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Detection of protein S-nitrosylation with the biotin-switch technique

Michael T. Forrester [a,b], Matthew W. Foster [c], Moran Benhar [c], Jonathan S. Stamler [a,c,*]

[a] Department of Biochemistry, Duke University Medical Center, Durham, NC 27710, USA
[b] Medical Scientist Training Program, Duke University Medical Center, Durham, NC 27710, USA
[c] Department of Medicine, Duke University Medical Center, Durham, NC 27710, USA

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ABSTRACT

Protein S-nitrosylation, the posttranslational modification of cysteine thiols to form *S*-nitrosothiols, is a principle mechanism of nitric oxide-based signaling. Studies have demonstrated myriad roles for S-nitrosylation in organisms from bacteria to humans, and recent efforts have greatly advanced our scientific understanding of how this redox-based modification is dynamically regulated during physiological and pathophysiological conditions. The focus of this review is the biotin-switch technique (BST), which has become a mainstay assay for detecting S-nitrosylated proteins in complex biological systems. Potential pitfalls and modern adaptations of the BST are discussed, as are future directions for this assay in the burgeoning field of protein S-nitrosylation.

Basics of S-nitrosylation

Protein S-nitrosylation—the covalent adduction of a nitroso group to a cysteine thiol side chain—has recently emerged as a principle mechanism by which nitric oxide (NO) mediates a wide range of cellular functions and phenotypes [1,2]. S-nitrosylation regulates diverse pathways such as G-protein-coupled receptor signaling [3–5], death receptor-mediated apoptosis [6–11], glutamate-dependent neurotransmission [12–15], vesicular trafficking [16–19], stimulation of prostaglandin synthesis [20–22], and the unfolded protein response [23]. In addition, aberrant S-nitrosylation is implicated in disease states such as tumor initiation and growth [24–28], neurodegeneration [23,29–32], and malignant hyperthermia [33]. Consequently, much effort is focused on understanding the role of S-nitrosylation in normal physiology and its contribution to pathophysiology. For example, several recent studies have shown that dysregulated S-nitrosylation of the ryanodine receptor (Ca^{2+}-release channel) may contribute to cardiac arrhythmias [34], heat stroke [33], and impaired exercise capacity [35]. As scientific interest in protein S-nitrosylation continues to intensify, an increasing number of studies are relying on the biotin-switch technique (BST) for the detection of endogenously S-nitrosylated proteins (protein–SNOs). The introduction of this assay by Jaffrey et al. in 2001 [36] has served as an impetus for studies probing S-nitrosylation in vivo, largely due to its superb compatibility with ubiquitous molecular methods (e.g., SDS–PAGE, immunodetection, mass spectrometry).

NO- vs sulfur-based assays of S-nitrosylation

The sulfur–nitrogen bond of an SNO is particularly labile and can undergo both homolytic and heterolytic cleavage reactions [37,38]. The lability of the S–NO bond has served as the cornerstone for numerous SNO-detection strategies, though the chemistries employed after SNO cleavage differ greatly between assays (Fig. 1). Most techniques detect the NO or nitrite (NO_2^-) liberated upon S–NO cleavage and hence can be considered "NO-based" strategies. In these assays, divalent mercury (e.g., $HgCl_2$) is often employed to heterolytically cleave the S–NO bond, producing a mercury–thiol complex and nitrosonium ion (NO^+); the latter is a potent nitrosant and undergoes rapid hydration to NO_2^- at neutral pH. Techniques (absorbance or fluorescence) that detect the NO_2^- product include the Saville [39–41], diaminonapthalene [39,42], and diaminofluorescein assays [42–45].

Another common NO-based technique employs homolytic or reductive conditions to cleave the S–NO bond, followed by chemiluminescence detection of the liberated NO via reaction with ozone. Such methods include Hg-coupled photolysis–chemiluminescence [46,47] and the copper–cysteine–carbon monoxide assay [48–50]. Though each of these NO-based methods is well suited to SNO quantitation (relative to SNO standards), they have limited use in

* Corresponding author. Fax: (919) 684-6998.
E-mail address: staml001@mc.duke.edu (J.S. Stamler).

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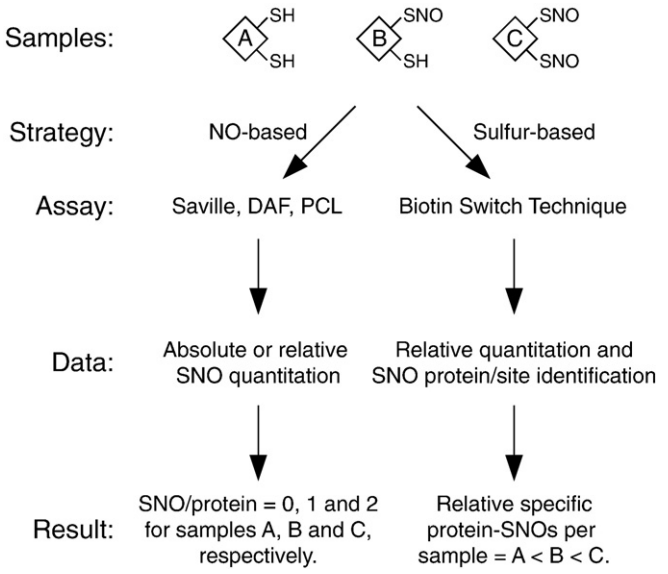

Fig. 1. A general comparison of NO- and sulfur-based strategies for detecting protein S-nitrosylation. As an example, three lysates containing various amounts of protein S-nitrosylation are subjected to both NO- and sulfur-based assays. NO-based strategies include the Saville and diaminofluorescein (DAF) assays, which employ a chemical probe, and Hg-coupled photolysis–chemiluminescence (PCL), which detects NO gas liberated by SNO homolysis, and can differentiate SNO from metal–NO. Importantly, this assay is highly sensitive (low nanomolar SNO concentrations can be detected) and has been well validated with genetic models of disrupted NO/SNO metabolism [108,109]. It therefore serves as a standard method for probing S-nitrosylation in vivo. With a complex biological sample (e.g., a lysate), these NO-based strategies can readily determine the absolute amount of SNO per sample, but cannot readily detect an individual protein–SNO. A sulfur-based strategy, such as the biotin-switch technique, employs covalent "tagging" at the sulfur atom of each SNO, thus facilitating relative quantitation and protein–SNO identification.

functional studies of S-nitrosylated proteins within complex mixtures because the proteins of interest must be purified (e.g., by immunoprecipitation) before SNO measurement. Although this method has been applied successfully in a number of cases—including S-nitrosylated hemoglobin [51–53], caspase-3 [11,54], thioredoxin-1 [55], c-Jun N-terminal kinase [56], G-protein-coupled receptor kinase 2 [5], ryanodine receptor [57,58] and prokaryotic OxyR [59]—the arduous nature of the approach has limited its application.

In contrast to NO-based assays, the BST is unique in that it targets the sulfur atom of an SNO without regard for the fate of any liberated NO species; it can thus be considered a "sulfur-based" strategy. As the BST employs covalent "tagging" of protein–SNOs, it can detect individual protein–SNOs in a complex mixture (because the tag is added to the protein of interest). For studies of a specific protein or class of proteins, the BST has therefore proved to be of great utility (>200 publications to date).

As described above, the major differences between NO- and sulfur-based strategies for SNO detection are summarized in Fig. 1. Importantly, the type of information gained from either approach is different. NO-based assays are capable of absolute quantitation (e.g., 10 nmol SNO per milligram protein), though they do not generally discriminate the source of each protein–SNO. In contrast, the BST tends to be more qualitative (relatively quantitative); its great advantage is its ability to detect individual protein–SNOs in a complex mixture, as well as to identify novel protein–SNOs.

Workflow of the biotin-switch technique

As illustrated by Fig. 2, the BST consists of three principal steps: (1) blocking of free cysteine thiols by S-methylthiolation with methylmethane thiosulfonate (MMTS; a reactive thiosulfonate); (2) conversion of SNOs to thiols via transnitrosation with ascorbate; and (3) in situ labeling by S-biotinylation of the nascent thiols with biotin–HPDP, a reactive mixed disulfide of biotin. The degree of biotinylation (and thus S-nitrosylation) is determined by either anti-biotin immunoblotting or streptavidin pulldown followed by immunoblotting for the protein(s) of interest.

The blocking step is typically initiated by the addition of SDS and MMTS, followed by heating at 50 °C and separation of proteins from excess MMTS by acetone precipitation. The combination of heat and SDS functions to fully denature proteins, thus granting MMTS optimal access to natively buried protein thiols. Effective blocking of free thiols is required to minimize "background" biotinylation (i.e., biotinylation that results from incomplete blocking) and to maximize assay sensitivity.

The second step in the BST converts the SNO to a free thiol so that it can be biotinylated. This is achieved by allowing ascorbate—a dienol

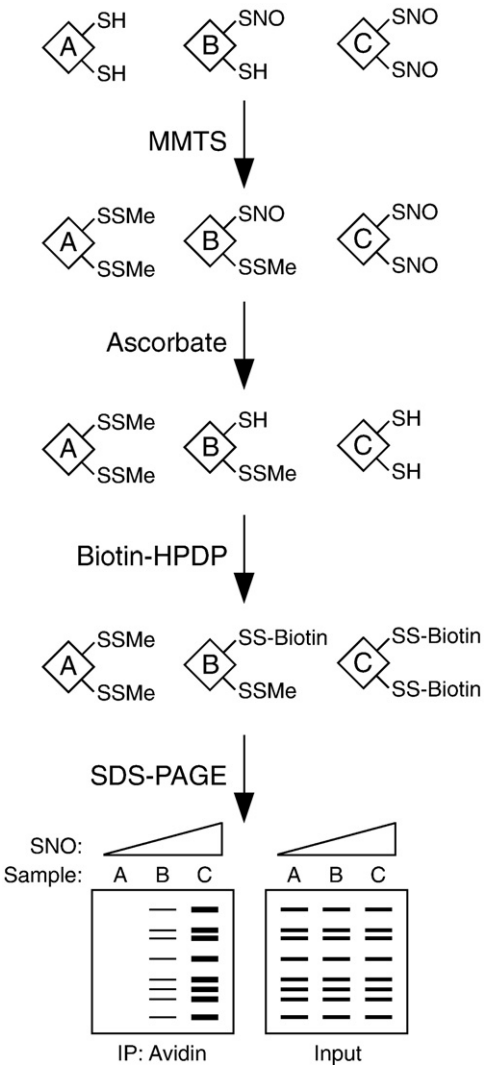

Fig. 2. Overview of the biotin-switch technique. In the example shown, three lysates with various degrees of S-nitrosylation are subjected to the assay. The blocking step involves *S*-methylthiolation of each cysteine thiol with *S*-methylmethane thiosulfonate (MMTS). Next, ascorbate is employed to convert each SNO to a free thiol via a transnitrosation reaction to generate *O*-nitrosoascorbate. In the labeling step, each nascent free thiol (previously an SNO site) is biotinylated with biotin–HPDP. Biotinylated proteins are enriched by avidin affinity medium and analyzed by SDS–PAGE/immunoblotting. Total protein–SNOs or an individual protein–SNO can be detected with avidin–HRP or with an antibody against a protein of interest, respectively. As illustrated, the degree of pulldown correlates with protein S-nitrosylation. Before avidin pulldown, a small fraction of each sample is analyzed to determine protein "input."

antioxidant with unique reactivity toward SNOs—to undergo a transnitrosation reaction with the protein–SNO. As initially reported by Holmes and Williams [60], this reaction proceeds via a concerted nitroso transfer mechanism whereby the SNO and ascorbate are converted to thiol and *O*-nitrosoascorbate. Notably, ascorbate is several orders of magnitude more reactive than other N- or O-based nucleophiles (e.g., amines, phenols) [61,62]. The reaction between SNOs and ascorbate also exhibits a critical pH dependence and is favored by transition metal chelation: alkaline conditions promote the nitroso transfer reaction, probably by increasing the fraction of monodeprotonated (i.e., nucleophilic) ascorbate, whereas transition metals may enable alternative chemistry (see below). The specificity of the BST for protein–SNOs is predicated on the fact that ascorbate will convert SNOs to free thiols without reducing other cysteine-based oxidations such as S-glutathionylation or S-oxides (sulfenic, sulfinic, and sulfonic acids). This specificity is supported by thermodynamic measurements (discussed below) and by several experimental validations [36,63].

The third step of the BST involves biotinylation of the nascent thiol (i.e., previous SNO site). This is performed concomitant with the ascorbate reaction, such that the newly liberated free thiols are immediately biotinylated. Excess biotin–HPDP is removed by acetone precipitation, and protein biotinylation (i.e., S-nitrosylation) is assessed by one of multiple routes. Avidin–agarose is frequently employed to enrich the biotinylated proteins, followed by elution and SDS–PAGE. It is important to note that the biotin tag is attached via a disulfide linkage, and therefore reductants (e.g., dithiothreitol (DTT)) will remove the tag. This reductive elution strategy is frequently employed to elute biotinylated proteins from avidin–agarose before SDS–PAGE, especially when the biotin tag is not employed for immunodetection (e.g., when blotting an individual protein). As demonstrated in Fig. 3A, the BST readily detects S-nitrosylated GAPDH in cytokine-stimulated macrophages, in which inducible nitric oxide synthase (iNOS) is upregulated [64–66]. Alternatively, total protein–SNOs can also be assessed by nonreducing SDS–PAGE and immunodetection of the biotin tag. As shown in Fig. 3B, this approach easily detects total protein–SNOs in HEK293 cells after treatment with *S*-nitrosocysteine (CysNO).

It is important to note that the BST can be used to assay S-nitrosylation by constitutive NOS isoforms (nNOS and eNOS; Fig. 4A), in addition to iNOS, and to examine S-nitrosylation dynamics in the context of signal transduction, as demonstrated for G-protein-coupled receptor kinase 2 (GRK2) after stimulation of multiple G-protein-coupled receptors (Fig. 4B). Thus, when performed appropriately it is highly sensitive as well as specific. Further, the BST can be used to identify the NOS isoform(s) involved (Figs. 4A and 4C) and to reveal the participation of denitrosylase activities or endogenous S-nitrosylating agents (Fig. 4D) across a wide range of cellular stimuli. Collectively, these examples illustrate the utility of the BST for assessing S-nitrosylation in vivo, particularly when used alongside the methodological, pharmacological, and genetic tools demonstrated in Figs. 3 and 4.

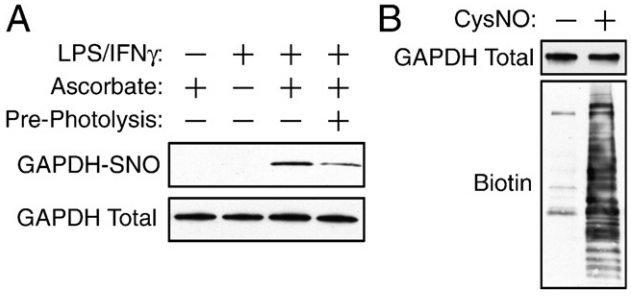

Fig. 3. A typical BST detects both endogenous and exogenous S-nitrosylation in cultured mammalian cells. (A) Murine RAW264.7 macrophages were either untreated or cytokine-stimulated with lipopolysaccharide (500 ng/ml) and IFN-γ (100 U/ml) for 16 h, which drives NO production. Cellular extracts were subjected to the BST and probed for S-nitrosylated GAPDH (GAPDH–SNO), along with ascorbate and prephotolysis controls. Notably, omission of ascorbate leads to nearly complete loss of biotinylation, and prephotolysis with a Hg vapor lamp greatly attenuates the same signal. (B) Whole cellular protein–SNOs are detected by treating HEK293 cells with 200 μM *S*-nitrosocysteine (CysNO) for 10 min. Cellular extracts were subjected to the BST and 5% of each biotinylation reaction (~40 μg) was analyzed by immunoblotting with avidin–HRP and anti-GAPDH antibody (for input).

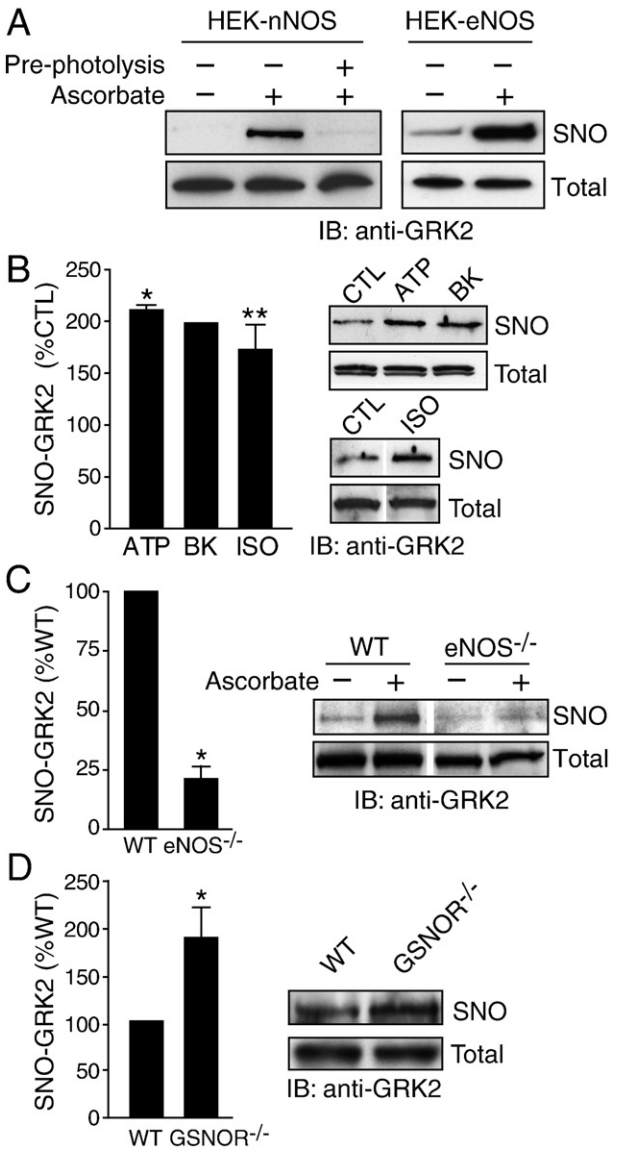

Fig. 4. The BST can be combined with pharmacological and genetic tools to study S-nitrosylation in primary cells and tissues in vivo. (A) GRK2 is S-nitrosylated in HEK293 cells that stably overexpress the constitutive NOS isoforms (eNOS and nNOS). (B) G-protein-coupled receptor-specific agonists (ATP, adenosine triphosphate; BK, bradykinin; ISO, isoproterenol; CTL, control), each of which leads to eNOS activation, increase GRK2 S-nitrosylation in human umbilical vein endothelial cells. (C) GRK2 S-nitrosylation is diminished in lungs from eNOS$^{-/-}$ mice relative to wild-type (WT) mice. (D) GRK2 S-nitrosylation is increased in lungs from mice lacking a major SNO-metabolizing enzyme, GSNO reductase (GSNOR$^{-/-}$). Adapted from Ref. [5].

Prephotolysis as an independent strategy in the BST

There is little doubt that the signals arising from a BST after treatment with CysNO, for example, represent bona fide S-nitrosylated proteins. Like all other biological assays, however, internal and external controls should be employed to verify data obtained with

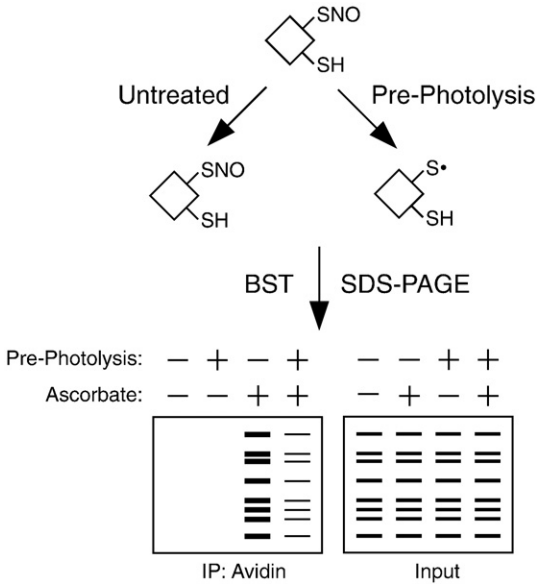

Fig. 5. An illustration employing UV prephotolysis and ascorbate controls within the BST for the analysis of a single lysate. One sample is split into two fractions, which are either untreated or exposed to a strong UV light source (prephotolysis) before the BST is performed. The prephotolysis step leads to homolytic cleavage of the S–NO into NO and an unstable thiyl radical, which is either reduced to a thiol or oxidized to a higher S-oxide. In either case, the signal from an SNO will be attenuated by prephotolysis, whereas free thiols or disulfides are unaffected. Inclusion or exclusion of ascorbate during the labeling step of the BST can also be employed to assay for protein–SNOs. This approach, which employs internal controls (e.g., prephotolysis and ascorbate), is ideal when external controls such as NOS inhibition or activation are not feasible (e.g., human tissue samples) or are ineffective (as in the case of reactions regulated by denitrosylation).

the BST, particularly when assaying endogenously S-nitrosylated proteins. These approaches frequently include: (1) omission of ascorbate during the labeling step of the BST, (2) overexpression or knockdown of a particular NOS isoform, and/or (3) pharmacological activation or inhibition of NOS. Though each of these approaches has been consistently applied to assay SNOs by the BST, we recently introduced an independent technique that involves photolysis of the SNO before the BST is performed (dubbed "prephotolysis") [5,63]. This methodology—a UV-based homolysis of the S–NO bond—is a simple and specific tool for assaying SNOs with the BST, particularly under conditions where NOS activation or inhibition is either unfeasible (e.g., human patient samples) or ineffective (e.g., NOS activity-independent, a characteristic of signaling activated by denitrosylation). This approach is based on Hg-coupled photolysis–chemiluminescence, in which the Hg-displaceable pool of NO groups (i.e., SNO) is photolyzed and detected via reaction with ozone. Photolysis eliminates SNO. Consequently, prephotolysis serves as a complementary approach to the BST. As shown in Fig. 3A, the signal from endogenously S-nitrosylated GAPDH is attenuated by a brief exposure to high-intensity UV light. A schematic illustration in Fig. 5 shows how prephotolysis and ascorbate can be combined to rigorously assess a complex sample for protein S-nitrosylation.

Design of the prephotolysis apparatus

Prephotolysis is performed by exposing the sample(s) to a strong UV radiation source within a light-sealed reaction chamber. Overall such an apparatus should: (1) deliver high-intensity radiation at 335 nm (the ideal wavelength for S–NO photolysis [47,67,68]), (2) prevent heating of the sample to avoid thermolytic reactions, (3) allow multiple samples to be photolyzed simultaneously, and (4) protect the operator from any harmful UV exposure. We employ a 200-W, 1.9-A Hg vapor lamp (Ace Glass, Catalog No. 7825-32), which delivers approximately 50% of its energy in the ultraviolet region. This lamp, along with an appropriate power supply, can be fitted into a sealed photochemical reaction cabinet (e.g., Ace Glass, Catalog No. 7836-20). Samples are generally photolyzed in borosilicate glass vessels (Pierce, Catalog No. 13504). Typically a 2-min exposure approximately 3 cm from the lamp is sufficient to photolyze at least 50% of a protein–SNO.

Several more economical sources of UV radiation may also suffice for prephotolysis, though they are likely to be less efficacious than a Hg vapor lamp. These include a UV transilluminator (often used to visualize DNA with ethidium bromide staining) and a UV crosslinker that provides radiation with wavelengths greater than 300 nm. In the latter case, a suitable apparatus would be the Ultraviolet Products (UVP) CL-1000 crosslinker (UVP, Catalog No. 81-0112-01) fitted with five long-range UV lamps (UVP, Catalog No. 34-0006-01).

Methodological issues and concerns raised about the BST

Despite its widespread use, the BST is technically challenging and labor intensive. Because each step contains potential sources of error, a rigorous experimentalist will include proper negative and positive controls to add confidence to their results. Numerous tools can be employed to check assay efficiency and to manipulate the stability or formation of protein–SNOs, thus adding great confidence to results obtained with the BST.

When comparing protein–SNOs across multiple conditions, it is imperative that each sample contain equivalent protein abundance, or inputs. Although some degree of normalization can be allowed, linearity (with respect to protein input and biotinylation) of the BST has not been demonstrated. Because proteins are subjected to multiple acetone precipitation steps and pellet washes, SDS–PAGE analysis should be performed on the biotinylated material before avidin pulldown. The final step of the BST is, for all intents and purposes, an affinity pulldown, which is almost always reported in the literature as both an "input" and a "pulldown." The BST should be held to the same standards.

The blocking step of the BST can also present a technical challenge, as some protein thiols can be resistant to complete blocking, resulting in high levels of SNO-independent biotinylation. In the absence of proper controls, incomplete blocking can be problematic because it may lead to misinterpretation of the data (i.e., "false-positive" signal). However, if the BST is performed correctly (i.e., +/– ascorbate), ineffective blocking will lower assay sensitivity (increase signal to noise) rather than specificity. Conversely, reverse strategies such as omitting ascorbate or employing prephotolysis can be used to determine the degree of blocking. Both of these treatments will attenuate a true SNO signal. Further, the blocking reaction can be lengthened to improve efficiency, though SNO stability may be compromised at 50 °C owing to thermolysis.

The specificity of the BST for SNOs (versus other cysteine-based modifications) had been questioned in several works. Here we review the actual data in these publications and explain why the observations are more likely artifact than a source of real concern. Landino et al. suggested that ascorbate reduces tubulin disulfides, a claim the authors use to challenge the validity of the BST [69]. However, as discussed below, reduction of alkyl (biological) disulfides by ascorbate is highly unfavorable thermodynamically, and an explanation for how "disulfides" were purportedly reduced was not provided. These authors in fact employed high concentrations of peroxynitrite to "oxidize" tubulin, though peroxynitrite also nitrosates thiols (albeit at low yields), thereby generating SNOs [70–72]; it is likely that S-nitrosylated tubulin was generated under these conditions. A later report by Huang and Chen argued that the BST yields "artifactual" ascorbate-dependent biotinylation of native reduced BSA [73] (i.e., SNO independent biotinylation). The authors did not provide a mechanistic basis for their observation, and subsequent work has reproduced the findings of Huang, which were

caused by a window-light artifact (BST should be performed in the dark) [63].

Most recently Giustarini et al. have challenged the BST mainly on the grounds that ascorbate reduces 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) [74]. However, this well-known reaction has no bearing on the reduction of biological (alkyl) disulfides: DTNB is a highly electrophilic, aryl disulfide that readily undergoes both hydrolysis and homolysis, the latter facilitated by ascorbate [75,76]. DTNB is therefore not representative of biological disulfides or in vivo Cys-based oxidation products. More perplexing, however, is these authors' suggestion that ascorbate reduces low-molecular-weight biological disulfides (e.g., glutathione, homocysteine, and cysteine disulfide) and protein mixed disulfides, but not intramolecular protein disulfides (under denaturing conditions). In fact, none of these reactions are favored thermodynamically, and there is no kinetic or thermodynamic basis for the difference between mixed and intramolecular protein disulfides under denaturing conditions. On closer inspection, the yield of reaction with cystine and glutathione disulfide was extremely low and seemed to saturate at a few percent over several hours, consistent with the presence of a contaminant (e.g., redox-active metals) or other artifact. Further, the authors measured disulfide reduction in the continuous presence of fluorescent thiol alkylators (bromobimanes), which irreversibly pull reactions by Le Chatelier's principle, and treated other samples with DTT to prevent "possible re-oxidation of thiols after their reduction by ascorbate" [74]. These approaches, which would artifactually generate thiols, cast doubt on the conclusions. These authors also do not provide a mechanistic explanation for their results or reference prior work to the contrary [77–81]. But most important of all, they do not perform the BST, whereas a comparative analysis of the BST across cell lysates derivatized by S-glutathionylation, S-oxidation, and S-nitrosylation revealed that only the S-nitrosylated proteins were detected [63].

Shared between all these claims of "artifactual signals" is the notion that ascorbate can reduce alkyl disulfides. However, this concept is both unsupported and difficult to reconcile experimentally with the use of MMTS, which converts protein thiols to mixed methyl disulfides, as a blocking agent. Clearly the BST would never work if ascorbate removed these "blocked" methyl disulfides. Further, the two-electron standard reduction potential of cysteine disulfides is −170 to −320 mV [77], indicating that this endergonic reaction will not likely couple to the two-electron oxidation of ascorbate, which is also unfavorable (the standard reduction potential of dehydroascorbate to ascorbate is +70 mV [82]). Thus, one would not expect ascorbate to directly reduce any biological protein cysteine oxidation products (except highly transient and unstable thiyl or sulfinyl radicals). In contrast, these electrochemical measurements favor the reverse reaction—thiol-dependent reduction of dehydroascorbate to ascorbate—a scenario supported by extensive in vitro and in vivo experimentation [78,80,81]. The same thermodynamic arguments hold for higher *S*-oxides as well [77].

Although the BST is highly specific for protein SNOs [63], we have noted that the presence of indirect sunlight (from an adjacent window) during the labeling step closely recapitulates the artifactual results of Huang et al. [73]. This SNO-independent biotinylation reaction is fully reversed by DTT, confirming that indirect sunlight indeed promotes an artifactual ascorbate-dependent protein–biotin disulfide during the labeling step. Under these conditions, biotin–HPDP (a synthetic aryl disulfide) undergoes quantitative reduction to biotin–thiol, though ascorbate exhibits no measureable reactivity in the absence of sunlight [63]. This production of high-micromolar biotin–thiol during the BST would lead to artifactual protein biotinylation via thiol/disulfide exchange with "blocked" (i.e., S-methylthiolated) proteins. The mechanism of this side reaction may involve homolysis of biotin–HPDP to generate an unstable alkyl thiyl radical of the biotin group and a resonance-delocalized aryl radical of 2-thiopyridine. In the presence of ascorbate, these thiyl radicals would be reduced to thiol [77] and therefore contribute to the observed artifact. Importantly, similar reactions between light, aryl disulfides and ascorbate have been reported [75,83]. These issues underscore the critical need to perform the labeling step of the BST in a light-free environment and to employ metal chelators (more below).

Potential problems associated with exogenous metals in the BST

Ascorbate will drive one-electron reductions of Cu^{2+} and Fe^{3+} ions, which may result in reactions that compromise BST specificity, including production of ascorbate and hydroxyl radicals. These reactions are readily mitigated through the use of metal chelators (e.g., EDTA, DTPA, neocuproine) [84–87]. Despite the widespread application of metal chelation with the BST, one recent report suggests a metal dependence for the BST to efficiently detect SNOs [88]. However, this conclusion overlooks the different chemistry operative in the presence and absence of transition metals: transition metals favor reductive chemistry by ascorbate over transnitrosation. Thus at low concentrations of ascorbate (<1 mM) as employed by Wang et al. [88], transition metals promote direct SNO reduction (metals serve as catalysts). Conversely, transnitrosation reactions, which form the basis of BST specificity, are favored by metal sequestration and higher ascorbate concentrations [60]. Increasing the amount of ascorbate (5–50 mM ascorbate) should remedy the reported "requirement" for exogenous metals that may increase assay sensitivity, but at the expense of specificity. In particular, redox-active metals are known to promote manifold reactions (both ascorbate dependent and independent), such as Cu^{2+}-mediated thiol oxidation [89–91] and Fenton chemistry [92–94]. Given the potential for such side reactions to compromise assay specificity, the addition of exogenous redox-active metals in the BST cannot be advocated without more rigorous validation in complex biological systems.

Given the known reactivity of Hg toward thiols and SNOs, numerous studies have attempted to employ Hg salts (e.g., $HgCl_2$) to quench SNOs before performing the BST (and thus serve as an internal assay control in lieu of omitting ascorbate). Despite the frequent application of Hg salts in NO-based assays (described above), the high affinity of Hg salts for free thiols [95–97] makes this strategy inherently problematic for the BST. As nicely demonstrated by Zhang et al. [98], $HgCl_2$ prevents biotinylation of free thiols and lowers any signal irrespective of S-nitrosylation status. Because all sources of biotinylation (i.e., signals) in the BST are attenuated by Hg salts, it is unclear how such a strategy could be used to distinguish an SNO from a false-positive signal due to incomplete blocking; therefore, Hg salts should not be used in lieu of other well-validated controls (e.g., prephotolysis, NOS inhibition, omitting ascorbate). By contrast, in an NO-based assay such as photolysis–chemiluminescence, Hg–thiol reactions identify the source of NO with Cys thiol by quenching the signal.

Modern permutations and adaptations of the BST

Various groups have adapted the BST to suit specific experimental goals. For example, several labs have trypsinized the biotinylated material before avidin pulldown to allow mass-spectrometry-based determination of protein–SNO sites (i.e., to identify specific cysteine residues targeted by S-nitrosylation) [5,99–101]. In addition, a thiol-reactive hexahistidine tag has been used in lieu of biotin–HPDP to facilitate mass spectrometric analysis [102]. These studies have further demonstrated the utility of the BST for SNO-oriented proteomics and bioinformatics. However, some of these strategies rely on a single peptide (containing the SNO site) for the identification of an entire protein, and thus stringent mass spectrometric criteria must be applied to these approaches to avoid incorrect protein (and SNO site) identification.

In another novel approach, Kettenhofen et al. substituted biotin–HPDP with thiol-reactive cyanine dyes to combine in-gel fluorescence

detection strategies with the BST [103,104]. Han et al. similarly employed coumarin-based fluorophores to the same end [105]. A major advantage of this approach is that it is readily adaptable to two-dimensional (2D) differential in-gel electrophoresis techniques in which multiple differentially fluorescence-labeled samples are coanalyzed on a single 2D electrophoresis gel [106,107].

Typical protocol for the biotin-switch technique

The overall steps of the BST are the following:

(1) Blocking,
(2) Labeling,
(3) Pulldown,
(4) SDS–PAGE and/or immunodetection.

General considerations for the BST

(1) A wide range of protein amount can be utilized (generally 0.3 to 5 mg of total protein per sample).
(2) A sensitive antibody with low background signal is critical for detecting an individual protein–SNO within a lysate.
(3) As discussed above, positive and negative controls should be included in every BST. For example, addition of 0.1 mM CysNO to one sample (before the BST) is an optimal positive control. A nonascorbate or photolyzed sample serves as a convenient negative control.
(4) Acetone precipitations are typically used to remove excess reagents and isolate proteins. However, for small sample volumes (<100 μl) or protein quantities (<300 μg), acetone precipitation is often inefficient or inconsistent. Under these conditions, P-6 desalting gel (Bio-Rad) or G-25 Sephadex (Amersham) can be substituted for acetone precipitations.

Necessary reagents and notes

MMTS (Fluka, Catalog No. 64306).
Biotin–HPDP (Pierce, Catalog No. 21341).
Streptavidin–agarose (Fluka, Catalog No. 85881).

Sodium ascorbate (Fluka, Catalog No. 11140). This ascorbate has been assayed for metal content, which is reported on the bottle. We have noted that this ascorbate is considerably more stable in aqueous solution than other preparations of ascorbate (i.e., yellowing due to slow oxidation to dehydroascorbate is undetectable).

Stock solutions

HEN buffer (100 mM Hepes, 1 mM EDTA, 0.1 mM neocuproine, pH 8.0). Alternatively, DTPA can be substituted for EDTA.
HENS buffer (HEN buffer with 1% SDS (w/v)).
HEN/10 buffer (HEN buffer diluted 10-fold with dH_2O).
HENS/10 buffer (HEN/10 buffer with 1% SDS (w/v)).
Neutralization buffer (25 mM Hepes, 100 mM NaCl, 1 mM EDTA, 0.5% Triton X-100, pH 7.5).
Wash buffer (neutralization buffer containing 600 mM NaCl).
25% SDS in dH_2O (w/v).
70% acetone in dH_2O (v/v).

Solutions to prepare immediately before assay

10% MMTS in *N*,*N*-dimethylformamide (v/v).
200 mM sodium ascorbate in HEN buffer (stored in the dark on ice).
2.5 mg/ml biotin–HPDP in DMSO.
Elution buffer (HEN/10 containing 1% β-mercaptoethanol (v/v)).

Sample protocol for the BST

(1) *Sample preparation:* Cells are lysed as desired. For example, a 6-cm plate of HEK293 cells can be lysed in 0.40 ml of 25 mM Hepes, 50 mM NaCl, 0.1 mM EDTA (DTPA may be a better metal chelator; note also that neocuproine is present in HEN buffer), 1% NP-40, 0.5 mM PMSF plus protease inhibitors, pH 7.4. Repeated passage through a 28-gauge needle will increase lysis efficiency. It is critical to avoid DTT (or other reductants) in the lysis buffer because they will destabilize SNOs and interfere with the BST. After centrifugation, protein concentrations are measured (e.g., using the BCA assay; Pierce). Samples are adjusted with lysis buffer to achieve equal concentrations of total protein.
(2) *Blocking:* For experiments employing 0.5–2 mg of protein, samples are diluted to 1.8 ml with HEN buffer. Next, 0.2 ml of 25% SDS is added along with 20 μl of 10% MMTS (final 2.0 ml volume with 2.5% SDS and 0.1% MMTS). Samples are incubated at 50 °C in the dark for 15–20 min with frequent vortexing. This step is frequently performed in 15-ml conical tubes. Alternatively, MMTS may be added directly to the lysis buffer to initiate blocking immediately after lysis, though it should be noted that MMTS interferes with the BCA protein assay. For some proteins, the blocking step can be efficiently performed at room temperature (e.g., S-nitrosylated caspase-3), though this should be determined empirically for each protein.
(3) *Precipitation:* Three volumes of cold acetone (6 ml) are added to each sample. Proteins are precipitated for 20 min at −20 °C and collected by centrifugation at 2000 *g* for 5 min. The clear supernatant is aspirated and the protein pellet is gently washed with 70% acetone (4×5 ml).
(4) *Labeling:* After resuspension in 0.24 ml HENS buffer, the material is transferred to a fresh 1.7-ml microfuge tube containing 30 μl biotin–HPDP (2.5 mg/ml). The labeling reaction is initiated by adding 30 μl of 200 mM sodium ascorbate (we tend to use a final concentration of 20 mM ascorbate, though smaller amounts may suffice [36] and larger amounts can be used as necessary [63]). Alternatively, an equivalent concentration of NaCl can be used as an ascorbate-free control. Samples are rotated at room temperature in the dark for 1 h. It is critical to avoid any sources of sunlight during this step.
(5) *Precipitation:* Three volumes of cold acetone (0.9 ml) are added to each sample. Proteins are precipitated for 20 min at −20 °C and collected by centrifugation at 5000 *g* for 5 min. The clear supernatant is aspirated and the protein pellet is gently washed with 70% acetone (4×1 ml).
(6) *Pulldown:* After complete resuspension in 0.25 ml HENS/10 buffer, 0.75 ml of neutralization buffer is added. A small fraction of each sample (e.g., 10 μl) is removed for analysis of protein input. The remaining material is transferred to a fresh 1.7-ml microfuge tube containing 25–50 μl of prewashed avidin-affinity resin. It is critical that bead volumes in each sample are the same. The samples are gently rotated for 12–18 h at 4 °C.
(7) *Washing:* Avidin beads are collected by centrifugation at 200 *g*×10 s in a swinging-bucket rotor, followed by washing with wash buffer (4×1 ml). After the final wash, the beads are fully dried via gentle aspiration with a 28-gauge needle.
(8) *Elution:* To each sample is added 30–50 μl of elution buffer. Proteins are eluted at room temperature with frequent agitation, followed by centrifugation at 5000 *g*×30 s. Supernatant is collected without disturbing the pelleted resin and mixed with 6× Laemmli loading buffer. For analysis of protein–SNOs via immunodetection of biotinylation, elution is performed by heating the beads to 95 °C in 30–50 μl of HENS/10 buffer containing nonreducing Laemmli loading buffer (all reductants must be avoided).

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