moreover, the photorelease should be performed with magnetic stirring to prevent precipitation of the streptavidin beads.

LC-MS/MS analysis—To generate high quality MS/MS data for the downstream informatics pipeline, we evaluated the capability of two individual fragmentation modes, HCD (higher-energy collision dissociation)⁶² and CID (collision induced dissociation) for analyzing the photoreleased S-sulphenylated peptides. The HCD mode not only yields higher numbers of identified sulphenylated peptides relative to CID mode, but also generates more abundant sequence fragment ions for modified peptide identification and site localization (Fig. 3a). Notably, a panel of diagnostic fragment ions (DFIs) with relatively high abundance can be observed in HCD MS/MS spectra of S-sulphenylated peptides (Fig. 3a), which can be utilized to virtually eliminate false-positive identifications, which have been a major problem in many proteome-wide PTM studies. Although DFIs are also present in CID spectra, their abundance is much lower (Fig. 3b). Numbers of peptide identifications

can also be increased with other practical approaches used in proteomic analyses, including increasing the peak capacity with long LC columns or gradients.

Informatics pipeline—We analyze the MS data from qualitative and quantitative S-sulphenylome analyses using the well-established informatics tools routinely used in our laboratory^{63, 64}. Typically, RAW data from LC-MS/MS is analyzed with the BumberDash search software suite, which incorporates the DirecTag and TagRecon algorithms^{65, 66}. DirecTag is used to generate short amino acid tags for each MS/MS in the RAW file⁶⁷. TagRecon is used to match the tags to candidate peptide sequences from the protein database⁶⁸. Typically, we query the peptide sequences against a decoy protein database consisting of forward and reversed human RefSeq database for anticipated modifications. The decoy database is necessary for calculating false discovery rates of identified peptide sequences and assessing the confidence of accepted peptide identifications. The resulting pepXML file from DirecTag-TagRecon search is then imported into IDPicker software for assigning each modified peptide to its corresponding protein sequence⁶⁹. Other informatics pipelines for proteomics should be also suitable for such analysis. Moreover, to obtain more comprehensive peptide identifications, one may utilize multiple database search engines or pipelines. Additionally, to manually validate peptide-spectrum matches and site localizations, we designed a decision-tree approach in our published work (See ref.34). For investigators new to MS-based proteomics, we strongly recommend two streamlined protocols for manual evaluation of automated peptides identifications from proteomic tandem mass spectra^{70, 71}.

Replicates—We suggest using at least two biological replicates per S-sulphenylome analysis experiment. If the sample amount permits, subject the sample to multiple replicate LC-MS/MS injections. Increasing the number of biological or technical replicates usually increases the number of identified S-sulphenylation sites.

Quantitative strategy

For quantitative determination of dynamic S-sulphenylome changes under redox perturbations, we typically use light and heavy(deuterated, d6) DYn-2 probe pairs to label cells with or without stimuli and measure the light to heavy ratio of modified peptides using MS1 filtering in a user-friendly software for quantitative proteomics, Skyline^{72, 73}. The accuracy and reproducibility of this approach for quantitative S-sulphenylome analysis has been demonstrated by our previous work³⁴.

Our chemoproteomic workflow should also be applicable to other quantitative proteomic pipelines using metabolic or chemical labeling strategies, such as SILAC⁷⁴, or even label-free methods. However, it is important to stress that the use of chemical labeling strategies, like ICAT⁷⁵ or iTRAQ⁷⁶, is likely to increase the complexity of their MS/MS spectra. For analyses in which label-free quantitative methods are employed, samples from different conditions should be prepared in parallel and normalized by spike-in of at least one synthetic alkyne modified peptide in tested samples to control for process variability.

An important consideration in quantitative PTM studies is the possible confounding effect of protein abundance changes. Our studies focused on very short-term oxidative perturbations

(i.e., treatments of a few minutes) of the S-sulphenylome in cell models, which preclude significant protein abundance changes. For studies involving treatments on longer time scales (e.g., greater than 1–2 hr), it may be necessary to quantify possible changes in protein abundance and then determine their contribution to altered levels of S-sulphenylated peptides.

Limitations of our approach

Although this chemoproteomic approach has dramatically expanded our ability to analyze the S-sulphenylome in cells, there are potential limitations to the approach. The first comes from this chemical labeling method, which unavoidably misses some S-sulphenylated targets, owing to steric effects or other factors such as modest reaction kinetics of DYn-2 probe that compromise the labeling reaction efficiency. Moreover, as with other large-scale PTM studies, this approach requires a relatively high amount of input protein, as well as experimental materials, such labeling reagent, trypsin and SCX spin columns, etc. Additionally, there are potential shortcomings for the quantitative strategy used in this protocol. For example, the use of a deuterated probe could complicate the quantitative analysis, since there is a slight difference in the elution times of light- and deuterated (heavy, d6)- tagged peptide pairs, with the deuterated isotopomer typically eluting 1–2 s before the light isotopomer. Moreover, MS1 filtered chromatograms are subject to interference by remaining CuAAC-related chemicals or unknown isobaric substances. To overcome these problems, several stringency criteria were applied to ensure the high accuracy and reproducibility of quantification (See ref.34 for more information).

Adaptations of our approach

Our site-centric chemoproteomic approach should find broad applications in both chemical biology and drug discovery, although the presented protocol mainly describes its application specifically for global and site-specific identification of protein S-sulphenylation in cells. For example, one may adapt our approach to study other post-translational modifications of interest (such as glycosylation, methylation and fatty acylation, etc.) by metabolic incorporation of cells with other alkyne tagged probes⁷⁷. One may also use this strategy to identify specific targeting sites of covalent inhibitors modified with alkyne tag⁷⁸.

MATERIALS

REAGENTS

- RKO cell line (ATCC, CRL-2577). **CRITICAL** To avoid cell contamination or stress, cells should be cultured carefully in high-quality medium with aseptic technique. All culture dishes, flasks, tubes, and pipettes need to be freshly unpacked or sterilized before use. Moreover, cells should be cultured in appropriate plating density, split and passaged regularly.
- McCoy's 5A medium (Life Technologies, cat. no. 16600)
- Fetal bovine serum (FBS; Atlas biologicals, F-0500-A)
- Potassium phosphate buffer (PBS, pH 7.2; Life technologies, cat. no. 20012)

- 0.25% Trypsin-EDTA (Life Technologies, cat. no. 25200)
- (Optional) Hydrogen peroxide (H_2O_2 ; Sigma-Aldrich, cat. no. 216763) **!CAUTION** H_2O_2 is toxic if swallowed. It also causes severe skin burns and eye damage. Handle it with appropriate protection.
- DYn-2 (Cayman chemical, cat. no. 11220)
- (Optional) DYn-2-d6 (KeraFast, cat. no. EST009. It can also be synthesized as described previously³⁴.)
- DMSO (Sigma-Aldrich, cat. no. D8418)
- Double-distilled, deionized water (Millipore Milli-Q system) **CRITICAL** Milli-Q water is used for the preparation of all aqueous solutions and other relevant applications, except for click chemistry reaction, HLB desalting, SCX cleaning and LC-MS/MS.
- HEPES buffer, 1M solution (Cellgro, cat. no. 25-0660-CI)
- Igepal (Sigma-Aldrich, cat. no. 18896) **!CAUTION** Contact will cause irritation to skin and eyes. Extremely hazardous. Handle carefully with protective gloves and eye protection.
- Sodium chloride (NaCl; Sigma-Aldrich, cat. no. S7653)
- Dithiothreitol (DTT; RPI, cat. no. 30500) **!CAUTION** DTT solid is harmful by inhalation, on contact with skin and if swallowed.
- Iodoacetamide (IAA; Sigma-Aldrich, cat. no. I1149) **!CAUTION** IAA is toxic if swallowed. It may cause sensitivity by inhalation and skin contact.
- Bicinchoninic acid assay reagent A (Thermo Scientific, cat. No. 23221)
- Bicinchoninic acid assay reagent B (Thermo Scientific, cat. No. 23224)
- HPLC-grade methanol (J.T. Baker, cat. no. 9093-02) **!CAUTION** Methanol is toxic and flammable. Handle it in a fume hood.
- HPLC-grade water (J.T. Baker, cat. no. 4218-02)
- Chloroform (Sigma-Aldrich, cat. no. C2432) **!CAUTION** Chloroform is toxic and flammable. Handle it in a fume hood.
- Ammonium bicarbonate (NH_4HCO_3 ; Sigma-Aldrich, cat. no. A6141)
- Urea (Sigma-Aldrich, cat. no. U6504) **!CAUTION** Exposure causes irritation to the skin and eyes. Handle it with appropriate protection.
- Sequencing grade trypsin, frozen (Promega, cat. no. V5113)
- Trypsin gold (Promega, cat. no. V28A)
- HPLC-grade acetonitrile (Fisher Scientific, cat. no. A998-1) **!CAUTION** Acetonitrile is toxic and flammable. Handle it in a fume hood.

- Formic acid (FA; Fluka, cat. no. 06440) **!CAUTION** FA is harmful by inhalation. It causes severe burns to skin and eyes. Handle it in a fume hood with protective gloves and eye protection.
- Azido-photocleavable-biotin (Az-UV-Biotin, KeraFast, cat. no. EVU102. It can also be synthesized as described in Supplementary Information.)
- Sodium ascorbate (Sigma-Aldrich, cat. no. A7631)
- Tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine, TBTA (Sigma-Aldrich, cat. no. 678937)
- Copper sulfate (CuSO₄; Fisher Scientific, cat. no. C493-500)
- Monosodium phosphate (NaH₂PO₄; Sigma-Aldrich, cat. no. S9638)
- Sodium acetate (NaAc; Sigma-Aldrich, cat. no. 236400)
- Acetic acid (EMD Millipore, cat. no. AX0073) **!CAUTION** Acetic acid is harmful by inhalation. It causes severe burns to skin and eyes. Handle it in a fume hood with protective gloves and eye protection.
- Streptavidin sepharose high performance (GE, cat. no. 17-5113-01)

EQUIPMENT

- HERACell 150i CO₂ incubator for cell culture
- Automated cell counter (Bio-Rad, cat. no. TC10TM)
- Beckman Coulter Allegra 6R centrifuge for pelleting cultured cells and streptavidin beads
- Branson Sonifier 450 for cell lysing **!CAUTION** Sonication causes damage to hearing. Wear ear protection.
- Eppendorf Centrifuge 5417R
- Microcentrifuge tube, 2.0 ml (Fisher Scientific, cat. no. 02-681-321)
- Eppendorf thermomixer
- SPE extraction manifold, 20 positions (Waters, cat. no. WAT2006) **!CAUTION** Vacuum should not exceed 22 in. Hg.
- HLB 3cc (60mg) SPE cartridges (Waters, cat. no. WAT094226)
- HLB 1cc (10mg) SPE cartridges (Waters, cat. no. 186000383)
- Vacuum centrifugation device (Thermo Scientific, SPD 121P)
- Refrigerated vapor trap (Thermo Scientific, RVT400) **CRITICAL** To maximize the efficiency of vacuum centrifugation, the cold trap as well as pump oil should be changed periodically.
- SCX Macro spin column (The Nest Group, cat. no. SMM HIL-SCX)
- UV irradiation lamp (UVP, UVLMS-38 EL Series)

- Thin walled borosilicate glass vial (VWR, cat. no. 66011-020)
- VWR Galaxy mini centrifuge (cat. no. C1213)
- Spin-X centrifuge tube filter, 0.45µm Nylon, 2.0 ml (Corning, cat. no. 8170)
- Target DP Vials for LC sampling (Thermo Scientific, cat. no. C4000)
- Vial insert (Thermo Scientific, cat. no. C4010)
- NanoLC system (Thermo Fisher Scientific, Easy-nLC 1000)
- NanoLC pre-column bulk packing material (Phenomenex, Jupiter C18, 5 µm, 300 Å)
- NanoLC analytical column bulk packing material (Dr. Maisch, ReproSil-Pur C18-AQ, 3 µm, 120 Å)
- QExactive Mass spectrometer (Thermo Fisher Scientific) **CRITICAL** We mainly use this instrument because it yields higher numbers of identified peptides relative to the Thermo Orbitrap XL or Elite systems. An instrument with HCD capability is important for detecting DFI features, which confirm identifications. Another important aspect to be considered is that LC-MS/MS instrument need to be routinely maintained and calibrated to maximize its sensitivity and accuracy.

Software

- Peptide identification: BumberDash (DirecTag-TagRecon) (<http://fenchurch.mc.vanderbilt.edu/software.php>).
- Protein assembly and MS/MS visualization: IDPicker 3 (<http://fenchurch.mc.vanderbilt.edu/software.php>)
- (Optional) Relative quantification experiments: Skyline (<https://skyline.gs.washington.edu/labkey/project/home/software/Skyline/begin.view>)

REAGENT SETUP

Cell culture medium Add 50 ml FBS solution to 450 ml McCoy medium. This medium should be stored at 4°C and warmed to room temperature (RT, ~25°C) before use.

DYn-2 stock solution (1M) dissolve 25 mg DYn-2 solid in 560 µl DMSO. This solution should be saved at –80°C. Under this condition, it can be stored for at least 6 months.

(Optional) DYn-2-d6 stock solution (1M) dissolve 25 mg DYn-2-d6 solid in 560 µl DMSO. This solution should be saved at –80°C. Under this condition, it can be stored for at least 6 months.

Labeling medium (5 mM DYn-2) add appropriate volume of 1M DYn-2 stock solution in serum-free McCoy medium and make sure the content of DMSO is lower than 5%.

CRITICAL this buffer should be freshly prepared immediately before use.

Lysis buffer 50 mM HEPES, 150 mM NaCl, 1% Igepal, 8 mM DTT, pH 7.5. **CRITICAL** DTT should be added just before use.

DTT reduction buffer (400 mM DTT) dissolve 18 mg DTT in 300 μ l water. **CRITICAL** this buffer should be freshly prepared immediately before use.

IAA blocking buffer (800 mM IAA) dissolve 75 mg DTT in 500 μ l water. **CRITICAL** this buffer should be freshly prepared immediately before use.

Trypsin digestion buffer (25 mM NH_4HCO_3) dissolve 200 mg NH_4HCO_3 in 50 ml water. The pH of this buffer is typically around 8. **CRITICAL** this solution should be made freshly before use.

Urea solution (6 M urea) dissolve 3.6 g urea solid in 10 ml water. **CRITICAL** this solution should be made freshly before use.

HLB solvent A 80% acetonitrile, 10% methanol in HPLC-grade water. This buffer is stable for at least 2 month at RT.

HLB solvent B 10% of 0.1% FA in HLB desalting solvent A. This buffer is stable for at least 2 month at RT.

Azido-photocleavable-biotin stock solution (40 mM Az-UV-Biotin) dissolve 18 mg azido-photocleavable-biotin in 50 μ l DMSO and dilute with 450 μ l acetonitrile. This solution should be saved at -80°C . Under this condition, it can be stored for at least 6 months.

Ligand stock solution (50 mM TBTA) dissolve 13 mg TBTA in 50 μ l DMSO and then diluted with 450 μ l acetonitrile. This solution should be saved at -80°C . **CRITICAL** This solution should only be thawed immediately before the click chemistry reaction is performed.

Copper Sulfate solution (100 mM CuSO_4) dissolve 25.0 mg Copper Sulfate solid (MW 250) in 1.0 ml water. **CRITICAL** this solution should be made freshly before use.

Sodium ascorbate solution (100 mM) dissolve 10.0 mg Sodium ascorbate (MW 198) solid in 0.5 ml water. **CRITICAL** this solution should be made freshly before use.

SCX conditioning buffer (0.2 M monosodium phosphate, 0.3 M sodium acetate) dissolve 6.9 g monosodium and 10.2 sodium acetate in 250 ml water. Typically, the pH will be around 5.5. This buffer is stable for at least 2 month at RT.

SCX loading buffer (5 mM NaH_2PO_4 , 25% acetonitrile, pH3.0) dissolve 103.5 mg monosodium phosphate in 150 ml water and adjust pH to 3.0 with HCl, and finally add 50 ml acetonitrile. This buffer is stable for at least 2 month at RT.

0.4M SCX elution buffer (5 mM NaH_2PO_4 , 0.4M NaCl, 25% acetonitrile, pH3.0) dissolve 4.6g NaCl in 200 ml SCX loading buffer. This buffer is stable for at least 2 month at RT.

0.2M SCX elution buffer (5 mM NaH₂PO₄, 0.2M NaCl, 25% acetonitrile, pH3.0) dilute 0.4M SCX elution buffer in equal volume of SCX loading buffer. This buffer is stable for at least 2 month at RT.

0.1M SCX elution buffer (5 mM NaH₂PO₄, 0.1M NaCl, 25% acetonitrile, pH3.0) dilute 0.2M SCX elution buffer in equal volume of SCX loading buffer. This buffer is stable for at least 2 month at RT.

Streptavidin binding buffer (50 mM NaAc, pH4.5) dissolve 6.8 g sodium acetate in 1l water, use acetic acid to adjust pH to 4.5. This buffer is stable for at least 1 month at RT.

Streptavidin washing buffer (50 mM NaAc and 2M NaCl, pH4.5) dissolve 29 g NaCl in 250 ml Streptavidin binding buffer. This buffer is stable for at least 1 month at RT.

Photorelease buffer (25 mM NH₄HCO₃) dissolve 100 mg NH₄HCO₃ in 50 ml water. **CRITICAL** this solution should be made freshly before use.

LC-MS sampling buffer 0.1% FA solution in HPLC-grade water containing 5% acetonitrile. This buffer is stable for at least 2 month at RT.

EQUIPMENT SETUP

NanoLC column A 360 µm outer diameter × 75 µm inner diameter microcapillary precolumn is connected to a 360 µm outer diameter × 50 µm inner diameter microcapillary analytical column containing 11-cm resin and equipped with an integrated electrospray emitter tip (New Objective).

NanoLC separation condition We typically use the LC gradients outlined in the following table at a constant flow rate of 300 nl min⁻¹.

Standard gradient		Longer gradient	
Time interval (min)	LC gradient (%B)	Time interval (min)	LC gradient (%B)
0	2	0	5
15	2	10	5
35	15	100	35
40	20	120	90
50	30	130	90
55	35	140	5
59	90	150	5
65	90		
80	2		
85	2		

Mass spectrometer The spray voltage is set to 1.5 kV and the heated capillary temperature to 250°C. Full scan MS are acquired from *m/z* 300–1800 with a resolution of 70,000, an AGC target of 3e6. Top 12 most intense precursor ions are selected in the data-dependent

mode for HCD MS/MS analysis. HCD spectra are recorded with a resolution of 17,500, an AGC target of 2e5, and normalized collision energy of 28. Peptide m/z that triggered MS/MS scans are dynamically excluded from further MS/MS scans for 20 s.

PROCEDURE

CRITICAL STEP All centrifugation steps are done at room temperature, unless otherwise stated.

Selective probe labeling of protein S-sulphenylation in cells TIMING 3–4h

- 1 Grow RKO cells in 15 cm plates with McCoy medium containing 10% FBS to 70–80% confluence. If you are not working with oxidant-perturbed samples, continue to Step 4.

CRITICAL STEP Typically, one 15-cm plate of RKO cells (70–80% confluence, $\sim 1 \times 10^7$ cells) should generate ~ 1.5 mg protein. Each S-sulphenylome analysis requires 30 mg of total protein. We typically obtain this amount of protein from 20–24 15-cm dishes of RKO cells. It is important to mention that cells should be cultured carefully (i.e., regular schedule for splitting with no over-growing, no more than 20 passages, mycoplasma testing) to avoid cell stress.

- 2 (Optional) Before cell stimulation experiments, deprive cultured cells from serum overnight (14–16 h) at 37°C.
- 3 (Optional) For cell stimulation experiments, remove medium from serum-deprived cells, wash plates quickly three times with PBS, and then treat cells with serum-free media containing 500 μ M H₂O₂ or vehicle (PBS). Incubate at 37°C for 5 min.
- 4 Remove medium, wash plates quickly three times with PBS, lift cells in each plate with 3 ml 0.25% trypsin-EDTA and then add 5 ml serum-free media. Then transfer lifted cells into four 50 ml centrifuge tubes.

CRITICAL STEP To avoid cell clumping, lifted cells should be transferred immediately to 50 ml tubes, and gently mixed by pipetting up and down.

- 5 Spin down cells by centrifugation at 200 g for 3 min. Remove the supernatant after centrifugation and wash pelleted cells with PBS twice. Combine cells into a 50 ml centrifuge tube.
 - 6 Resuspend the pelleted cells with freshly made labeling medium (See Reagent Setup) containing 5 mM DYn-2 or DYn-2-d6 (Optional). For $\sim 2 \times 10^8$ RKO cells from ~ 20 15-cm plates, 40 ml 5 mM DYn-2 is typically needed.
- CRITICAL STEP** Make sure the density of the cell suspension is no more than 5×10^6 cells for the labeling experiment. For quantitative S-sulphenylome study, typically, un-stimulated cells are labeled with DYn-2 and H₂O₂-stimulated cells are labeled with DYn-2-d6.

- 7 Incubate the cell suspension at 37°C for 2 h with periodically (20–30 min) mixing by gently pipetting up and down.
- 8 Collect cells by centrifugation at 200 g for 3 min at 4°C. Wash the pelleted cells with cold PBS three times.

? TROUBLESHOOTING

PAUSE POINT Cells can be stored at –80°C for several weeks

Prepare lysates **TIMING 2h**

- 9 Add 2 ml cold lysis buffer to the cell pellet (See Reagent Setup).
CRITICAL STEP Typically, one volume of cell pellet is lysed by mixing with three volumes of lysis buffer. To avoid sample-preparation related modification, we typically add DTT to the lysis buffer. If another lysis buffer system is used, it should also contain DTT or other reducing reagents.
- 10 Lyse the cells on ice with sonication and then leave them on ice for 30 min.
- 11 Incubate the cell lysate for 30 min at 55°C with vortex mixing and then cool to room temperature (RT, ~25°C).
- 12 Carbamidomethylate reduced cysteine residues by adding 100 µl IAA blocking buffer (See Reagent Setup) at a final concentration of 32 mM. Incubate the sample for 20 min at RT with light protection and rotation.
- 13 Use a BCA assay to determine the protein concentration. If you are not working with DYn-2-d6 labeled samples, continue to Step 14. The typical protein concentration of cell lysate should be around 15 mg ml⁻¹. Dilute the lysate solution with PBS to achieve a protein concentration of 7.5 mg ml⁻¹.
- 14 (Optional) For quantitative S-sulphenylome analysis, prepare a single solution by mixing an equal amount of protein (by weight) from DYn-2 and DYn-2-d6 labeled samples.
- 15 Transfer the protein sample (7.5 mg ml⁻¹) to five 2.0 ml centrifuge tubes (800 µl each). Add 800 µl cold methanol and 200 µl chloroform to each tube to quench the alkylating reaction and precipitate proteins (lysate: methanol: chloroform = 4: 4: 1, vol/vol/vol).
CRITICAL STEP To maximize recovery of proteins, we tested different solvent systems, including methanol, acetonitrile, and methanol/chloroform mixture. Acetonitrile generally allows most efficient protein precipitation, but the pelleted proteins are extremely difficult to resuspend. Methanol-precipitated protein can be more easily resuspended, but the precipitation is incomplete. The methanol/chloroform mixture not only allows efficient protein precipitation, but also generates a protein pellet that can be resuspended.
- 16 Pellet proteins by centrifugation at 1700 g for 20 min at 4°C. Save the solid at the phase interface, and then wash the pellet with methanol/chloroform (1:1) twice (sonicate, then centrifuge at 20,000 g for 10 min at 4°C).

- 17 Evaporate protein pellet briefly to near dryness at 37°C by thermomixer.

PAUSE POINT Protein pellet can be stored at –80°C for several weeks

Tryptic digestion TIMING 1d

- 18 Dissolve protein pellet in each centrifuge tube with 1 ml trypsin digestion buffer (See Reagent Setup) by sonication. After this point, pool all aliquots in a 15 ml tube.

CRITICAL STEP Add 0.1 M urea as final concentration to dissolve proteins if necessary.

- 19 Use the BCA assay to determine the protein concentration. Dilute the protein suspension with trypsin digestion buffer (See Reagent Setup) to achieve a protein concentration of 2 mg ml⁻¹. Typically, the final volume of protein sample made for tryptic digestion is ~12 ml.

- 20 For the first trypsin digestion, use an enzyme-to-substrate ratio of 1:50 (wt/wt) and incubate at 37°C overnight (typically 14–16 h). For the second trypsin digestion, use an enzyme-to-substrate ratio of 1:100 (wt/wt) and incubate at 37°C for 4 h.

? TROUBLESHOOTING

- 21 Centrifuge at 20,000 g for 5 min to remove any material that did not go into solution and save the supernatant (>10 ml). Reduce the volume of digests to about 2 ml by vacuum centrifugation.

Peptide desalting TIMING 4–5h

- 22 Use four 60 mg HLB SPE cartridges with a vacuum manifold to desalt peptide samples.

CRITICAL STEP The choice of cartridge size should be based on sample input amount. The maximum mass capacities and typical sample volumes for 60 mg HLB SPE cartridges are 2–10 mg and 0.2–2 ml, respectively.

- 23 Condition the cartridges with 2 ml HPLC-grade acetonitrile by gravity flow.

- 24 Equilibrate the cartridges with 4 ml HPLC-grade water by gravity flow.

- 25 Load the sample (~500 µl) onto each cartridge by vacuum.

- 26 Wash the cartridges with 2 ml HPLC-grade water by vacuum.

- 27 Elute peptides with 1 ml HLB solvent A and 0.5 ml HLB solvent B (see Reagent Setup) by gravity flow.

? TROUBLESHOOTING

- 28 Evaporate the eluted peptide samples to dryness by vacuum centrifugation.

CRITICAL STEP In order to get a dry, fluffy peptide sample that is easily reconstituted, freeze the peptides samples at -80°C first and then dry these samples (1.5–2 ml per tube) by vacuum centrifugation for approximately 3 h.

PAUSE POINT Dried peptide samples are stable and can be stored at -20 or -80°C for several weeks.

CuAAC TIMING 3h

- 29** For CuAAC reaction, use the reaction formulation outlined in the table below.

Order	Reagent	Concentration (mM)	Adding volume (μl)
1	H ₂ O	-	360
2	Acetonitrile	-	210
3	Az-UV-Biotin	40	15
4	Sodium ascorbate	100	60
5	TBTA	50	15
6	CuSO ₄	100	60
Total Volume			720

CRITICAL STEP Before adding Az-UV-Biotin, check the pH of the mixture using a pH indicator strip. If the last desalting step is performed properly, the pH of the reaction mixture should be typically around 6. However, if not, adjust pH with FA or NH₄OH. It is worth mentioning that this reaction formulation is typically used for peptide samples generated from 30 mg input material. If different input sample materials are used and/or other click reagents are used for this reaction, the solvent type or volume as well as ratios of these reactants will need to be adjusted.

? TROUBLESHOOTING

- 30** Incubate the reaction mixture at RT for 2 h with rotation; protect the sample from light by wrapping in foil.

CRITICAL STEP Although our photocleavable linker (benzoin ester) is selectively sensitive to certain wavelength near UV, it is still necessary to protect it from direct light exposure, thereby avoiding photochemistry with low quantum efficiency. After 2 h, the color of the reaction mixture will turn into dark green because of Cu^{II} is reduced to Cu^I by sodium ascorbate.

- 31** Quench the CuAAC reaction by adding 4 volumes of SCX loading buffer (See Reagent Setup)

PAUSE POINT Centrifuge at 20,000 g for 5 min at RT and use the supernatant for Step 37, or save them at -80°C for up to several weeks.

Remove excess click reagents by SCX TIMING 3h

- 32** Use MacroSpin SCX columns with an Eppendorf 5417R centrifuge to remove excess click reagents. For sample generated from 30 mg starting material, we typically use 24 MacroSpin SCX spin columns (See Supplemental Fig. 1a). We could typically process 24 SCX columns in two separate runs using an Eppendorf 5417R centrifuge (See Supplemental Fig. 1b). A check list for this step is provided in Supplementary Information. Moreover, in order to minimize the processing errors, we typically use a VIAFLO electronic multi-channel pipette to aspirate and transfer solutions or buffers used for SCX cleaning steps (See Supplemental Fig. 1c).
- 33** Condition the SCX spin-columns with 500 μ l of HPLC-grade methanol by centrifugation at 110 g for 5 min in several 2.0 ml Eppendorf micro centrifuge tubes.
- 34** Condition the SCX spin-columns with 500 μ l of HPLC-grade water by centrifugation at 110 g for 5 min to prevent salt precipitation. Repeat once.
- 35** Condition the SCX spin-columns with 500 μ l SCX conditioning buffer (See Reagent Setup) by centrifugation at 110 g for 30 sec, and let stand in the tube for at least one hour prior to its initial use.
- 36** Remove the excess SCX conditioning buffer by centrifugation at 110 g for 4 min and equilibrate the SCX spin-columns with 500 μ l SCX loading buffer (See Reagent Setup) by centrifugation at 110 g for 5 min. Repeat two more times.
- 37** Load the samples by adding 150 μ l of quenched click reaction mixture onto each SCX spin-columns. Centrifuge the columns at 110 g for 2 min.

? TROUBLESHOOTING

- 38** Wash the SCX spin-columns with 150 μ l SCX loading buffer by centrifugation at 110 g for 2 min. Repeat this step twice to wash out any trace impurities or un-retained detergent.
- 39** Remove the collecting tube and blot dry any moisture on the exterior of the column. Add 150 μ l SCX elution buffers containing different concentration of NaCl (0.1, 0.2, and 0.4 M, See Reagent Setup) and place it in new 2 ml centrifuge tubes. Spin as above.

CRITICAL STEP For quantification experiment, we typically elute peptides from SCX columns only with 0.4M SCX elution buffer to reduce the complexity of data analysis.

- 40** Dilute the eluted liquid with streptavidin binding buffer (See Reagent Setup) by at least 8 fold.

CRITICAL STEP The relatively high concentration of acetonitrile in the SCX elutes is likely to affect the following streptavidin capture and enrichment. Thus, they need to be diluted. We typically combine the same elutes from 12 SCX

columns together (1.8 ml), transfer to six 15 ml tubes, and add 10 ml streptavidin binding buffer to each tube.

Streptavidin Capture and Photorelease TIMING 5–6h

- 41** Wash 2 ml streptavidin slurry with PBS twice and resuspend the streptavidin in streptavidin binding buffer (See Reagent Setup, final volume ~3 ml).
- 42** Add 500 µl prewashed streptavidin slurry to each 15 ml tube containing the SCX elutes and incubate for 2h at RT with end-to-end rotation and light-protection.
- 43** Wash streptavidin beads with 10 ml streptavidin binding buffer (See Reagent Setup) and incubate for at least 15 min at RT with light-protection. Repeat this step once.
- 44** Wash streptavidin beads twice with 10 ml streptavidin washing buffer (See Reagent Setup).
- 45** Wash streptavidin beads twice with 10 ml water.
- 46** Resuspend streptavidin beads with 5 volumes of photorelease buffer (See Reagent Setup).
- 47** Transfer the streptavidin suspensions to several thin walled glass tubes. Irradiate with 365 nm UV light for 2h with stirring.
- 48** Spin down streptavidin beads at 1700 g for 3 min using Eppendorf centrifuge and save the supernatant.
- 49** Remove excess streptavidin beads in the supernatant by Spin-X centrifuge tube filters with 0.45 µm nylon membrane using benchtop centrifuge.
- 50** Reduce the volume of each photoreleased sample to about 100 µl by vacuum centrifugation.

Final Desalting TIMING 3–4h

- 51** Use 10 mg HLB SPE cartridges to desalt peptide samples.
- 52** Condition the cartridges with 1 ml HPLC-grade acetonitrile by gravity flow.
- 53** Equilibrate the cartridges with 2 ml HPLC-grade water by gravity flow.
- 54** Load the sample (~100 µl) onto the cartridges by gravity flow.
- 55** Wash the cartridges with 1 ml HPLC-grade water by gravity flow.
- 56** Elute peptides with 0.5 ml HLB solvent A and 0.25 ml HLB solvent B (see Reagent Setup) by gravity flow.
- 57** Dry the eluted peptide samples by vacuum centrifugation for 2–3h.

PAUSE POINT Dried peptide samples are stable and can be stored at –20 or –80°C for several weeks.

LC-MS/MS TIMING variable (dependent on the number of samples and runs)

- 58** Reconstitute the dried peptide samples in 15 µl LC-MS/MS sampling buffer (See Reagent Setup)
- 59** Centrifuge the samples at 20,000 g for 10 min to remove any material that did not dissolve and place the vials in the LC autosampler, which is cooled to 4°C.
- CRITICAL STEP** After centrifugation, aspirate carefully the supernatant from the sample without touching the bottom of the Microcentrifuge tube.
- 60** Analyze each sample using the LC-MS/MS method parameters outlined in the Equipment Setup. For each analysis, we typically inject 2 µl of each sample at a flow rate of 300 nl min⁻¹.

? TROUBLESHOOTING

Peptide and protein Identification TIMING variable (dependent on the number of samples)—Major parameters used for S-sulphenylated peptide identification and protein assembly are listed in the table below.

Parameters	Settings
Database	Decoy human RefSeq database
Precursor ion mass tolerance	0.01 Da
Fragment ion mass tolerance	0.1 Da
Maximum number of modifications	3
Maximum number of missed cleavages	3
Maximum number of charge	4
Dynamic modification	Methionine oxidation (+15.9949 Da) Carbamidomethylation (+57.0214 Da) DYN-2-triazohexanoic acid modification (DTH, +333.1689 Da, optional DTH-d6, +339.2065)
Maximum Q value of PSM	0.02
Maximum spectra per match	1
Maximum spectra per peptide	1
Maximum distinct peptides	1
Maximum additional peptides	0

- 61** Import RAW data from LC-MS/MS analysis into BumberDash search software suite (Supplementary Fig. 2). Choose DirecTag-TagRecon as the peptide sequence search engine under “Type of search” and load the decoy protein database. The typical configuration dialogue and parameter settings of DirecTag-TagRecon are shown in Supplementary Fig. 3–4.
- 62** Import the resulting pepXML file from DirecTag-TagRecon search into IDPicker software. The maximum Q value or FDR of peptide-spectrum matches (PSMs) is typically set as 0.02 to achieve either a peptide or a protein false discovery rate (FDR) no greater than 5%. The typical configuration dialogue and parameter settings of IDPicker are shown in Supplementary Fig. 5.

Manual inspection TIMING variable (dependent on the number of identifications)

- 63 Manually inspect the spectra using a streamlined decision-tree approach (See ref.34). We typically visualize the automatically annotated MS/MS spectra with the SeeMS tool embedded in IDPicker software. We require that the matched spectra to the identified S-sulphenylated peptides contain the major three diagnostic fragment ions (m/z 368.16, 366.15, and 336.19, see Fig. 3a) and can be properly annotated. Moreover, if the identified peptides contain more than one cysteine residue, we require at least three DYN-2-triazohexanoic acid modification-specific fragment ions appeared in the corresponding spectrum for unambiguous site localization.

CRITICAL STEP Although most of current strategies for estimating site FDR such as the probability-based method are simple and effective, they are more applicable to very large-scale PTM dataset (10,000s matched IDs, e.g., phosphorylation and ubiquitination sites). When the number of matches being evaluated is relatively small (hundreds of IDs), these methods become inaccurate because of the inadequate sample size. In order to ensure accurate site localization, we therefore perform manual evaluation of all the automatically identified MS/MS spectra, rather than automatic estimation of site FDR. Investigators new to MS-based proteomics can also refer to several streamlined guidelines for manual evaluation of PTM identifications (See ref. 67, 68) or expert systems for computer-assisted annotation of MS/MS spectra.

(Optional) Quantification TIMING variable (dependent on the number of samples)

- 64 Use the isotopic signatures of light and heavy isotope-labeled peptides to quantitatively compare protein S-sulphenylation levels between two conditions (e.g., treated with or without oxidants). We analyze data from quantification experiments of protein S-sulphenylation using MS1 filtering module in Skyline software (Version 1.2 or later) according to its tutorial on the following website: https://skyline.gs.washington.edu/labkey/wiki/home/software/Skyline/page.view?name=tutorial_ms1_filtering. In this protocol, major parameters or criteria used for MS1 filtering are listed in the table below. Following data extraction, the ratios of peptide areas of heavy modified peptides to their light isotopomers ($R_{H:L}$) can be calculated automatically and manually evaluated if necessary. Typically, only the most abundant isotope for each peptide is used for quantitation. Moreover, quantitation results are usually obtained from at least two biological replicates. Of note, there are a number of other in-house built or commercially available programs that can process the quantification results in a similar manner.

Parameters/criteria	Settings
Precursor/Product ion mass	Monoisotopic
Ion types	P
Precursor charges	2,3,4
Precursor mass analyzer	Orbitrap
Resolving power	70,000 at 400 Th
Retention time window	3 min
Minimum isotopic dot product scores	0.8
Minimum signal to noise (S/N)	5

CRITICAL STEP As mentioned in the Experimental Design, the use of a deuterated probe could complicate the quantitative analysis, but this limitation could be largely overcome by a cut-off setting of the Skyline isotopic dot product scores of the top three isotopic chromatographic peaks of MS1 filtered peptides. Moreover, quantifiable peptides are not necessarily identified by a peptide-spectrum match (PSM) in every replicate or run, since MS/MS spectra are obtained in data-dependent acquisition mode. In cases where a probe-modified peptide was identified in one run, but not in another, the above settings can enable accurate localization and proper integration of the corresponding peak in the run without the peptide identification.

? TROUBLESHOOTING

Troubleshooting advice can be found in Table 1.

TIMING

Step 1–8, selective labeling protein S-sulphenylation in cells: 3–4 h

Step 9–17, prepare lysates: 2 h

Step 18–21, tryptic digestion: 1 d

Step 22–28, peptide desalting: 4–5 h

Step 29–31, CuAAC: 3 h

Step 32–40, remove excess click reagents by SCX: 3 h

Step 41–50, streptavidin Capture and Photorelease: 5–6 h

Step 51–57, final desalting: 3–4 h

Step 58–64, LC-MS/MS and data analysis: >3 d

ANTICIPATED RESULTS

By using our protocol, we previously showed that up to 1000 of S-sulphenylated sites on over 700 proteins can be identified in RKO cells without exogenous stimuli or treatment³⁴. Figure 3 shows an example for identification of Cys238 of human N- α -acetyltransferase 15 (NAA15) as a target for protein S-sulphenylation, which is demonstrated by the fully

annotated HCD MS/MS spectrum with diagnostic fragment ions. For identified S-sulphenylated peptides containing more than one cysteine residue, at least three modification-specific fragment ions are required for unambiguous site localization. For example, S-sulphenylated sites on Cys152 or Cys156 from the same peptide sequence of GAPDH can be distinguished by the sequence fragment ions from y7 to y10 in their corresponding spectra (Fig. 4). All the fully-annotated HCD MS/MS spectra for all identified S-sulphenylated peptides from RKO cells can be obtained from our previous publication (Ref.34). It is noteworthy to mention that the HCD MS/MS spectra of modified S-sulphenylated peptides are often more complex and noisier than those for unmodified peptides.

In combination with isotopically labeled DYn-2 probe, our chemoproteomic approach enables simultaneous measurements of hundreds of dynamic protein S-sulphenylation events in cells in response to redox perturbations³⁴. We observed dramatic changes in the S-sulphenylome in cells upon H₂O₂ (Fig. 5a). More importantly, for the first time, this approach allows us to see markedly different responses of multiple S-sulphenylated sites on the same protein to exogenous and endogenous redox perturbations. The example summarized in Figure 5b shows the ratios of R_{H/L} of S-sulphenylation events on nine cysteines of fatty acid synthase (FASN) distributed broadly over a ~60-fold range in response to H₂O₂ treatment. With regard to the accuracy and reproducibility of quantitative S-sulphenylome analysis, we recommend using at least two biological replicates with two or three technical LC-MS/MS runs for each.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Selective labeling and detection of protein S-sulphenic acid in cells. **(a)** Cyclohexanedione group of DYn-2 probe selectively reacts with protein sulphenic acid modifications in cells. To the best of our knowledge, this probe has no reactivity toward other functional groups in cells. Moreover, this probe does not change cell viability and redox balance. **(b)** Alkyne group of DYn-2-labeled proteins can be conjugated to azide-bearing tags (e.g. fluorescent or biotin tag) via click chemistry and detected by immunofluorescence, western blotting (WB), or proteomics.

Figure 2.

Two workflows for proteome-wide identification of targeted sites of alkyne tagged probes. **(a)** Protein capture. Proteome labeled with an alkynyl probe is first conjugated with an azido biotin with cleavable linker via CuAAC chemistry. The biotinylated proteins are then enriched with streptavidin beads and subjected to sequential on-beads tryptic digestion and released by an enzymatic or chemical cleavage mechanism. The resulting peptides are analyzed by mass spectrometry based proteomics for site-localization. **(b)** Peptide capture, as described in the Procedure. Proteome labeled with an alkynyl probe is first digested into tryptic peptides and then conjugated with an azido biotin with photocleavable linker via CuAAC chemistry. The biotinylated peptides are then enriched with streptavidin beads and released by an enzymatic or chemical cleavage mechanism. Sequential LC-MS analysis and informatics pipeline provide identification of labeled peptides, proteins and their sites of modification.

Figure 3.

Identification of Cys238 of NAA15 as the target of S-sulphenylation in RKO cells. **(a)** Fully annotated HCD MS/MS spectrum with diagnostic fragment ions (zoom window) of a DYn-2-triazo-hexanoic-acid modified peptide from S-sulphenylated NAA15. The highlighted cysteine in the peptide sequence represents the S-sulphenylated site (Cys238) of NAA15. **(b)** CID MS/MS spectrum of the same peptide.

Figure 4. Identification of Cys152 (upper) and Cys156 (lower) of GAPDH as the targets of S-sulphenylation in RKO cells. Fully annotated HCD MS/MS spectra of the same peptide sequence with different sites of DYn-2-triazo-hexanoic-acid modification are shown. The cysteine in purple color in the peptide sequence represents the S-sulphenylated site.

Figure 5.

Quantitative S-sulphenylome analysis. **(a)** The proteome-wide and site-specific changes in S-sulphenylation in intact RKO cells in response to exogenous H₂O₂ (500 μ M, 5 min), where nine S-sulphenylated cysteines on the FASN protein are shown in red, and all other cysteines by white symbols. The relative change in protein S-sulphenylation between two conditions is determined by the ratio of extracted ion current peak intensities for heavy and light isotope-labeled DYn-2-tagged peptides, using the MS1 filtering function of Skyline software. Ratios are displayed on a log₂ scale on the y axis. **(b)** Extracted ion chromatograms (XIC) for nine S-sulphenylated peptides/sites from FASN are shown, with the profiles for light- and heavy- labeled peptides in red and blue, respectively. Light labels correspond to untreated cells; heavy labels correspond to H₂O₂-treated cells. The mean calculated experimental ratios from at least four replicates are displayed below the individual chromatogram, respectively. Figure reproduced from ref.34.

Table 1

Troubleshooting table

Step	Problem	Possible reason	Solution
8	Cells in suspension cannot be pelleted by normal centrifugation force	Density of cell suspension is too high, resulting in cell clumping	Increase the volume of labeling medium used for suspending the cells and mix cells more frequently during labeling process. Meanwhile, make sure that the concentration of sulphenic acid probe stays the same
20	Incomplete tryptic digestion	Precipitated protein pellet has not been thoroughly dissolved	Sonicate the protein suspension more times (step 18) or increase the concentration of urea (Don't forget to make sure that samples are diluted to <0.5M urea before digestion)
		The first overnight digestion dramatically changes the pH of the digest	Adjust the pH of the digest to 8.0
27	Low yield of eluted peptides from HLB cartridges	HLB cartridges are overloaded	Use larger size of HLB cartridges or desalt samples in more same-size cartridges
		Flush rates during sample loading and washing steps are too high	Reduce the charge force of the SPE extraction manifold by controlling the valve
29	Incomplete CuAAC reaction	Salt is not removed completely	Repeat HLB desalting step one more time
		Click reagents do not work properly (e.g. ascorbate has become oxidized)	Always perform this reaction in fresh sodium ascorbate and CuSO ₄ solution. Add the click reagents into the digested peptide samples in the same order as indicated in the in-text table and in a timely manner. We typically add all the reagents for one sample, before moving onto the next one.
37	Failed SCX cleaning	SCX spin columns are overloaded	Clean samples into more SCX spin columns
60	Failed LC-MS/MS analysis	LC column clogs during sample running	Remember to remove insoluble substances from the sample
	Fuzzy chromatographic peak shape	Unstable electrospray	Manually adjust the spray voltage or use metal-coated electrospray tip
		Salt is not removed completely	Repeat the final desalting step one more time