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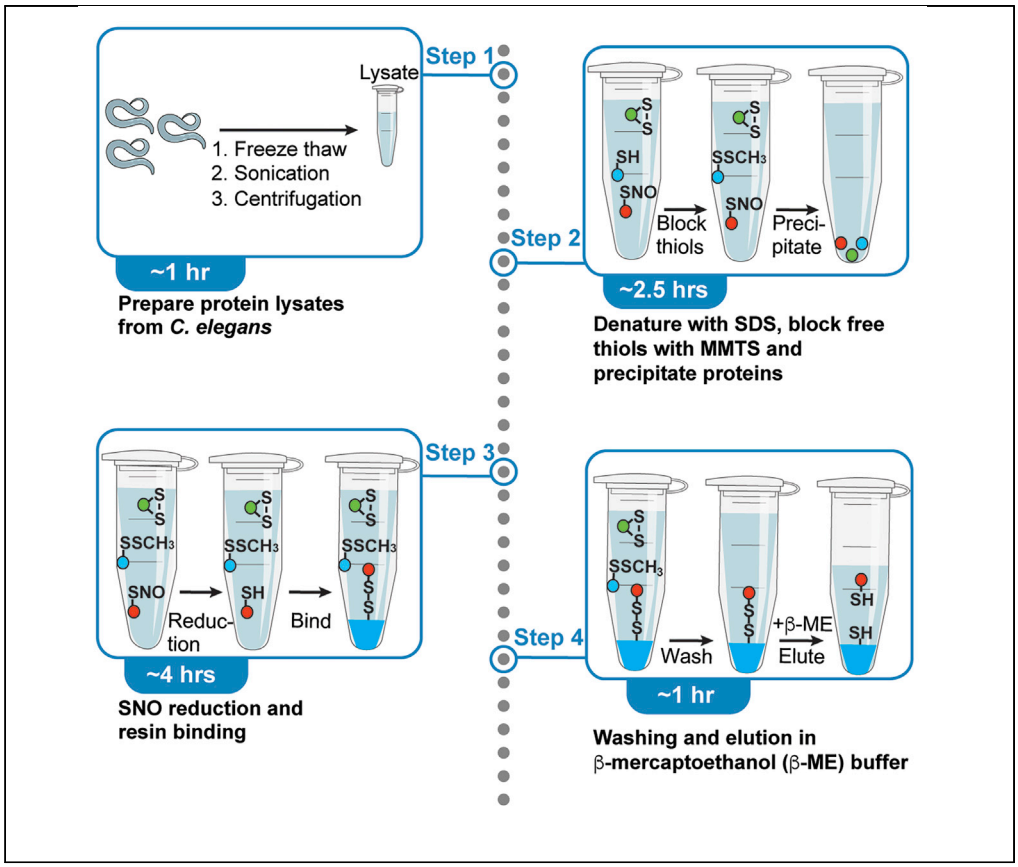
An optimized protocol for isolation of S-nitrosylated proteins from *C. elegans*



Post-translational modification by S-nitrosylation regulates numerous cellular functions and impacts most proteins across phylogeny. We describe a protocol for isolating S-nitrosylated proteins (SNO-proteins) from *C. elegans*, suitable for assessing SNO levels of individual proteins and of the global proteome. This protocol features efficient nematode lysis and SNO capture, while protection of SNO proteins from degradation is the major challenge. This protocol can be adapted to mammalian tissues.

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Highlights

Protocol for isolating S-nitrosylated proteins from *C. elegans*

Assesses global changes in S-nitroso-protein levels

Also assesses S-nitrosylation changes in individual proteins of interest

Protocol is adaptable to mammalian tissues

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An optimized protocol for isolation of S-nitrosylated proteins from *C. elegans*

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SUMMARY

Post-translational modification by S-nitrosylation regulates numerous cellular functions and impacts most proteins across phylogeny. We describe a protocol for isolating S-nitrosylated proteins (SNO-proteins) from *C. elegans*, suitable for assessing SNO levels of individual proteins and of the global proteome. This protocol features efficient nematode lysis and SNO capture, while protection of SNO proteins from degradation is the major challenge. This protocol can be adapted to mammalian tissues.

For complete information on the generation and use of this protocol, please refer to Seth et al. (2019).

BEFORE YOU BEGIN

S-nitrosylated (SNO) proteins can be captured using the biotin-switch assay (Jaffrey and Snyder, 2001; Forrester et al., 2009a) or by the SNO Resin-Assisted Capture (SNO-RAC) method that was first developed by our laboratory (Forrester et al., 2009b). Compared to biotin-switch, the SNO-RAC method involves fewer steps due to direct conjugation of SNO-site cysteines to a resin, rather than direct biotinylation of SNO-sites followed by capture with an avidin resin. Additionally, it is more efficient especially for high molecular-weight SNO-proteins and more versatile. That is, because of the reversible Cys–resin bond, it can be used in conjunction with isobaric Tag for Relative and Absolute Quantitation (iTRAQ), Tandem Mass Tag (TMT) or other molecular mass markers compatible with mass spectroscopy, in order to quantify the relative abundance of captured proteins under diverse experimental conditions.

While performing the SNO-RAC procedure, it is important to protect the samples from direct exposure to light [sunlight or other sources of ultraviolet (UV), including fluorescent bulbs] as the S–NO bond in the SNO-moiety has been shown to be light sensitive (Forrester et al., 2007). Additionally, trace metal contamination must be avoided by using Milli-Q water for the preparation of all the reagents and buffers, and by adding the metal chelators Ethylenediaminetetraacetic acid (EDTA) or Diethylenetriaminepentaacetic acid (DTPA) plus Neocuproine, since transition metals such as copper and iron can also attack the S–NO bond.

The protocol below describes the SNO-RAC procedure optimized for capturing SNO-proteins from *C. elegans*. It has been shown that NO from bacteria, the food source for *C. elegans*, has a profound effect on the physiology of *C. elegans* mediated by S-nitrosylation (Gusarov et al., 2013; Seth et al., 2019). Since nitric oxide-generating enzymes have yet to be identified in *C. elegans*, bacteria are the

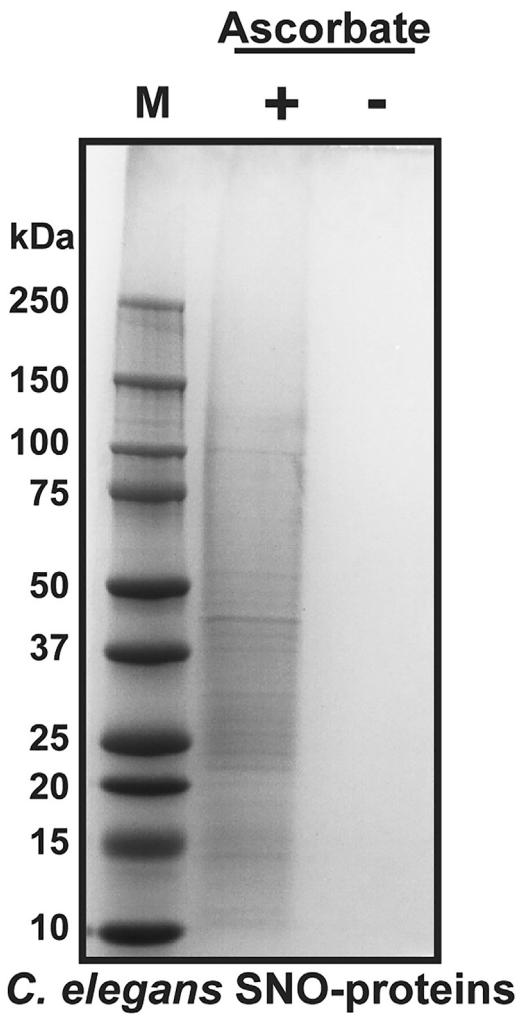

Figure 1. Robust S-nitrosylation is observed in *C. elegans*

A silver-stained SDS-PAGE gel showing abundant *C. elegans* SNO-proteins isolated using the above protocol, from wildtype young adult worms under standard growth conditions using wildtype *E. coli* as the food source. Many SNO-proteins are visible in the plus Ascorbate (+ Ascorbate) lane but not in the minus Ascorbate (−Ascorbate) lane, verifying that the thiol-blocking step was effective. M= molecular weight marker.

only source of NO that can be manipulated in these nematodes (Gusarov et al., 2013; Seth et al., 2019). Nonetheless, this protocol for SNO-RAC can be easily adapted to other cells and tissues, including those from mammalian sources.

The *C. elegans* wild isolate (*C. elegans* var Bristol) N2 nematodes were obtained from the Caenorhabditis Genetics Center at the University of Minnesota. They were maintained at 20°C on nematode growth medium (NGM) plates, which were prepared as described (Stiernagle, 2006) with one modification: Before pouring our NGM plates we added filter-sterilized arginine to a final concentration of 1 mM arginine as it serves as the substrate for the bacterial NOS. One day prior to adding the nematodes, these NGM plates were seeded with a lawn of bacteria as a food source. *B. subtilis* 1A1 (strain 168), obtained from the Bacillus Genetic Stock Center (BGSC) at The Ohio State University, are standard feeder bacteria, but in the laboratory *C. elegans* also feed on *E. coli* (as in Figure 1; obtained from Coli Genetic Stock Center at Yale University), especially in cases where genetic modification of the bacteria is desirable. Bacteria were grown 16–18 h in lysogeny broth (LB) medium at 37°C until they reached optical density A_{600} of 0.8, and were then added to each plate to

create the bacterial lawn. Seeded plates were dried 16–18 h at 20°C. The nematodes were then transferred onto these dried plates and maintained at 20°C.

Before you begin the protocol, prepare the following: freshly-equilibrated SNO-RAC resin, frozen or freshly-pelleted *C. elegans* with appropriate treatments for your desired experiment, ice-cold lysis buffer with protease inhibitors (all of which are described below), and also prepare the standard M9 buffer as has been described elsewhere (Stiernagle, 2006). For quantitative comparisons, it is best to perform SNO-RAC assay on all comparator samples for a complete experimental replicate in the same batch, rather than to compare samples assessed in separate assays.

Preparation of the SNO-RAC resin

Timing: ~1 day

(Perform this on the day before the experiment)

1. Using a wooden/plastic spatula (avoid metal), aliquot the desiccated commercial Thiopropyl Sepharose 6B resin in a microfuge tube. Powder enough to fill a tube to its 0.1 mL mark will suffice for 3–4 samples.
2. Hydrate the resin by adding 1 mL of Milli-Q water and incubating 16–18 h at 4°C with constant inversion in a tube-rotator (Barnstead International, Model #415110).
3. The next day, spin the hydrated resin at 1000 × *g* for 1 min at 4°C and aspirate the supernatant.
4. Add 1 mL of HEPES - EDTA - Neocuproine (HEN) buffer (pH 8.0), composed of 100 mM HEPES (pH 8.0), 1 mM EDTA and 0.1 mM Neocuproine. Incubate the resin at 4°C with constant inversion for 1 hr in a tube-rotator (Barnstead International, Model #415110) to equilibrate the resin with the working buffer.

   Note: When preparing 1L of HEN buffer, dissolve the required amount of Neocuproine in 500 µL methanol and add directly to the buffer. HEN buffer can be stored at 20°C–25°C for at least 1 month.

5. Spin the hydrated resin at 1000 × *g* for 1 min at 4°C and aspirate the supernatant. Repeat the equilibration with HEN buffer for another hour as described in step 4.
6. Spin the equilibrated resin at 1000 × *g* for 1 min at 4°C and aspirate the supernatant. Add sufficient HEN buffer to the packed bead pellet to obtain a ~50% slurry and store at 4°C until use.

   Note: The commercial Thiopropyl Sepharose 6B resin (GE/Cytiva, Cat #17-0420-01, 18-31 µmol activated thiol/mL swollen beads) has been discontinued recently, and equivalent alternatives are not yet available commercially (GE also supplied numerous other resellers). Commercial resins with less than 5 µmol activated thiol/mL yield weak and inconsistent results in our hands. The detailed protocol for the in-laboratory preparation of suitable thiopropyl-agarose SNO-RAC resin is found in (Forrester et al., 2009b). With the following modifications to that protocol, we find that the binding capacity of laboratory-made SNO-RAC resin, when used in a non-limiting amount of 100 µl of 50% slurry for each sample, is similar to that of the discontinued GE/Cytiva resin: Instead of 50 mM cystamine, use 200 mM cystamine; instead of 100 mM DTT use 200 mM DTT; and instead of 100 mM 2-pyridyl disulfide use 200 mM 2-pyridyl disulfide. Alternatively, biotin switch or iodoacetyl-utilizing TMT based methods can be used (Chung et al., 2015; Jaffrey and Snyder, 2001).

Preparation of frozen or freshly harvested nematodes

Timing: ~20 min

Note: This step can be performed days before the experiment if freezing the samples, or on the same day if using fresh samples

7. Grow ~30,000 synchronized N2 nematodes on standard nematode growth medium plates supplemented with 1 mM arginine (plus whatever other modifications to growth conditions that your specific experiment may require) until they reach the young adult stage. Arginine in the growth plate serves as a substrate for the bacterial nitric oxide synthase that is required for the production of nitric oxide (Adak et al., 2002).

Note: 3–5 times the number of worms will be required if analysis of the nematodes at earlier larval stages is desired.

8. Rinse nematodes off the plate with 10 mL of standard M9 buffer by repeatedly washing the liquid down the plate held at an angle, then transfer to a 50 mL tube (Stiernagle, 2006). Rinse the plate again repeatedly with another 10 mL of M9 buffer and transfer that liquid to the same 50 mL tube to collect the remaining worms from the plate. Incubate the tube at 20°C–25°C for 20 min while rocking the tube gently in a rocking shaker (Research Products International, Model 55).
9. Centrifuge the tube at 2000 × *g* for 2 min at 20°C–25°C to pellet the worms and remove the supernatant by aspiration.
10. Wash the worm pellet by adding 20 mL of Milli-Q water and inverting the capped tube gently until the pellet is dispersed, followed by centrifugation at 2000 × *g* for 2 min at 20°C–25°C to pellet the worms. Remove the supernatant by aspiration. Repeat this wash step, for two washes in total with Milli-Q water.
11. After the final wash, the intact worm pellets can either be flash-frozen and stored at −80°C (up to a week) or can be used immediately in the next step.

Note: Incubating the worms at 20°C–25°C for 20 min in M9 buffer, in step 8, provides the *C. elegans* sufficient with time to digest bacteria remaining in their gut, which helps to reduce the bacterial SNO-signal in subsequent steps.

Preparation of the lysis buffer

Timing: ~15 min

Reagent	Final concentration	Amount
5M NaCl	50 mM	0.1 mL
NP-40	1%	0.1 mL
100 mM PMSF	1 mM	0.1 mL
Protease inhibitor tablets	n/a	1
HEN buffer	n/a	9.7 mL
Total		**10 mL**

12. Prepare fresh lysis buffer by taking 9.7 mL of HEN buffer in a 15 mL tube and adding 100 µL of 5 M NaCl (final concentration 50 mM), 100 µL of NP-40 (final concentration 1%), 100 µL of 100 mM PMSF (final 1 mM) and an EDTA-free protease inhibitor tablet. Final volume is 10 mL HEN/lysis buffer. Keep the lysis buffer on ice and use within 2–4 h.

Note: The 100 mM PMSF stock solution is made using ethanol as the solvent, and NP-40 is viscous so use a wide-bore or cut pipet tip.

13. Store this lysis buffer on ice until needed.

⚠ CRITICAL: Use only Milli-Q water to make all the buffers and reagents.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
ALG-1	Invitrogen	Cat#PA1-031; RRID:AB_2539852
Bacterial and virus strains		
B. subtilis 1A1	Bacillus Genetic Stock Center (BGSC) at The Ohio State University (OSU)	BGSCID: 1A1
B. subtilis 1A1 (Δ*nos*)	BGSC, OSU	BGSCID: BKE07630
E. coli strain BW25113 WT	Coli Genetic Stock Center (CGSC) at Yale University	CGSC#7636
Chemicals, peptides, and recombinant proteins		
Thiopropyl Sepharose 6B Resin	GE/Cytiva	Cat #17-0420-01
S-Methyl methanethiosulfonate (MMTS)	Sigma-Aldrich	Cat #64306
HEPES	Fisher	BP310-500
Ethylenediaminetetraacetic acid (EDTA)	Fisher	O2793-500
Diethylenetriaminepentaacetic acid (DTPA)	Sigma-Aldrich	D6518-50G
Protease inhibitor tablets	Roche	04693159001
NP-40	Sigma	74385-1L
Phenylmethylsulfonyl fluoride	Sigma	P7626-25G
Sodium dodecyl sulfate	Bio-Rad	1610302
Dimethyl sulfoxide	Fisher	BP231-100
Acetone	Fisher	A18P-4
Sodium L-ascorbate	Sigma	11140-50G
Laemmli sample buffer	Bio-Rad	161-0737
β-Mercaptoethanol	Sigma	M3148-100 mL
Neocuproine hydrochloride hydrate	Sigma-Aldrich	Cat #N1626
Sodium L-ascorbate	Sigma-Aldrich	Cat #11140
Sodium chloride	Fisher	BP358-212
Agar	BD	DF0145070
Peptone	AMRESCO	J636-500G
Potassium phosphate monobasic	Arcos Organics	205925000
Potassium phosphate dibasic	Arcos Organics	424195000
Magnesium sulfate	RPI	M65240-100
Ethanol	Fisher	BP2818-500
Calcium chloride	Fisher	C77-500
Sodium phosphate dibasic	Fisher	S374-1
L-Arginine monohydrochloride	Sigma	A6969-25G
Lysogeny broth (LB)	Fisher	BP1426-500
Critical commercial assays		
BCA Kit (Regant A, Reagent B)	Thermo Scientific	23223, 23224
Imperial Protein Stain	Thermo Scientific	24615
SilverQuest Staining Kit	Invitrogen	45-1001
Spin-X Cellulose Acetate Tube Filters	Costar	8161
Experimental models: organisms/strains		
C. elegans wild isolate (*C. elegans* var Bristol)	Caenorhabditis Genetics Center	Cat #N2 WormBase ID: WBStrain00000001
Other		
Petri dishes for NGM	VWR	25384-302
50 mL Tubes	Falcon	352098
15 mL Tubes	Falcon	352196
1 mm Diameter zirconia beads	BioSpec Products	11079110zx
Tube rotator	Barnstead International	Model #415110

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Rocking shaker	Research Products International	Model 55
Benchtop non-refrigerated centrifuge	Eppendorf	5417C
Benchtop refrigerated centrifuge	Thermo Scientific	Legend Micro 21R
Tabletop refrigerated swinging bucket centrifuge	Beckman Coulter	Allegra X-15R

STEP-BY-STEP METHOD DETAILS

Performing worm lysis

Timing: ~1 h

1. Keep the worm pellets on ice (or thaw on ice) and add an appropriate amount of lysis buffer (~1 mL for ~30,000 worms); incubate on ice for 15 min.
   a. Resuspend the worms in the lysis buffer by trituration using a 1 mL pipette, and transfer to a microfuge tube pre-cooled on ice.
   b. Rupture the worms in the lysis buffer by:
      i. Snap-freezing the tubes in liquid nitrogen followed immediately by quickly thawing in a 37°C water-bath. Repeat this freeze-thaw cycle four times.
      ii. Enhance worm lysis by subjecting the sample to four sonic pulses of 30 s each at the setting 4 on the VirSonic sonicator (VirTis, SP Industries, Warminster, PA), with cooling on ice between pulses.

(After the sonication, the lysate can be visualized under a light microscope to confirm worm lysis)

Note: After this stage, the lysates are always kept away from direct light due to the light sensitivity of SNO-proteins.

Blocking the free thiols

Timing: ~45 min

2. Centrifuge the sonicated worm lysate at 14,000 × *g* for 10 min at 4°C to pellet any worm-debris, and transfer the lysate supernatant to a 15 mL conical tube at 20°C–25°C.
3. Make up the total volume of the worm lysates to 1.8 mL by adding an appropriate volume of the HEN buffer and perform protein quantification of an aliquot by the bicinchoninic acid assay (BCA) method.

Note: Total protein amount should be in the range of 1–4 mg per tube; lesser amounts will yield inconsistent signals.

4. Add 200 μL of 25% Sodium dodecyl sulfate (SDS) to a final concentration 2.5% to the quantified worm lysate to denature the proteins and add 30 μL of 10% (v/v) of S-Methyl methanethiosulfonate (MMTS) to a final concentration 0.15% to block all free thiols. Incubate in a water-bath at 50°C for 30 min to fully denature proteins so all free thiols are blocked. Vortexing every 5 min improves the blocking efficiency.

Note: Dilute the MMTS to 10% (v/v) in Dimethyl sulfoxide (DMSO) for use in step 4. Also, if the supernatant seems turbid after centrifugation in step 2, an additional spin at 20,000 × *g* for 10 min can be performed. Other thiol-modifying agents can be used to block cysteine residues in place of MMTS. These include iodoacetate, iodoacetamide and *N*-ethyl maleimide

(NEM) (Jaffrey, 2005; Kenyon and Bruice, 1977). However, each of these blocking agents has different reactivity and accessibility toward protein thiols, which will be influenced by assay conditions, and more than one blocking agent may be required for complete blocking (Seth and Stamler, 2015). We prefer use of MMTS for this protocol as it leads to complete blocking of free thiols quickly under our described conditions, as demonstrated by the –Ascorbate control (Forrester et al., 2009b).

Protein precipitation, resuspension, and quantification

Timing: ~1.5 h

5. Add 3 volumes (6 mL) of ice-cold acetone to the blocked worm lysates to precipitate proteins, and mix well by inverting a few times. Incubate the samples for 20 min at −20°C to aid the precipitation. This step removes the excess free MMTS that would interfere with downstream steps.

Pause point: Acetone precipitation can be left 16–18 h at −20°C.

6. The precipitated proteins are pelleted by centrifugation at 4,000 × *g* for 10 min at 4°C in a swinging bucket centrifuge.

Note: If the pellet is not tight and moves when the tube is inverted, centrifuge longer, up to an additional 30 min at 4,000 × *g*.

a. Carefully decant the supernatant without disturbing the pellet, and gently rinse the pellet (without dislodging it) 4 times by gently adding 2 mL of ice-cold 70% acetone along the walls of the tube and then removing it by tilting the tube at an angle.
b. After the final rinse, remove any remaining 70% acetone with a P-200 tip, followed by a P-20 tip, and then air dry the pellet for 15 min by leaving the uncapped tube at 20°C–25°C, but protected from light.
c. Resuspend the pellet in 500 μL of HENS buffer (HEN buffer + 1% SDS) by triturating up and down with a P-200 pipet tip. Alternatively, the pellet can be dissolved by sonicating three times for 1 s each.

7. Perform protein quantification using the BCA assay as in step 3.

Reduction of SNOs to free thiols and binding to the resin

Timing: ~4 h

8. To a microfuge tube, add lysate to contain 2 mg of total blocked protein, and make up its volume to 400 μL with HENS buffer.
   a. Then add the following:
      i. 50 μL of 500 mM sodium ascorbate, to a final concentration of 50 mM.
      ii. 50 μL of SNO-RAC resin (from a 50% slurry, mix well and transfer using wide-orifice tips) from step 6 under PREPARATION OF THE SNO-RAC RESIN.

Note: In this step, addition of ascorbate specifically reduces the S–NO group on proteins, leading to the formation of free thiols (wherever NO was bound), which then chemically react with the SNO-RAC resin to form covalent disulfide.

b. Simultaneously, for the minus ascorbate control sample, in a separate tube place an equal amount of protein plus HENS buffer up to 400 μL, but instead add 50 μL of HEN buffer (i.e., instead of the ascorbate) plus 50 μL of SNO-RAC beads.

Note: In the absence of ascorbate, there should not be any available free thiols in the sample for reaction with the SNO-RAC resin. Thus, this sample serves as a control for the thiol blocking step, and should be performed for every condition.

⚠ CRITICAL: Protect the samples from direct sunlight and UV light.

9. Cover each tube with aluminum foil and incubate at 20°C–25°C for 4 h on a tube-rotator (Barnstead International, Model #415110) in the dark.

Washing and elution

Timing: ~1 h

10. Centrifuge the sample tubes at 1,000 × *g* for 1 min at 20°C–25°C in a swinging bucket centrifuge and remove the supernatant by aspirating with a 18G needle attached to a vacuum line.

Note: Do not centrifuge at lower temperature as that might cause SDS precipitation.

11. Wash the resin by:
    a. Adding 1 mL of HENS buffer and inverting the tube three times to gently disperse the pellet, followed by centrifugation at 1,000 × *g* for 1 min at 20°C–25°C. Remove the supernatant by aspirating with a 18G needle attached to a vacuum line. Repeat this wash step more three times, for four total washes.
    b. Wash the resin with 1 mL of 10-fold-diluted HENS buffer (HENS buffer diluted 10-fold with Milli-Q water) followed by centrifugation at 1,000 × *g* for 1 min at 20°C–25°C. Aspirate the supernatant. Repeat this step once more.
    c. Aspirating all the remaining wash buffer from tube with a 27G needle attached to a vacuum line. Completely air dry the pelleted resin, which now contains the chemically-bound SNO-proteins, for ~15 min at 20°C–25°C.
12. Elute the intact SNO-proteins by adding 35 μL of elution buffer: 1× Laemmli sample buffer + 10% β-mercaptoethanol, followed by:
    a. Gentle agitation of the samples (flick with finger intermittently) at 20°C–25°C for 30 min followed by centrifugation at 1,000 × *g* for 1 min to separate the eluted proteins in the supernatant from the pelleted resin.
    b. Using thin gel-loading pipet tips (which will exclude the resin beads), remove all the supernatant liquid and place it in a fresh tube at 20°C–25°C.
    c. The eluted SNO-RAC samples can now be stored in −20°C until they are ready to be run on a denaturing SDS-Polyacrylamide (SDS-PAGE) gel or used immediately. Load on gel after heating at 94°C for 4 min to denature.

EXPECTED OUTCOMES

This protocol describes the isolation of SNO-proteins from *C. elegans*. The captured SNO-proteins can then be run on a denaturing SDS-PAGE gel for two distinct uses. First, as shown in Figure 1, the entire gel can be stained, using silver stain or Coomassie blue (depending on the abundance of SNO-proteins in the sample), to visualize the entire SNO-proteome from the total lysate. Second, as shown in Figure 2, which includes select panels reproduced from our earlier published work (Seth et al., 2019), the SNO-proteins can be isolated from nematodes grown under different treatment conditions. These are then run in a gel so that they can be compared to each other globally, or they can be transferred to a nitrocellulose membrane and then probed with a specific antibody to detect changes in the level of SNO-protein(s) of interest by immunoblotting. Comparing distinct samples from different treatment groups reveals how the S-nitroslyation of protein(s) changes under selected conditions.

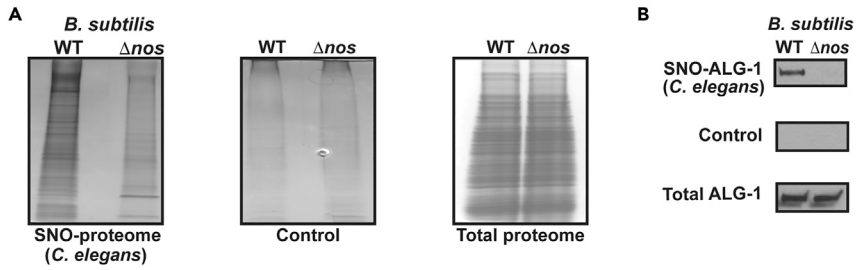

Figure 2. Relative abundance of *C. elegans* SNO-proteome and specific SNO-proteins

(Figure reprinted with permission, from (Seth et al., 2019)). *C. elegans* lysates were prepared and SNO-RAC was performed from the young adult N2 worms using the protocol detailed here.

(A) Left-panel shows a silver stained gel where the SNO-proteins have been isolated from *C. elegans* grown either on wild-type *B. subtilis 1A1* (WT) or *B. subtilis* mutants lacking bacterial nitric oxide synthase (Δnos). Middle panel shows the "minus-ascorbate" controls and the right panel shows the Coomassie blue staining of the *C. elegans* total proteome loading controls.

(B) Immunoblot of the exemplary *C. elegans* protein ALG-1 after SNO-RAC was performed on lysates prepared from *C. elegans* grown as in (A).

The samples produced by this protocol also can be used to identify and quantify the SNO-proteome of *C. elegans* by mass-spectroscopy (MS) analysis. For MS, elute the captured SNO-proteins in the final resin pellet (step 12) instead in 10-fold-diluted HEN buffer containing 10% β-mercaptoethanol, and remove resin beads from the eluates by centrifuging in Spin-X Cellulose Acetate Tube Filters (Costar, Catalog #8161) at 10,000 × *g* for 5 min and collecting the flow through. The cleaned-up samples can then be analyzed without further modification after protease digestion by MS using standard techniques for simple identification, or peptides resulting from protease treatment in individual samples can be reacted with specific mass-tags (iTRAQ, TMT) before sample mixing for relative quantitation of SNO-site peptides under differing conditions.

The specific details of the MS identification procedure were outlined in our earlier publication (Seth et al., 2019), which also includes an Excel file containing a representative list of ~1000 SNO-proteins identified using this protocol. Additionally, our earlier published work contains figures that demonstrate global and specific changes in the S-nitrosylation of the *C. elegans* proteome under different conditions (Seth et al., 2019).

LIMITATIONS

This protocol needs high amounts of starting material, in the range of 1–4 mg of total protein lysate. But even starting with 4 mg of total protein lysate does not ensure the detection of low abundance SNO-modified proteins by immunoblotting after SNO-RAC. This is because only a small fraction of a protein of interest may become modified by S-nitrosylation under specific experimental conditions, plus an antibody must exist that detects the native protein readily by western blotting in 10–50 μg of starting lysate (because there may be less of the protein present in the SNO-RAC eluate). In this situation, this protocol may be unsuccessful in detecting the S-nitrosylation of the specific protein of interest.

Additionally, this protocol does not differentiate between proteins that are heavily S-nitrosylated at multiple cysteine residues or proteins that are S-nitrosylated at just a single cysteine residue. In both instances, SNO-proteins will be pulled down to a similar extent. The amount of a protein pulled-down by the resin depends on its relative abundance and on the fraction of the protein that is S-nitrosylated up to one SNO. Once one SNO-site cysteine is captured by the resin, additional SNO-site cysteines on a protein do not increase the amount of protein captured.

TROUBLESHOOTING

Problem 1

Low protein yield in *C. elegans* lysate (step 1)

Potential solution

To enhance the protein lysate yield from *C. elegans*, the worms collected can be smashed open by adding 1 mm diameter zirconia beads (Catalog # 11079110zx BioSpec Products) to the tube containing the freeze-thaw worms, prior to the sonication step 1c ii under "Performing Worm Lysis". The beads and worms are mechanically agitated in a bead beater (Mini beadbeater-8, BioSpec Products) for 1 min with the control set at halfway between the "mix" and "homogenize" settings, before proceeding to the sonication step. Alternately, one can start with a greater number of *C. elegans*, by combining worms harvested from multiple plates that have been treated identically. However, while the amount of protein that can be processed in a single assay tube is limited, it is possible to combine eluates from multiple assay tubes treated identically into a single measurement (by gel/blot or MS).

Problem 2

Low signal in eluted SNO-RAC samples following SDS-PAGE or Western blot (step 12)

Potential solution

The signal strength may be improved by increasing the starting amount of *C. elegans* lysate, or by making sure that all the excess MMTS is washed away by increasing the number of acetone rinsing steps in step 6a. Residual MMTS might interfere with the protein quantification by the BCA, giving erroneous readings, and would certainly interfere with resin capture by blocking SNO-site thiols freed upon ascorbate treatment. Since *C. elegans* depend on their food source bacteria for most of their SNOs, it is also important to make sure that worms have not been starved, but have ample supply of bacterial food available prior to harvest. Another important consideration is that Western blot signal intensity varies with the avidity of the antibody used; where possible, be sure to use the most avid antibody available (although for many *C. elegans* proteins of interest, options may be limited in this regard). Some proteins simply may be expressed at too low a level endogenously for reliable detection using available antibodies.

Problem 3

Visible bands in the eluted minus-ascorbate sample lanes (step 12c)

Potential solution

No-ascorbate control samples, with no ascorbate added in the reduction and binding steps (step 8b), should have no or minimal appreciable signal. Visible bands in the no-ascorbate samples are usually indicative of incomplete blocking of free thiols by MMTS, and intense bands demonstrate assay failure. Efficiency of the blocking step can be enhanced by increasing the blocking time to 45 min, increasing the amount of MMTS used for blocking to 0.2%, or by decreasing the lysate concentration by diluting it two-fold with HEN buffer (while keeping the final concentrations of the ascorbate and MMTS components of the blocking mix the same). In all cases, the lysate concentration should be kept below 2 mg/mL during blocking and we do not recommend performing this protocol with less than a total of 1 mg lysate protein per sample tube.

Problem 4

Loose sample pellet during acetone precipitation (step 6)

Potential solution

If the pellet is still loose after centrifugation, it can be tightened and consolidated by a longer centrifugation, for 30 min at 4,000 × *g*. If using a fixed angle rotor, turning the sample tube 180° before the additional spin will yield a tighter pellet. Alternately, to aid complete protein precipitation, the supernatant may be decanted carefully as much as possible, without disturbing the loose pellet, and then 3 volumes (~6 mL) of ice-cold acetone can be added and the tube incubated at −20°C for an additional 2 h. After this incubation, a longer centrifugation for 30 min at 4,000 × *g* should be performed.

Problem 5

Turbid sample after resuspension in HENS buffer (step 6c)

Potential solution

A turbid sample after this step is usually indicative of the presence of residual acetone. This can be prevented by removing all traces of acetone in the preceding step by careful aspiration, followed by air-drying to completion at 20°C–25°C. Alternately, acetone precipitation can be repeated by adding 3 volumes of ice-cold acetone to the sample and proceeding to step 6.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Jonathan S. Stamler (jss156@case.edu).

Materials availability

This study did not generate new unique materials or reagents.

Data and code availability

The study did not generate/analyze datasets or codes.

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AUTHOR CONTRIBUTIONS

P.S. performed the experiments. P.S. and J.S.S. designed the research. P.S., R.T.P., and J.S.S. interpreted the data. P.S. and R.T.P. did the technical troubleshooting. P.S., R.T.P., and J.S.S. wrote the manuscript. J.S.S. supervised the research.

DECLARATION OF INTERESTS

Jonathan Stamler is a co-founder of the company SNO bio, which develops SNO-based therapeutics.

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