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In-Gel Detection of S-Nitrosated Proteins using Fluorescence

Methods

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Abstract

Gel-based detection of S-nitrosothiols has relied on the biotin-switch method developed by Jaffrey et al (*Nat. Cell. Biol.*, 3, 193–197, 2001). This method attempts to replace the nitroso group with a biotin label to allow detection and isolation of S-nitrosated proteins, and has been extensively used in the literature. Here we describe a modification of this method that differs from the original in two major ways. First, it uses a combination of copper ions and ascorbate to achieve selective reduction of the S-nitrosothiol. Second, it replaces the biotin label with fluorescent cyanine dyes in order to directly observe the modified proteins in-gel and perform comparative studies using difference gel electrophoresis (DIGE) analysis in two dimensions.

Introduction

While S-nitrosation has become increasingly recognized as an important post-translational modification of cysteinyl residues, the detection of such modifications has been somewhat troublesome (Gladwin et al., 2006). It is relatively straight forward to detect total S-nitrosothiol level, as has been described in detail elsewhere (Samouilov and Zweier, 1998; Yang et al., 2003; Bryan and Grisham, 2007; Fang et al., 1998), however, the detection of S-nitrosation on specific proteins (PSNO) relies on indirect methodology (Jaffrey et al., 2001). The reasons for this are that (i) the nitroso group is not appropriate for radiolabeling and (ii) there is no reliable antibody for the direct detection or immunoprecipitation of S-nitrosated proteins. In addition, no strategies for directly and stably modifying the S-NO group have been devised.

Consequently, the only strategy so far described involves the complete destruction of the S-nitroso functional group and its replacement by a label. This was first exemplified by the biotin-switch assay in which the S-nitroso group is replaced by a biotin label (Jaffrey et al., 2001). The three steps that comprise this method (Figure 1) involve the blockade of all free thiol groups, the ascorbate-mediated reduction of S-nitrosothiols to thiols, and the labeling of the nascent thiols with a biotinylating agent. While this technique has been used for many studies, it remains somewhat controversial due to (i) the possibilities of false positives due to incomplete thiol blocking, (ii) the slow kinetics of S-nitrosothiol reduction by ascorbate, (iii) the possibility of disulfide reduction by ascorbate and (iv) the widespread (though anecdotal) inconsistency experienced by many investigators.

Here we describe the modifications made to the original method to allow sensitive detection of protein S-nitrosothiols both traditional biotin-based methods and novel fluorescence methods.

The role of trace metal ions in ascorbate-mediated reduction of S-nitrosothiols

We have previously investigated the biotin switch method in detail and have reported that ascorbate is a very poor reducer of S-nitrosothiols. In order to get significant biotin labeling much higher levels of ascorbate are required than originally proposed (Zhang et al., 2005). This has recently been confirmed by others (Forrester et al., 2007), and raises the question of how other investigators achieved positive results in the biotin switch assay using the original low ascorbate conditions. We recently examined this issue and observed that the presence of contaminating metal ions can dramatically enhance the ability of ascorbate to reduce S-nitrosothiols, and the addition of copper ions and the removal of metal ion chelators allows the biotin switch assay to work at the originally proposed ascorbate levels (Wang, Kettenhofen, Hogg and Gladwin, manuscript in preparation). It is therefore not ascorbate that is reducing the S-nitrosothiol but contaminating transition metal ions. In the case of copper ions, the role of ascorbate is to reduce the cupric ions back to the cuprous state which can then rapidly reduce the S-nitrosothiol

to form the parent thiol, nitric oxide and cupric ion (Equations 1 and 2) (Singh et al., 1996; Askew et al., 1995). It is likely that other metal ions may also catalyze ascorbate-dependent S-nitrosothiol reduction in a similar mechanism as copper. Consequently, in the protocols described below we have removed metal ion chelators from the buffers and supplemented the buffers with copper ions.

Detection of protein S-nitrosation using fluorescent labeling methods

While the replacement of protein RSNO with a biotin tag provides a handle for affinity-precipitation experiments that have been successfully utilized for the identification of modified proteins (Greco et al., 2006; Hao et al., 2006), it has the disadvantage that Western analysis is required for on-gel visualization of the modified proteins. We have modified the original biotin-switch method to develop a direct in-gel fluorescence method for the determination of S-nitrosated proteins using cyanine-dye technology and difference gel electrophoresis (DIGE) (Alban et al., 2003) in order to allow direct in-gel visualization of the labeled proteins using fluorescence scanning. The availability of two spectrally-resolvable versions of maleimide CyDyes (Maeda et al., 2004) allows us to exploit DIGE technology for comparative proteomic studies of protein S-nitrosation. In addition to the advantage of directly visualizing the labeled proteins in gel, this technology allows direct comparisons of samples to be made within the same gel, therefore eliminating gel-to-gel variations that commonly plague 2D-gel proteomic studies. The labeling scheme is shown in Figure 2. Protein thiols exist in various states of modification, and the basic approach of the DIGE method is to examine the difference in modification before and after exposure to an experimental condition (e.g. exposure to a nitric oxide donor or to an inflammatory stimulus). Both control samples and experimental samples are treated in an identical manner with the exception that the control and experimental samples

are labeled with different cyanine dyes. In scheme 2, control samples are labeled with the green fluorescence dye Cy3 and the experimental sample is labeled with the red fluorescence dye Cy5. However, it is useful to also run the opposite labeling scheme to ensure that any observations are not dye specific. In an identical manner to the biotin switch assay (Figure 1), the first step is to block thiol groups. Any thiol groups that escape this blockade will be labeled in both the control and the experimental sample. The next step is to reduce the modified thiols to the parent thiol and label them with the appropriate dye. Various reduction strategies can be employed. For example, ascorbate alone, ascorbate with copper ions, DTT, and other reducing agents such as triphenylphosphine, can all be used to reduce different pools of modified thiol. For the specific detection of S-nitrosothiols we employ an ascorbate/Cu(II) mixture are described below. As shown in Figure 2, any background thiol modifications present in the control sample that are reduced to thiols in the reduction step will be labeled green. For the treated sample, these same proteins will also be labeled red, but in addition, any thiol modifications that occur as a result of treatment, that are also reduced by the specific reduction strategy employed, will also be labeled red. Consequently the difference between the red fluorescence and the green fluorescence will report the treatment-dependent modifications. After labeling, both control and experimental samples are pooled at equal protein load and run in the same lane of a gel, which is then scanned twice – once for red and once for green fluorescence. The difference between the red and green fluorescence scans will then reveal the treatment-dependent modifications. As mentioned above, and illustrated below, it is advisable to do an identical experiment with reverse labeling to make sure that the differences observed are not a function of the labeling order.

The bottom panel of Figure 2 shows some possible outcomes for this experimental paradigm when using both an ascorbate/Cu(II) mixture or DTT as the reducing agent. The top line represents a protein that is S-nitrosated upon treatment and shows red in ascorbate/Cu(II) but red and green in DTT. The overlay of the two scans is highly informative as, in this case the ascorbate/Cu(II) treatment will show red and the DTT yellow. The second line illustrates a S-nitrosated protein with incomplete thiol blocking (on either the same or a different thiol on the protein that contains the S-nitrosothiol). In this case, a yellow band is observed in the absence of a reducing agent as a result of equal read and green labeling. This yellowish color in the overlay image will also be present in all reduction schemes due to the green background fluorescence in the control sample. However, it may still be possible to distinguish this modification from the difference between the intensity of the red and green fluorescence. Nitrosative/oxidative treatment of cellular proteins can cause disulfide formation as well as S-nitrosation. If the disulfide is not reduced by ascorbate/Cu(II) then it will only show up upon DTT reduction (line 3 in Figure 2B), however if it is reduced by ascorbate/Cu(II) then it will be indistinguishable from an S-nitrosothiol (line 4 in Figure 2B). Finally, any basal disulfides that are also reducible by ascorbate/Cu(II) will show up yellow on the overlay and therefore will not show a false positive. In summary, this illustration shows that only disulfides that are formed as a result of the experimental treatment and which are then reducible by ascorbate/Cu(II) will show up as ‘false positive’ S-nitrosothiols. Consequently, before a positive identification of protein S-nitrosation can be made, it is important to rule out this possibility. This is not an easy task, but can be done either by attempting to S-thiolate cellular proteins with an oxidant stress or diamide/thiol mixtures, or preferably (if the protein is known and is available in a pure form) specifically synthesizing and testing S-thiolated forms of the protein.

Current Protocol for CyDye-Switch method

Our recommended protocol for the CyDye-Switch method is as follows:

1. **Sample Preparation and Free Thiol Blocking:** Throughout the entire CyDye switch protocol, as well as any other RSNO analysis, samples should be kept in the dark whenever possible. A vast excess of NEM (50 mM) or any other thiol-reactive

molecule of choice should be present either in the lysis buffer (HDN: 250 mM HEPES with 1 mM DTPA and 100 μ M Neocuproine, pH 7.7 containing protease inhibitors) for cellular or tissue studies, or added immediately following the protein treatment for *in vitro* studies. Immediate thiol-blocking is recommended to prevent any additional PSNO formation during sample processing as well as quench the low molecular weight thiol (GSH) pool that may react with PSNO to 'reverse' the modification. Complete blocking is accomplished by denaturing proteins with SDS (2.5% w/v final) and incubating samples at 50° C for 30–60 minutes with frequent vortexing. Complete blocking is essential to prevent nonspecific protein labeling that may create a high background or result in false positive signals.

2. Separate sample proteins from excess blocking agent: Excess thiol-blocking agent (NEM) must be removed in order to prevent any competition for the labeling reaction. This can be done by various techniques of protein precipitation (TCA, EtOH, acetone, etc.) or size-exclusion column chromatography (also possibly dialysis). For precipitation, add 2 volumes of 15% TCA and spin down protein pellet (2,000 $\times$ g for 10 min). Aspirate all supernatant and wash the pellet 2 times with 15% TCA with repeated spins to ensure complete NEM removal. Resuspend the pellet in HS (25 mM HEPES with 1% SDS) as no metal chelators should be present for any samples using copper-mediated reductions.
3. PSNO Reduction/CyDye Labeling: Specific reduction of PSNO is mediated by the addition of ascorbate (1 mM final) and copper sulfate (1 μ M final) in the presence of a thiol-reactive maleimide CyDye in order to label the resulting free thiols at cysteine residues that were previously S-nitrosated. Cu(I) salts (e.g. CuCl) can be used in place of Cu (II) and may be preferable as the metal ion is already reduced. However, solutions of Cu(I) salts are generally more unstable. CyDye (Cy3 or Cy5) is added to a level of 100 pmoles dye/ μ g of protein (as determined by BCA assay). The reduction/labeling reaction is allowed to proceed for 15–60 minutes at room temperature. Samples for 1D analysis are mixed 1:1 with Laemmli buffer containing β -mercaptoethanol while samples for 2D analysis must undergo buffer exchange with IEF-compatible rehydration buffer.
4. Pool matched sample pairs prior to protein separation for DIGE analysis: Mix equal amounts of Cy3-labeled control sample and Cy5-labeled treatment sample (or vice versa) to allow for direct in-gel comparisons. Load pooled sample on a single lane or single IPG strip for 1D or 2D analysis respectively. Samples can then be co-separated by electrophoresis using gels that are cast in low-fluorescence glass plates. The band/spot intensities for Cy3 and Cy5 fluorescence can be compared following gel fluorescence scanning using a Typhoon Trio Imager or a similar instrument. Alternatively, a single CyDye can be used for side-by-side comparison of samples as is done with the classic biotin switch.

DIGE analysis of a model protein mixture

In order to test these protocols we have selected a model mixture of four proteins with differing thiol/disulfide content and molecular weight. The four proteins used were aldolase, which has a high thiol/disulfide ratio (8 total cys, 3.6 free thiols as determined by DTNB assay), catalase (4 total cys, 0.8 free thiols), bovine serum albumin (35 total cys, 0.4 free thiols) and lactoferrin (34 total cys, 0.2 free thiols). The thiol levels were determined using Elman's reagent. In each case we have generated S-nitrosated versions of these proteins, pooled them and subjected them to the CyDye switch method. In this experiment we have used both senses of labeling and only show the overlay of the two fluorescent images. As shown in Figure 3, in the absence of reducing agents no bands were visible. This indicates that thiol blocking was complete. In the presence of ascorbate alone, a faint band was seen for aldolase, but only in the treated

samples. This indicates that the S-nitrosothiol of aldolase is particularly sensitive to reduction and illustrates that ascorbate alone will emphasize such proteins. Reduction by ascorbate/Cu (II) shows very clear labeling of aldolase, catalase and BSA, but not lactoferrin. In all cases the labeling is only present for the S-nitrosated protein and not for the control protein, indicating that in all cases this treatment did not reduce endogenous disulfides. Finally, reduction using DTT shows that aldolase and to some extent catalase still present a clear distinction between the treated and control samples, suggesting that disulfide reduction and labeling is not a big issue with these proteins. In contrast, lactoferrin and BSA show indistinguishable labeling between control and S-nitrosated samples (yellow in the overlay). This nicely illustrates that in proteins that contain many disulfides such as BSA, the ascorbate/Cu(II) mixture is still able to detect S-nitrosation.

The Sensitivity of Detection of the CyDye label

In order to examine the sensitivity of the CyDye method we prepared S-nitroso HSA and diluted it with untreated HSA to varying degrees so that the total protein content remained equal. These mixtures were subjected to the CyDye switch protocol using only Cy3 as the labeling agent. Figure 4A shows both the fluorescence gel and the Scion-Image densitometric band intensity as a function of the calculated amount of original protein S-nitrosothiol loaded onto the gel. These data are recapitulated in Figure 4B with a conventional evenly spaced x-axis. This illustrates that the CyDye switch method can detect down to about 40 fmol of S-nitrosated protein and that the response is relatively linear with the exception of the highest level examined where the response tails off. This illustrates that fluorescence imaging is at least as sensitive as the biotin-switch method. Of course, these results are specific for the particular reduction condition (i.e. copper concentration and time) used as well as the sensitivity setting on the fluorescence scanner. This data using HSASNO represents a conservative estimate of sensitivity given the low Cu(II) concentration (0.1 μ M) and short reduction/labeling time (15 min). Additionally, as seen in the data from our simple protein mixture, sensitivity will most likely be dependent upon the individual protein being analyzed.

Fluorescence detection of S-nitrosated proteins in plasma

We have employed the CyDye switch method to examine S-nitrosation of plasma proteins after the treatment of plasma with S-nitrosocystiene. Figure 5A illustrates the level of total RSNO observed in plasma after treatment with S-nitrosocystiene, as measure by tri-iodide-based chemiluminescence. Plasma proteins were then subjected to CyDye switch labeling, and under the conditions we have employed, only a band representing S-nitrosoHSA was observed; perhaps unsurprising as this is the major thiol-containing protein in plasma. Again as low as 40 fmol of protein S-nitrosothiol could be clearly detected above background levels. This also is a conservative estimate as identical reduction/labeling conditions were used for the plasma samples and the pure HSASNO samples presented above. Similar results were obtained for the Cy5 label (not shown).

2D DIGE-detection of S-nitrosated proteins

The DIGE technology works best when run in conjunction with 2D gels. In figure 6 we illustrate a 2D- gel of normal human bronchial epithelial (NHBE) cell lysate proteins that were S-nitrosocysteine-treated ex-vivo. The total protein S-nitrosothiol content for this sample was an extremely high and non-physiological level (>10 nmoles/mg protein). For this experiment we used the ascorbate/Cu(II) reduction scheme for labeling. As can be seen, ascorbate/Cu(II) reduction revealed a multiplicity of spots that were labeled in the treated sample (Cy5, red) but not present in the control (Cy3, green). The relative sparseness of labeling in the control sample is very encouraging as it suggests thiol blocking is complete and endogenous disulfides are not reduced by the ascorbate/Cu(II) treatment.

Modifications of the Original Biotin Switch Method

The original biotin switch method described by Jaffrey et al (Jaffrey et al., 2001) consisted of the following steps (See Figure 1). First, sample proteins were incubated with the thiol blocking agent S-methylmethanethiosulfonate (MMTS). The efficiency of this step dictates the specificity and sensitivity of this method as any unblocked thiols will show up as false positives. By a simple calculation, it can be seen that if 1% of protein thiols are nitrosated, blocking efficiency needs to be 99% for a signal:background ratio of 1:1. S-nitrosated proteins are generally much lower in abundance than this, and signal:background needs to be much higher than 1:1, suggesting blocking efficiency needs to be much higher than 99%. Although blocking efficiency is crucial, it is relatively easy to test and account for if appropriate control samples are available. Our experience is that alternative blocking agents, such as N-ethylmaleimide (NEM) or iodoacetamide (IAA) work as well as MMTS. In fact MMTS can be problematic as it blocks thiols through the formation of a disulfide, which is generally a more easily reducible modification than a thioether. The second step is the reduction of S-nitrosothiols by incubation with 1 mM ascorbate for 1 hour in the presence of EDTA and neocuproine in HEPES buffer. This treatment aims to selectively reduce the S-nitrosothiols but not reduce disulfides so that all thiol groups generated by this reduction process derive from protein RSNO. It is this step that has generated the most problems with this procedure. At first glance this step appears completely untenable as the rate constants for the reduction of RSNO by ascorbate have been established and predict that 1 mM ascorbate for 1 hour would reduce only a small fraction of the total S-nitrosothiol (Holmes and Williams, 2000; Kashiba-Iwatsuki et al., 1996). For example, the reaction between GSNO and ascorbate has a rate constant of about $12 \text{ M}^{-1}\text{s}^{-1}$ at pH 7.3 (Holmes and Williams, 2000), giving a pseudo-first order half time for GSNO decay of about 16 hours with 1 mM ascorbate. S-Nitroso bovine serum album appears to be reduced even more slowly than GSNO (Zhang et al., 2005). In the original studies, the efficiency of this reduction step was never examined, but our studies have demonstrated that this level of ascorbate hardly makes a dent in the total RSNO level in a complex mixture of cellular S-nitrosothiols (Kettenhofen et al., 2007). This was consistent with our experience with the biotin switch assay which only gave positive signals at ascorbate levels of 30 mM and above (Zhang et al., 2005), and others have clearly experienced the same problems (Forrester et al., 2007). Interestingly, in our previous studies we consistently used DTPA in place of EDTA in the buffers as DTPA is far better at diminishing the oxidative effects of trace iron and copper than is EDTA. We recently closely examined the effects of iron chelators on the ability of ascorbate to promote RSNO decay, and showed the 'dirtier' the experiment, the better the result. In other words, if buffers were chelex-treated, or if sufficient quantities of freshly prepared metal chelators were used, the degree of biotin labeling was greatly diminished (Wang et al, submitted manuscript). However, if copper ions were purposely added to the incubation mixture, the biotinylation signals were greatly enhanced. This led us to propose that the reduction/labeling step of the biotin-switch assay should be preformed in the absence of metal chelators and with the addition of copper ions to the buffers (see below). The final step is the labeling of the newly formed thiols. (N-(6-(Biotinamido)hexyl)-3'-(2'-pyridyldithio)-propionamide (Biotin-HPDP) was originally proposed as the biotinylation agent as it forms a disulfide with the protein thiol, which can aid the release of the protein from agarose beads after a precipitation experiment. However, as with MMTS, the disulfide is a weaker link than a thioether, and the attachment of biotin through a maleimido or an iodoacetamide linking agent are other possibilities.

Current Procedure for the Biotin Switch Assay

Our recommended procedure for the biotinylation of protein S-nitrosothiols is as follows:

1. Protein thiols should be blocked as soon as possible after the cessation of the experiment. Protein mixtures should not be acidified or precipitated before all thiols are blocked. The blocking buffer contains 250 mM HEPES, pH 7.7 containing 1 mM

EDTA (or DTPA), 0.1 mM neocuproin, 2.5% SDS and 50 mM MMTS (or NEM). Amber tubes should be used for all steps of the reaction, and direct light should be avoided, to prevent photolytic decomposition of S-nitrosothiols. While S-nitrosothiols are not acutely light-sensitive, prolonged exposure to light source will result in some photolysis of the S-N bond. In addition, problems with direct sunlight have been reported in the ascorbate reduction step (Forrester et al., 2007). This mixture is incubated for 30 minutes at 50 °C with occasional vortexing.

2. Excess blocking agent can be removed by either passage down a G25 microspin column (three times) or protein precipitation with ethanol or acetone. Both methods allow buffer exchange to a metal chelator-free buffer consisting of HEPESs (25 mM, pH 7.7) and 1% SDS. Ascorbate (1 mM), biotin-HPDP (2 mM, or alternative biotinylation agent) and copper ions are added to this mixture and incubated for 1 hour. Our experience suggests that the oxidation state of the copper salt is not particularly crucial, and we have achieved good results with both Cu(I) chloride and Cu(II) sulfate. This is because ascorbate reduces the Cu(II) to Cu(I) which effectively reduces the S-NO bond. The amount of copper required may be significantly system dependent and it is essential that the ability of the Cu/ascorbate solution to reduce S-nitrosothiols is checked by an independent method (such as tri-iodide chemiluminescence) in each system. With pure proteins as low as 10 nM copper was able to reduce protein RSNO whereas in complex mixtures up to 1 μ M may be required. It should be noted that copper ions are catalytic and are undergoing a Cu(I) to Cu(II) redox cycle during ascorbate-dependent S-nitrosothiol decay. Although we have not specifically examined this, it is likely that too much copper may become problematic, as copper(II) can oxidize thiols and potentially lead to a reduction in labeling. Also, addition of high levels of copper(I) may also result in superoxide and hydrogen peroxide generation through the reduction of oxygen and thus affect the integrity of the proteins.
3. After labeling, proteins are detected on Western blots using either a peroxidase conjugated anti-biotin antibody or peroxidase conjugated streptavidin. For quantitative determination of the level of biotinylation, biotinylated cytochrome c can be used as an internal standard as previously described (Landar et al., 2006). It should be stressed that the use of a biotinylated standard does not quantify the amount of S-nitrosothiol, but allows the determination of the amount of biotinylated proteins. Other controls (discussed below) and the use of standard S-nitrosated proteins are required to fully understand the relationship between the level of biotinylation and the original level of S-nitrosothiol. Instead of Western analysis, labeled proteins can be isolated using streptavidin-agarose for direct mass analysis.
4. It is essential for every experiment that a number of controls are included. First, a control needs to be performed in the absence of ascorbate/Cu in order to determine the success of the blocking step. Second, the experimental sample should be fully reduced with (e.g.) DTT before the blocking step to ensure that labeling is specific. Third, it is advisable to run a positive control consisting of a DTT-reduced sample in which the blocking step is omitted. This will give an idea of the total amount of a particular thiol-containing protein and can be used not only to test if the methods are working, but also to ascertain the fraction of a particular protein that has been modified.

While these modifications of the method allow reproducible biotinylation of S-nitrosated protein, the method still contains an intrinsic problem in regards to possible false-positive signals from disulfides that may be reduced by the ascorbate/Cu step. While thiol blockade is generally very efficient, suggesting the reduction of endogenous disulfides is not a major issue, the detection of mixed disulfides generated during NO/RSNO exposure cannot be fully ruled

out. While we see no reduction of pre-formed mixed-disulfides on human serum albumin using this technique (Wang et al submitted manuscript), this needs to be tested on a protein-by-protein basis, and a positive identification of an S-nitrosothiol can only be confirmed if the possible formation of mixed disulfides is ruled out. Despite this limitation, it should be emphasized that mixed disulfide formation is also an important post-translational NO-dependent thiol modification, and so even if the type of modification is not 100% clarified, this method still provides valuable information.

Conclusion

This article describes our current protocols for both the original biotin switch method and a novel CyDye-Switch method to detect S-nitrosated proteins. The major modification to the original method is the use of ascorbate/Cu(II) mixtures to facilitate the S-nitrosothiol reduction. Under these conditions reduction appears facile and relatively specific. Although we cannot rule out signals from other modifications that may be generated from treatment of cells with oxidants, such as mixed disulfides, as least in the case of serum albumin, such modifications do not give false positives (Wang et al manuscript in preparation and data presented here). The CyDye switch method allows comparative assessment of two states of cellular proteins directly in-gel without the necessity of a Western blot. It is highly sensitive and specific (with the same caveat mentioned above for the conventional biotin-switch method). The fact that proteins can be directly detected in-gel removes the necessity of spot registration and allows for the automated processing of positive spots for protein identification. These fluorescence-based methods should provide an additional and in many ways superior method for the detection and identification of S-nitrosated proteins.

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Figure 1. The Biotin Switch Method

The biotin switch assay involves selectively replacing the nitroso group with a biotin label. This is accomplished in three steps. The first step involves blockade of all thiol groups. The second step involves the selective reduction of S-nitrosothiols to their parent thiols, avoiding reduction protein-protein disulfides or protein-mixed disulfides. The third step involves labeling of the nascent thiols with a thiol-specific biotinylation agent thus tagging only the S-nitrosated proteins.

Figure 2. The Cy-Dye Switch Method

A) Protein cysteine residues can exist in protein disulfides as mixed disulfides and as free thiols. The experimental treatment (e.g. addition of NO donor or activation of NOS) will convert some of these thiols to S-nitrosothiols and some to disulfides. In the CyDye switch method, the control sample is treated with a thiol blocker and the resulting proteins are reduced with ascorbate/cu and labeled with green Cy3. The proteins that show up as green fluorescence will therefore represent unblocked thiols and endogenous disulfides that were reduced (e.g. by the ascorbate/Cu treatment or by DTT). The experimental sample is treated in the same way, but labeled with red Cy5. In this case red fluorescence will be associated with the same proteins that are labeled in the control experiment, but in addition, both S-nitrosothiols, and potentially

some mixed disulfides that were formed from the experimental treatment, will also be labeled in red. The labeled protein from both control and experiment are then pooled at equal protein concentration and run on a gel. B) Typical patterns that may be expected in various scenarios.

Figure 3. CyDye switch analysis of a simple protein mixture

An equimolar mix (1 mg/ml total protein) of aldolase, catalase, BSA, and lactoferrin was treated with either 100 μM S-nitrosocysteine (CysNO) or buffer alone (control) for 30 min. Samples were then labeled with Cy3 (green) or Cy5 (red) by CyDye switch using various reduction conditions as indicated. Equal amounts of experimentally paired samples were then pooled and separated by SDS-PAGE. An overlay image of Cy3 and Cy5 fluorescence is presented.

Figure 4. Sensitivity of HSASNO detection by Cydye switch

S-nitroso-HSA samples (constant total protein, varied SNO) were Cy3-labeled by CyDye switch with ascorbate (1 mM)/Cu(II) (0.1 μ M) reduction. 1 μ g protein containing varying amounts of SNO (indicated below graphs) was resolved in each lane by SDS-PAGE. The gel was imaged for Cy3 fluorescence (top panel) and band intensities were quantified using Scion Image and plotted versus the amount of SNO loaded (bar and scatter plots).

Figure 5. Detection of S-nitrosated proteins in plasma

Human plasma was treated with various concentrations of CysNO for 60 min. Total levels of SNO were determined by tri-iodide based chemiluminescence and normalized to protein content (top panel). Samples were labeled with Cy3 using CyDye switch with ascorbate (1 mM)/Cu(II) (0.1 μM) reduction. Proteins were then resolved by SDS-PAGE and Cy3 gel fluorescence was imaged (middle panel). The gel image was analyzed using Scion Image and the band intensities for HSASNO were plotted versus the total amount of SNO loaded per lane (bottom panel).

Figure 6. 2D-DIGE detection of S-nitrosated proteins in a complex mixture

NHBE cells were lysed and cytosolic proteins were treated with either 100 μ M S-nitrosocysteine (CysNO) or buffer alone (control) for 30 min. Samples were then labeled using the CyDye switch protocol with ascorbate (1 mM)/Cu(II) (10 μ M) reduction. The control sample was labeled with Cy3 (green) while the treated sample was labeled with Cy5 (red). Equal amounts of protein from each sample were then pooled and co-separated by 2D electrophoresis. The gel was imaged for both Cy3 and Cy5 fluorescence. Cy3 (top), Cy5 (middle) and overlay (bottom) images are presented.