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PROTEOMIC METHODS TO EVALUATE NOX-MEDIATED REDOX SIGNALING

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Abstract

The NADPH oxidase (NOX) family of proteins is involved in regulating many diverse cellular processes, which is largely mediated by NOX-mediated reversible oxidation of target proteins in a process known as redox signaling. Protein cysteine residues are the most prominent targets in redox signaling, and to understand the mechanisms by which NOX affect cellular pathways, specific methodology is required to detect specific oxidative cysteine modifications and to identify targeted proteins. Among the many potential redox modifications involving cysteine residues, reversible modifications most relevant to NOX are sulfenylation (P-SOH) and S-glutathionylation (P-SSG), as both can induce structural or functional alterations. Various experimental approaches have been developed to detect these specific modifications, and this chapter will detail state-of-the-art methodology to selectively evaluate these modifications in specific target proteins in relation to NOX activation. We also discuss some of the limitations of these procedures and potential complementary approaches.

Keywords

NADPH oxidases; DUOX; H₂O₂; Redox signaling; Sulfenylation; S-glutathionylation; Dimedone

1 Introduction

The NADPH oxidases (NOX) were originally recognized as enzymes predominantly involved in host defense, but are now also widely recognized to have more diverse roles in many aspect of cell biology and cellular signaling (1). Generally, this relates to regulated production of reactive oxygen species (ROS) by activated NOX/DUOX enzymes, such as superoxide (O₂⁻) or hydrogen peroxide (H₂O₂), which in turn act as second messenger molecules that can reversibly modify proteins at susceptible redox sensitive amino acids, analogous to reversible phosphorylation/dephosphorylation, a process known as redox signaling (2–4). The concept of redox signaling applies to oxidation of redox-active metal ions in metalloproteins or oxidant-sensitive amino acid side chains such as cysteine and

⁶This buffer should be similar in composition to the lysis buffer used, but additional components (e.g. as protease or phosphatase inhibitors) are not necessary at this stage.

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methionine. Since cysteine thiols (P-SH, where P denotes the rest of the protein) are typically the most redox-sensitive among the amino acids, particularly when localized in areas in which interactions with neighboring amino acid side chains can lower the cysteine pK_a and increase its nucleophilicity (5), most attention has been paid to analysis of protein cysteine oxidation as a central mechanism of redox signaling. In addition to direct oxidation of protein cysteines by NOX-derived ROS, such redox modifications can also occur in a “relay” fashion, in which initial oxidation of highly susceptible cysteines (in e.g. peroxiredoxins) is propagated by transferring oxidizing equivalents to target proteins through thiol-disulfide exchange mechanisms (6).

The most widely recognized example of redox-based signaling involves the reversible inactivation of protein tyrosine phosphatases, which possess a redox-sensitive active site Cys residue. Oxidation of this Cys inactivates phosphatase function, thereby allowing for extended phosphorylation of target proteins and their prolonged (in)activation (7, 8). X-ray crystallography data indicate that oxidatively inhibited phosphatase PTP1B actually contains a sulfenyl amide (Fig. 1), likely formed by reaction of initially generated P-SOH with an adjacent amide bond, which may be responsible for its inactivation (9). Alternatively, others argue that PTP1B is also inactivated by *S*-glutathionylation (10). Recent studies by us and others have indicated that tyrosine kinases themselves can also be regulated in a more direct fashion by oxidative mechanisms mediated by NOX/DUOX activation. For example, activation of the epidermal growth factor receptor (EGFR) by its cognate ligand epidermal growth factor (EGF) was found to result in NOX2-mediated oxidation of a conserved cysteine within the ATP-binding region (C797) to P-SOH, which is thought to alter electrostatic interactions within the active site and enhance kinase activity by (11, 12). Similarly, the non-receptor tyrosine kinase Src was found to be susceptible to oxidative events induced by e.g. NOX activation (13–15). Our recent studies demonstrate that activation of both EGFR and Src in airway epithelial cells in response to protease allergens or other injurious triggers occurs in coordination with cysteine oxidation within these proteins, and is mediated by activation of the epithelial NOX isoform DUOX1 (16–19). Importantly, while NOX/DUOX activation can induce formation of both P-SOH and P-SSG within these proteins in a sequential mechanism, evidence suggests that it is P-SOH rather than P-SSG that enhances their kinase activity (12, 15). In other cases, *S*-glutathionylation may be the primary mechanism involved in oxidant-mediated enzyme inactivation, as was demonstrated in the case of IKK- β in the context pro-inflammatory signaling (20) or in oxidative processing of ER-localized pools of cellular receptors such as Fas (21).

A main complicating factor in the analysis of protein cysteine oxidation is its diverse nature (Fig. 1). In the context of NOX-dependent signaling, oxidation of a cysteine (typically by H_2O_2) initially generates a sulfenic acid (P-SOH). Such formation of P-SOH impairs the function of catalytic cysteines and can also alter protein function by other electrostatic mechanisms (12, 15). However, this product is typically not stable within proteins and readily reacts with other cysteine thiols to form a disulfide bond (P-S-S-R, where R is another protein or small molecule), either by reaction with another cysteine within the same protein (to form an intramolecular disulfide), reaction with P-SH in different protein (to form an intermolecular disulfide), or reaction with low-molecular weight thiols such as free cysteine or glutathione (GSH) (resulting in *S*-cysteinylation; P-S-S-Cys or *S*-

glutathionylation; P-SSG). These various disulfide intermediates can be reduced back to the original reduced thiol (P-SH) by oxidoreductases such as thioredoxins and glutaredoxins (22). P-SOH is also subject to further oxidation to sulfinic and sulfonic acids (P-SO₂/₃H; Fig. 1), but this is mostly relevant to conditions of severe oxidative stress and is likely not of major significance in the context of physiological cell signaling. It is often unclear which oxidative cysteine modification is most critical for regulating protein function. Formation of P-SOH may be considered a gateway intermediate to facilitate disulfide formation, which often is thought to be the most relevant modification that regulates protein function, but in some cases P-SOH rather than P-SSG may be primarily responsible for the redox signal, and conversion of P-SOH to P-SSG instead serves to prevent overoxidation and allow regeneration of the initial cysteine thiol (12, 15). It is important to note that the biochemistry of thiol-based redox signaling is more complex, and is not restricted to the actions of NOX-derived ROS, but can also involve reversible modifications related to production of nitric oxide (NO), a process known as *S*-nitrosylation (23), alkylation by various cellular electrophiles (24), and formation of polysulfides (P-S-S_(n)-H) due to cellular formation of e.g. hydrogen sulfide H₂S or other polysulfide species (25, 26). Although these modifications are typically not thought to be directly regulated by NOX/DUOX activation, they could be affected by NOX/DUOX in more indirect ways. Indeed, recent studies indicate that NOX activation can also promote oxidation of polysulfide species resulting in so-called perthiosulfenic acids (P-S-S_(n)OH) (27).

Based on these various considerations above, there is a strong need for reliable methodology that allows for identification and quantification of specific protein targets that are oxidized in response to NOX/DUOX activation, as well as the specific oxidative modification(s) (e.g. P-SOH vs P-SSG) within these targets. Much of our current understanding in this regard is based on indirect approaches to assess protein cysteine oxidation, such as isotope coded affinity tag (ICAT) proteomics approaches (28) or so-called switch techniques in which oxidized proteins are subject to chemical reduction followed by labeling with thiol-reactive detection reagents (e.g. biotin). While some of these biotin switch techniques are based on putatively selective chemistry to reduce specific forms of oxidized cysteine, e.g. ascorbate for *S*-nitrosothiols (29) or glutaredoxin-based reversal of P-SSG (30), such approaches are not always specific and may detect alternative modifications such as P-SOH. Detection of P-SOH has over the years mostly relied on the use of cell-permeable 5,5-dimethyl-1,3-cyclohexanedione (commonly known as dimedone) or related reagents, which are thought to preferentially react with the P-SOH and are unreactive with P-SH, in contrast with previously used reagents such as 4-chloro-7-nitrobenzofurazan (31). Identification of dimedone adducts in specific proteins can then be confirmed by mass spectrometry or by immunochemical detection with specific antibodies raised against dimedone-adducted Cys residues (32). To expand analytical approaches, dimedone has also been synthesized as a biotin-conjugated derivative, which allows for facile purification (15, 33) or other utilities taking advantage of biotin-streptavidin interactions, such as fluorophore labeling for overall quantification or subcellular localization of sulfenylated proteins. Many of these reagents are now commercially available (e.g. from Kerafast or Cayman Chemicals). While many research groups, including our own (15, 17, 18), have successfully utilized such biotin-conjugated dimedone probes, an intrinsic limitation is their limited cell permeability, which

means that they are best used during cell lysis or tissue homogenization, requiring careful steps to avoid artificial oxidation and labeling during such lysis/homogenization procedures. To circumvent this, dimedone has been conjugated to alkyne or azide moieties, which improves cell permeability and allows for their use in intact cells, to probe oxidation in a more *in situ* manner. Protein adducts with dimedone-alkyne or -azide can then be evaluated using click chemistry reactions after cell lysis (11, 34).

With respect to analysis of protein *S*-glutathionylation (35), many studies have relied on use of an α -GSH antibody (e.g. ViroGen α -GSH) to detect *S*-glutathionylation in specific proteins or to perform immunoprecipitation. While this has allowed for successful detection of *S*-glutathionylated proteins in some cases (36, 37), it is unclear whether this approach works equally well for all *S*-glutathionylated proteins (38), and affinity may in some cases be limited. Another approach is to use a biotin switch method in which reduced cysteines in protein mixtures are first alkylated, after which P-SSG is reduced to the corresponding thiol using an enzymatic glutaredoxin-based reduction step, and subsequently recovered by thiol-specific detection methods (39). While this method can be selective for P-SSG, it is cumbersome and critical controls are needed, since the GSH that is included in the glutaredoxin-based step could also reduce other oxidized forms of cysteine. An alternative approach to assess formation of P-SSG *in situ* is by preloading cells with e.g. biotin-GSH conjugates, after which incorporation of biotin into proteins after e.g. cell stimulation reflects increased *S*-glutathionylation. The use of esterified forms of biotin-GSH (BioGEE) enhances cell permeability and allows for facile loading into cells prior to experimentation (40, 41). Another advantage of this labeling approach is that subsequent purification of biotin-tagged proteins is facilitated by the fact that the Biotin-G-SS-protein adduct is DTT-cleavable, to allowing for easy elution of proteins and minimizing contamination with non-specifically bound proteins.

In the following sections, we will describe specific methodology for analysis of P-SOH or P-SSG in specific cell proteins in the context of NOX/DUOX signaling (Fig. 2). Approaches to detect P-SOH will utilize two specific dimedone-based probes: 1) the biotin conjugated DCP-Bio1 probe, and 2) the alkyne-containing DYn-2 probe (which can be linked to Biotin-azide via click reactions). Biotin tagging of P-SOH is then followed by avidin-based purification for analysis by MS (for unbiased protein screening) or by Western blot analysis of suspected target proteins. The methods described below are largely based on protocols developed by Nelson and co-workers (42) and the Carroll group (2, 12), with some modifications. Analysis of PSSG, modeled after the method developed by Sullivan and co-workers (40), will be based on cell preloading with BioGEE, after which adduction of cellular biotin-tagged GSH to proteins will be determined as a reflection similar adduction on endogenous (*S*-glutathionylation). We will present some examples in the context of NOX-mediated signaling and discuss some limitations and pitfalls in the notes section. It is important to recognize that NOX enzymes could directly oxidize a target protein but may also work by more oxidative mechanisms (e.g. by activating of other NOXes or mitochondria), and therefore it is important to also consider complementary approaches that address spatial considerations, using e.g. fluorescent imaging strategies to localize specific oxidative events in proximity to a specific NOX enzyme (43).

2 Materials

Prepare all aqueous solutions in Milli-Q H₂O, or water of comparable purity. All reagents should be of the highest available purity and should be stored per manufacturer's recommendation.

2.1. Materials for Identification of Targets of Sulfenylation

1. Cultured cells of interest (*see* Note 1)
2. Cell stimulus of choice (e.g. 10 µg/mL EGF in H₂O, or 10 mM ATP in H₂O)
3. Phosphate Buffered Saline pH 7.4
4. Dimethyl Sulfoxide (DMSO)
5. 100 mM DCP-Bio1 (Kerafast) or 100 mM-1.0 M DYn-2 (Kerafast) in DMSO (*See* Notes 2 and 3).
6. Catalase (~40,000 Units/mL; Worthington)
7. 1 M N-ethylmaleimide in ethanol
8. Western Solubilization Buffer (WSB) (50 mM HEPES, 250 mM NaCl, 1.5 mM MgCl₂, 1% Triton X-100, 10% glycerol, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 2 mM Na₃VO₄, 10 mg/ml aprotinin, and 10 mg/ml leupeptin (pH 7.4)) (*see* Note 4)
9. DYn-2 Lysis buffer (50 mM HEPES pH 7.4, 150 mM NaCl, 1% NP-40, 0.1% SDS, 200 u/mL catalase, EDTA-free protease and phosphatase inhibitor cocktails (e.g. Thermo Scientific Halt protease and phosphatase inhibitor cocktail) (*see* Note 5)
10. WSB Wash Buffer (50 mM HEPES, 250 mM NaCl, 1% Triton-X100, 10% Glycerol, pH 7.4) (*see* Note 6)

¹In principle this can be applied to any cultured type, either grown as adherent cultures or in suspension culture, although methodology presented here are based on studies with adherent cultures.

²While dimedone is not known to react with reduced thiols or most other oxidized forms, it is also reactive with sulfenyl amides, which are typically formed after initial P-SOH formation, but potentially via other mechanisms as well (6, 49, 50). Kinetic studies suggest that reaction of dimedone with P-SOH is rather slow, and may be limited by other cellular reactions of P-SOH, e.g. with other thiols, which may be faster (51). To overcome this, high concentrations of dimedone are needed to outcompete such reactions, which is only feasible in cases where dimedone-based traps are used in the context of cell lysis buffer (such as DCP-Bio1). Cell preloading with high concentrations of cell-permeable dimedone-based probes (e.g. DYn-2) may impact cellular pathways and even viability, or could disrupt functions of e.g. peroxidase enzymes that form intermediate P-SOH (6).

³Reactivity of P-SOH may be highly variable, and factors other than intrinsic P-SOH reactivity (e.g. steric factors) may influence labeling. Indeed, recent studies using several diverse molecules to trap P-SOH have shown that their ability to trap P-SOH in cells does not always overlap (52), suggesting that different available probes may be needed to cover the full P-SOH proteome.

⁴This lysis buffer is chosen because it optimizes solubilization of transmembrane proteins such as EGFR, but other standard lysis buffers can be used as well. For best results, ensure that the pH is adjusted prior to the addition of protease inhibitors. For application to tissue homogenates, it is recommended that homogenization buffers similarly contain NEM and catalase to minimize artefactual protein sulfenylation during homogenization.

⁵Comparable lysis buffer can be used here as well, as long as they don't contain metal chelators or other compounds that may interfere with the click reaction (e.g. EDTA).

⁶This buffer should be similar in composition to the lysis buffer used, but additional components (e.g. as protease or phosphatase inhibitors) are not necessary at this stage.

11. Neutravidin High Capacity Beads (Thermo Scientific) or comparable streptavidin agarose beads
12. Click reaction mix (1 mM Tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl]amine (TBTA) in 9:1 Acetonitrile:DMSO, 5 mM sodium ascorbate in PBS, 500 μ M CuSO₄ in PBS, and 500 μ M biotin azide in DMSO (Kerafast)). Add 10 μ L 100 mM TBTA, 10 μ L 500 mM sodium ascorbate, 10 μ L 50 mM CuSO₄ and 900 μ L water. Check the pH of the solution with a pH strip. If the pH is 7.0, add 50 μ L water and 50 μ L 10 mM biotin azide.)
13. 1% Sodium Dodecyl Sulfate (SDS) in H₂O.
14. 6 M Urea in PBS.
15. 1 M NaCl in H₂O.
16. 100 μ M Ammonium Bicarbonate + 10 mM DTT in H₂O.
17. 100 μ M Ammonium Bicarbonate in water.
18. Elution buffer (50 mM Tris, 2% SDS, 1 mM EDTA, pH 7.4) in H₂O.
19. Laemmli Buffer (The concentration prepared depends on preferred sample volume. The final, diluted concentration that we use is 0.063 M Tris-Cl, 2% SDS, 10% v/v Glycerol, 0.02% β -mercaptoethanol, 0.01% Bromophenol Blue, pH 6.8).

2.2 Materials for Identification of S-Glutathionylated Proteins

1. Cultured cells of interest (*see* previous section and Note 1)
2. Stimulus of choice (*see* previous section)
3. Phosphate Buffered Saline, pH 7.4
4. RIPA Lysis Buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na₂EDTA, 1 mM EGTA, 1% NP-40, 1% sodium deoxycholate, 2.5 mM sodium pyrophosphate, 1 mM β -glycerophosphate, 1 mM Na₃VO₄, 1 μ g/ml leupeptin, and 1 mM PMSF)
5. 1 M N-ethylmaleimide in ethanol
6. Neutravidin High Capacity Resin (Thermo Scientific) or comparable streptavidin agarose resin
7. 500 mM NaHCO₃ in H₂O
8. 110 mM Glutathione ethyl ester (GEE) in NaHCO₃ (*see* Note 7)
9. 100 mM EZ link Sulfo-NHS-Biotin
10. 500 mM NH₄HCO₃

⁷These reagents are used to synthesize BioGEE, as is described in Section 3.1, but BioGEE can also be purchased commercially (Thermo Scientific).

11. PD MidiTrap G-25 spin columns
12. 10 mM DTT in H₂O
13. PBS + 0.1% Sodium dodecyl sulfate
14. Amicon spin filters (0.5 mL, 3 kDa cutoff)
15. Laemmli Buffer (The concentration prepared depends on preferred sample volume. *See Note 8*).

3 Methods

The methods described below are broadly applicable to cell-based studies of cell signaling pathways induced by e.g. cytokines or growth factors, and the role of NOX enzymes can be assessed by e.g. siRNA-mediated deletion, CRISPR/Cas9 gene editing approaches, or pharmacological inhibitors (although NOX-isoform specific inhibitors are still largely lacking (44)), or by using cells isolated from different genetic mouse models of NOX-deficiency. These methods to identify P-SOH or P-SSG have been used successfully in identifying targets of protein oxidation (40, 42, 45–47), and also in various studies by our group that address redox signaling by DUOX1 (15, 17, 18, 41). Approaches have also been developed for quantitative analysis of e.g. P-SOH through mass spectrometry utilizing isotope-labeled dimedone approaches, either with or without biotin purification (46, 48), but these are particularly challenging for large proteins with multiple cysteines (requiring diverse protein digestion procedures) or low-abundance proteins that are often involved in cell signaling pathways. The importance of specific cysteines could alternatively be addressed by mutating these cysteines in suspected or confirmed target proteins.

3.1 Cell Culture and Pre-treatments

1. Culture cells of interest to desired confluence. Typically, 100,000–200,000 cells per treatment group are sufficient for successful detection of target proteins by Western blotting, but considerably more cells will be needed for MS analysis (see Note 9).
2. If applicable, perform cell transfections to manipulate e.g. NOX/DUOX enzymes according to established protocols. Alternatively, pretreat with small molecule NOX/DUOX inhibitors as appropriate.
3. Prior to cell treatments, replace culture medium with serum-free medium.
4. For analysis of P-SOH using DYn-2, pre-incubate cells with 5 mM DYn-2 reagent in serum-free medium for 30 min (*see Note 10*).

⁸Laemmli buffer is either used at 2X or 6X (to avoid unnecessary sample dilution), to yield final 1X concentrations of 0.060 M Tris-Cl, 2% SDS, 10% v/v Glycerol, 0.02% β-mercaptoethanol, 0.01% Bromophenol Blue (pH 6.8).

⁹Adherent cells in this case can be substituted for any protein mixture desired, with different considerations, depending on the desired outcome. For example, when analyzing lung homogenates with DCP-Bio1, we will homogenize utilizing the lysis buffer containing whichever probe is desired. Ultimately, this depends on the experimental question and limitations regarding the specific sample type.

¹⁰It is important to restrict the amount of added DMSO to <0.1% of total media volume. If needed, make intermediate dilutions with PBS or media. Additionally, if desired, a control mixture of the DYn-2 vehicle (DMSO) can be used to assess nonspecific protein detection.

5. For analysis of P-SSG, pre-incubate cells with BioGEE, by changing to serum-free media containing 250 μ M BioGEE and incubating for 1 hr at 37°C.
 - a. BioGEE can be purchased commercially (Thermo Scientific), but can also be easily prepared as follows: Mix 250 μ L 110 mM GEE with 250 μ L 100 mM Sulfo-NHS-biotin (see Materials) and react at RT for 1 hr with mixing. Quench the reaction by addition of 2 mL 500 mM NH_4HCO_3 (see Note 11). The final concentration of BioGEE is ~10 mM (see Note 12).
6. Treat cells with appropriate stimulus to activate NOX/DUOX-dependent redox signaling (e.g. ATP to stimulate DUOX1 or EGF to activate NOX2 (15)).
7. At appropriate time points, place cells on ice, remove medium and wash with PBS, and add appropriate lysis buffer (see next section).
 - a. In the case of cells preloaded with BioGEE, wash cells with PBS containing 50 mM NEM prior to cell lysis. This NEM step removes residual unreacted BioGEE and prevents its reaction with proteins during cell lysis.

3.2 Cell Lysis and Avidin Purification of DCP-Bio1- or DYn-2-Tagged Proteins

1. Lyse cells on ice by adding 100 μ L WSB (DCP-Bio1) or DYn-2 lysis buffer (See Materials) supplemented with 10 mM NEM (10 μ L of 1 M Stock/ per mL buffer) and 200 units/mL catalase (5 μ L 40,000 units/mL stock per mL buffer). (Optional) If using DCP-Bio1, add 10 μ L of a 100 mM stock in DMSO to the WSB buffer for a final concentration of 1 mM. Incubate on ice for 1 h with gentle rocking. If adding protease inhibitors (e.g. for DYn-2 buffer) do so before adding to cells.
2. Scrape cells, collect lysate into an eppendorff tube, and sonicate briefly. Centrifuge at $21,000 \times g$ for 5 min to remove cell debris. Lysates can be stored at -20°C until use.
3. Determine protein concentration using a BCA assay and collect equal amounts of protein from each sample for purification. This amount can range from ~100 μ g to ~10 mg (see Note 13). Ensure that 30–50 μ L of lysate is saved for analysis of input controls and/or total streptavidin analysis.
4. Wash neutravidin beads (25–50 μ L bead slurry/sample) with 20 mM Tris pH 7.4 buffer 3x using a rotisserie mixer. Collect beads between washes via centrifugation at $1,200 \times g$ for 5 min. After the final wash, resuspend beads in ~2

¹¹This should be in at least a 5-fold molar excess, ensuring that the amine reactive NHS is quenched by the NH_4HCO_3 .

¹²BioGEE concentration may need to be optimized, but 250 μ M seems to work best in epithelial cells. Loading cells with high concentrations of BioGEE might alter cellular processes, and thus induce artifacts. Also, biotin-tagged GSH may not be equally capable of reaching its protein target compared to endogenous GSH.

¹³Lysate aliquots containing 100–500 μ g of total protein are typically sufficient for detection of biotin-tagged proteins of interest by western blot analysis. For proteomic identification, larger sample sizes (>1 mg protein) will be required.

volumes of Tris buffer to generate fresh neutravidin bead slurry for use in subsequent steps.

5. If using DYN-2, preclear lysates with around 30 μ L fresh neutravidin slurry to remove endogenously biotinylated protein. Centrifuge samples at $1,200 \times g$ and add supernatant to new tubes while discarding the beads. Following this preclearing step, perform the click reaction with a 1:1 solution of lysate to click reaction mix (see Materials). React for 1h at RT with mixing. Quench the reaction with 1 mM EDTA.
6. Add protein sample to 0.5 mL Amicon centrifuge filters (3 kDa molecular weight cutoff) and perform buffer exchange with WSB wash 3 times by centrifugation at $13,500 \times g$ for 20–30 min. Discard the flow-through from each spin. This step removes excess unconjugated biotin reagents.
7. Collect retained liquid containing the protein sample (typically $\sim 50 \mu$ L), rinse the top of the filter with ~ 0.2 mL WSB wash and combine with protein sample.
8. Distribute washed beads evenly between new tubes. To the beads, add the buffer-exchanged protein sample from steps 6 and 7, and dilute sample/slurry mix to a total volume of 0.5–1 mL with Tris buffer.
9. Rotate beads overnight at 4°C on a rotisserie mixer.
10. The following day, spin beads at $1,200 \times g$ for 5 min and collect supernatant (*see* Note 14). Wash beads with 1 mL 0.1% SDS for 30 min with rotisserie mixing. Spin beads and wash with new 1% SDS again for 5 additional minutes. Spin beads and remove 1% SDS.
11. Wash beads with 4M urea for 30 min with rotisserie mixing. Spin beads, discard supernatant, and wash for an additional 5 min with 4M urea. Spin beads and discard supernatant.
12. Wash beads with 1M NaCl for 30 min with rotisserie mixing. Spin beads, discard supernatant. Wash for an additional 5 min with new 2M NaCl. Spin beads and discard supernatant.
13. Wash beads with 100 μ M Ammonium Bicarbonate + 10 mM DTT for 5 min and rotisserie mixing. Spin beads and discard supernatant. (*see* Note 15)
14. Wash beads with 100 μ M Ammonium bicarbonate for 5 min and rotisserie mixing. Spin beads and discard supernatant.
15. Wash beads with water for 5 min and rotisserie mixing. Spin beads and discard supernatant.

¹⁴The proteins of interest should be bound to the beads at this point, however it is beneficial to save the supernatant at this step as a means of assessing any potential issues that may have arisen upon final protein analysis.

¹⁵During the final washes, beads may get stuck in the bottom of the tube. If this happens, simply mix the samples by vortexing before putting them on the rotisserie mixer.

- a. Wash steps 10–14 serve to remove proteins that are bound non-specifically to the beads or to biotin-tagged proteins (and would represent false-positives; see Note 16).
16. Add 100 μ L Elution buffer. Boil samples at 100°C for 10 min. Briefly vortex samples and spin at $21,000 \times g$ for 5 min. Remove and keep supernatant.
17. Add Laemmli buffer to supernatant and mix briefly (*see* Note 8). Samples can be stored at -20°C until analysis.

3.3 Cell lysis and Avidin Purification of BioGEE-Tagged Proteins

1. Lyse cells with RIPA buffer supplemented with 50 mM NEM (to avoid thiol exchange and loss of P-SSG during lysis) on ice for 30–60 min with gentle rocking.
2. Scrape cells, collect lysate and sonicate. Centrifuge to remove cell debris. Lysate can be stored at -20°C until use.
3. Determine protein content in cell lysate (e.g. using BCA assay), save 30–50 μ L lysate for input and total Streptavidin analysis.
4. Dilute equal amounts of each sample (*see* Note 13) to 1 mL with RIPA Buffer + 50 mM NEM.
5. Condition G25 columns with 3 washes of RIPA buffer via centrifugation at $1000 \times g$ for 2 min.
6. Load sample on to column with centrifugation at $1000 \times g$ for 2 min. This step is needed to remove unreacted Bio-GSH, as proteins in the sample will be in the flow through.
7. In a separate tube, add 150 μ L high-capacity neutravidin bead slurry per sample and wash twice with 1 mL PBS.
8. Add 50 μ L bead slurry to each sample and rotisserie mix for 30 min at 4°C
9. Spin sample at $1,200 \times g$ for 5 min. Discard the beads, save the supernatant and add it to a new vial.
10. To the supernatant, add an additional 100 μ L bead slurry and rotisserie mix overnight at 4°C .
11. The next day, spin the sample at $1,200 \times g$ and save the supernatant (*see* Note 14).
12. Wash the beads five times for 10–20 min with 1 mL cold RIPA buffer with rotisserie mixing. Spin and discard the supernatant after each wash. (*see* Note 17)

¹⁶Since avidin pull-downs could still contain proteins that co-precipitated and were not directly biotin-tagged, it is recommended to confirmation identification of biotin-tagged proteins by first immunoprecipitating proteins of interest (using specific antibodies) and then performing analysis by SDS-PAGE and streptavidin-HRP blotting.

¹⁷As in Note 6, a simplified RIPA buffer without protease and phosphatase inhibitors can be used.

13. Wash the beads with 1 mL PBS + 0.1% SDS with rotisserie mixing. Spin and discard the supernatant.
14. Resuspend the beads in 500 μ L PBS+ 0.1% SDS, mix for 30 min at room temperature.
15. Spin the beads down and save the supernatant for analysis of non-glutathionylated proteins (“-DTT”; *see* Note 18).
16. To elute *S*-glutathionylated proteins, add 500 μ L PBS with 0.1% SDS containing 10 mM DTT. Mix for 30 min at room temperature.
17. Spin the sample again and save the supernatant. To ensure all proteins are removed successfully, add another 500 μ L PBS with 0.1% SDS and wash. After spinning, add this to the DTT eluted samples. This sample (“+DTT”) contains the *S*-glutathionylated proteins (*see* Note 19).
18. Concentrate samples using 3 kDa Amicon filters (0.5 mL capacity), spin at $21,000 \times g$ for 30 min until all sample is $\sim 100 \mu$ L in volume. If the sample is below 100 μ L, dilute with buffer.
19. Add Laemmli buffer to samples and heat at 95°C for 5 min (*see* Note 8). Samples can be stored at -20°C until analysis.

3.4 Analysis of Avidin-Purified Proteins

Avidin-purified proteins representing either P-SOH or P-SSG (based on sections 3.3 or 3.4) can be analyzed by LC-MS/MS (for unbiased analysis) or by SDS-PAGE and Western blot analysis of candidate proteins of interest. For LC-MS/MS identification, separate proteins by SDS-PAGE and stain gel with Coomassie brilliant blue or silver stain, excise bands, and work up according to standard in-gel digestion protocols. SDS-PAGE allows for identification of major protein bands and facile sample desalting prior to tryptic digestion, but is not strictly required, and standard solution-based digestion methods are also suitable as long as peptides are desalted prior to LC-MS/MS, using e.g. C18 ZipTips (Millipore, according to manufacturer’s instructions).

For analysis of candidate proteins by Western blot, separate proteins by standard SDS-PAGE, transfer separated proteins to nitrocellulose membranes, and probe membranes with either Streptavidin-HRP (to visualize all biotin-tagged proteins) or antibodies against specific proteins of interest, according to established protocols. Importantly, cell lysates (*see Step 3* in Section 3.2 or 3.3) should be analyzed similarly as input loading controls. Fig. 3 shows results from a representative analysis of P-SOH in NCI-H292 cells in response to stimulation with either 100 μ M ATP or 100 ng/ml EGF, using DYn-2. Fig. 4 shows an example of P-SSG analysis in EGFR or Src in murine tracheal epithelial cells in response to stimulation with EGF.

¹⁸Supernatant samples eluted in the absence of DTT can be analyzed similarly as “negative controls” to confirm specificity of the DTT elution step in steps 16 and 17.

¹⁹Application of BioGEE for identification of specific cysteine targets within proteins/peptides by MS will be limited if they contain multiple cysteines, since the tag will be removed during reducing conditions during purification or prior to MS analysis.

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Fig. 1:
Schematic representation of potential oxidative intermediates involved in ROS-dependent signaling.

Fig. 2:
Flowchart illustrating the different derivatization steps to detect P-SOH or P-SSG by either MS approaches or Western blot of candidate proteins.

Fig. 3:

Increased sulfenylation of EGFR and Src in response to NOX/DUOX stimulation by ATP or EGF. NCI-H292 cells were preloaded with DYN-2, stimulated with 100 μ M ATP or 100 ng/ml EGF, conjugated with biotin post-lysis, and streptavidin purified. Avidin-purified samples were subjected to SDS-PAGE and Western blot against Src or EGFR (top panel), and cell lysates were probed with streptavidin-HRP to assess overall protein sulfenylation, or with phosphospecific antibodies (pY1068 EGFR, pY416 Src, Cell Signaling; bottom panel). As a control, DYN-2 omitted to assess background sulfenylation in unstimulated control.

Fig. 4: S-glutathionylation of EGFR and Src in response to EGF stimulation. Primary mouse tracheal epithelial cells were preloaded with BioGEE and stimulated with EGF (0–200 ng/mL) for 10 min. Biotin-tagged proteins were purified and analyzed by Western blot for Src and EGFR (top panel). Additionally, whole cell lysates were probed for kinase activation (pY1068 EGFR, pY416 Src) compared with unphosphorylated forms using (phosphor)specific antibodies (Cell Signaling, bottom panel).