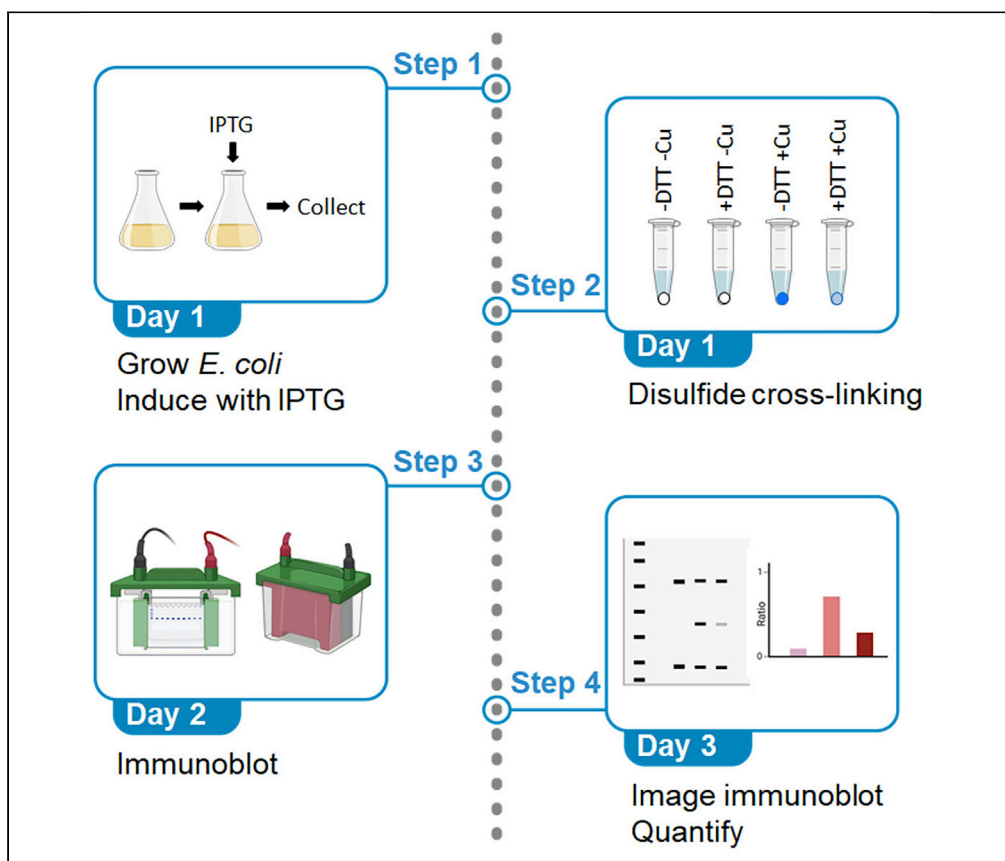


Protocol

An optimized disulfide cross-linking protocol to determine interactions of proteins produced in *Escherichia coli*



Protein-protein interactions play important roles in regulating cellular functions. We present an optimized disulfide cross-linking protocol for testing predicted interactions of soluble or membrane proteins. Coexpression in *E. coli* of proteins with a single cysteine residue results in disulfide bond formation upon treating the cells with oxidants if the two proteins interact and the cysteine residues are near each other. Quantification of cross-linked proteins after immunoblot sensitively and reproducibly measures the interaction.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Disulfide cross-linking *in vivo* allows determination of protein-protein interactions

Applicable to predicted interactions of soluble or membrane proteins in *E. coli*

Cells coexpressing proteins with a single cysteine residue are treated with oxidants

Cross-linked proteins are separated by SDS-PAGE and detected by immunoblot

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Protocol

An optimized disulfide cross-linking protocol to determine interactions of proteins produced in *Escherichia coli*Sandra Olenic^{1,2,*} and Lee Kroos^{1,3,*}¹Department of Biochemistry and Molecular Biology, Michigan State University, East Lansing, MI 48824, USA²Technical contact³Lead contact*Correspondence: sandra.olenic@tufts.edu (S.O.), kroos@msu.edu (L.K.)
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SUMMARY

Protein-protein interactions play important roles in regulating cellular functions. We present an optimized disulfide cross-linking protocol for testing predicted interactions of soluble or membrane proteins. Coexpression in *E. coli* of proteins with a single cysteine residue results in disulfide bond formation upon treating the cells with oxidants if the two proteins interact and the cysteine residues are near each other. Quantification of cross-linked proteins after immunoblot sensitively and reproducibly measures the interaction. For complete details on the use and execution of this protocol, please refer to Olenic et al. (2022).¹

BEFORE YOU BEGIN

⌚ Timing: 0.5–1 h

1. Prepare stock solutions of the following reagents in single-use microfuge tubes: 360 mM 1,10-phenanthroline, 240 mM CuSO₄, 0.5 M neocuproine, 12 mM *N*-ethylmaleimide (NEM) and store at –20°C.
2. Use fresh 360 mM 1,10-phenanthroline stock solution that is < 3 months old.
3. The day before the start of the experiment (day 0), inoculate 4–5 colonies from a freshly transformed plate (e.g., *E. coli* strain BL21DE3 transformed with a pET-derived plasmid for protein production) into 10 mL of Luria-Bertani (LB) medium with selection. Grow overnight in shaker (200 rpm, 37°C). For best results, start overnight after 4 pm and grow for 16–20 h.

Note: To determine if two proteins of interest can be cross-linked, use a strain that produces single-Cys variants of each protein. The single-Cys variants are derived from functional Cys-less variants, which are typically made by replacing native Cys residues with either Ser or Leu using site-directed mutagenesis. Ser substitution is typically chosen for Cys residues predicted to be located in soluble protein domains, due to the side chain similarity of Ser and Cys. Leu substitution is typically chosen for Cys residues predicted to be in transmembrane segments of membrane proteins, because the hydrophobic Leu side chain often preserves the structure of transmembrane α -helices. For more information on successfully creating functional Cys-less variants, please refer to Zhang et al.² and the QuikChange kit protocol. Resulting Cys-less variants (after testing in functional assays) are then further mutagenized to create variants with a single Cys in each protein at positions predicted to be in proximity based on



computational modeling of the protein complex. The modeling may rely on structural data, protein homology, co-evolutionary couplings, and/or prior cross-linking results.¹

Note: Each protein of interest should contain a different epitope tag (e.g., His₆ or FLAG). High quality antibodies are available for these tags, which facilitates the visualization by immunoblot and quantification of the cross-linked protein complex. This protocol was optimized for the use of FLAG and His₆ tags and anti-FLAG and anti-penta-His antibodies, respectively, however it should be applicable for other antibodies as well.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-FLAG (1:10,000 dilution)	Sigma-Aldrich	Cat# A8592
Anti-penta-His (1:10,000 dilution)	Qiagen	Cat# 34460
Bacterial and virus strains		
<i>Escherichia coli</i> BL21(DE3) (genotype: <i>E. coli</i> B <i>dcm ompT hsdS(r_B m_B) gal</i> with λDE3 prophage)	Novagen	Cat# 69450
Chemicals, peptides, and recombinant proteins		
Acteone	Sigma-Aldrich	Cat# 179124-4L-PB
Bromophenol blue	Bio-Rad	Cat# 161-0404
Chloramphenicol (Cm)	Roche	Cat# 10 634 433 001
CuSO ₄ ·5 H ₂ O	Sigma-Aldrich	Cat# C7631
Dithiothreitol (DTT)	Sigma-Aldrich	Cat# D0632-25G
Dimethyl sulfoxide (DMSO)	J.T. Baker	Cat# 9224-01
Ethylenediaminetetraacetic acid (EDTA)	Invitrogen	Cat# 15576-028
Methanol	Sigma-Aldrich	Cat# 179337-4L-PB
Neocuproine	Sigma-Aldrich	Cat# N1501
N-ethylmaleimide (NEM)	Sigma-Aldrich	Cat# E3876
1,10-Phenanthroline monohydrate (2-phenanthroline)	Sigma-Aldrich	Cat# P9375
Trichloroacetic acid (TCA)	J.T.Baker	Cat# 0414-01
Tris base	Invitrogen	Cat# 15504-020
Sodium dodecyl sulfate (SDS)	Bio-Rad	Cat# 161-0302
Western Lightning Plus, Chemiluminescent Substrate	PerkinElmer	Cat# NEL105001EA
Critical commercial assays		
QuikChange II Site-Directed Mutagenesis Kit	Agilent	Cat# 200523
Software and algorithms		
Image Lab	Bio-Rad	Version 5.2.1
Other		
Ambient temperature (20°C–25°C) microcentrifuge for 1.5–2.0 mL tubes	N/A	N/A
Cooled (4°C–12°C) microcentrifuge for 1.5–2.0 mL tubes	N/A	N/A
Incubator shaker	N/A	N/A
Klett-Summerson photoelectric colorimeter or spectrophotometer	N/A	N/A
Orbital shaker	N/A	N/A
Vortexer	N/A	N/A
Water bath or heat block	N/A	N/A

MATERIALS AND EQUIPMENT

This protocol uses single-use tubes of the following reagents: 360 mM 1,10-phenanthroline, 240 mM CuSO₄, 0.5 M neocuproine, 12 mM NEM. Single-use tubes are used to prevent multiple freeze

thaws. If you are cross-linking multiple samples, take what you need and discard leftover. For example, if you need to cross-link one sample, you will need the following: 20.8 μL of 360 mM 1,10-phenanthroline, 2.1 μL of 240 mM CuSO_4 , 25.2 μL of 0.5 M neocuproine and 100 μL of 12 mM NEM. Thus, we recommend using 1 tube of 1,10-phenanthroline, 1 tube of CuSO_4 , 1 tube of neocuproine and 1 tube of NEM.

1,10-phenanthroline stock solution

Reagent	Final concentration	Amount
1,10-phenanthroline	360 mM	97.5 mg
50% methanol	N/A	1.5 mL
Total	N/A	1.5 mL

Add 50% methanol to 97.5 mg 1,10-phenanthroline in a microfuge tube up to 1.5 mL total volume. Dissolve by vortexing. Make 150 μL aliquots in microfuge tubes and store at -20°C for up to 3 months.

CuSO_4 stock solution

Reagent	Final concentration	Amount
$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	240 mM	60 mg
dd H_2O	N/A	1 mL
Total	N/A	1 mL

Add H_2O to 60 mg $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ in a microfuge tube up to 1 mL total volume. Dissolve by vortexing. Make 50 μL aliquots and store at -20°C for up to 12 months.

Neocuproine stock solution

Reagent	Final concentration	Amount
Neocuproine	0.5 M	156 mg
DMSO	N/A	1.5 mL
Total	N/A	1.5 mL

Add 100% DMSO to 156 mg neocuproine in a microfuge tube up to 1.5 mL total volume. Note that the DMSO stock does not need to be strictly anhydrous and should be stored in a 20°C – 25°C flammable cabinet until retailer's expiration date. Dissolve by vortexing. Make 150 μL aliquots in microfuge tubes and store at -20°C for up to 6 months.

Note: Dissolving neocuproine in DMSO is time consuming. To speed up the process, you can heat the solution for 1–2 min in a 37°C incubator or water bath.

NEM stock solution

Reagent	Final concentration	Amount
NEM	12 mM	15.5 mg
dd H_2O	N/A	1 mL
Total	N/A	1 mL

Add H_2O to 15.5 mg NEM in a microfuge tube up to 1 mL total volume. Dissolve by vortexing. Make 200 μL aliquots in microfuge tubes and store at -20°C for up to 6 months.

△ CRITICAL: NEM is considered hazardous. NEM is fatal if swallowed, toxic in contact with skin, causes severe skin burns and eye damage, may cause respiratory irritation and may cause an allergic skin reaction. Wear protective gloves and wash any exposed skin thoroughly after handling. Do not breathe dust and work in a well-ventilated area.

Resuspension Buffer

Reagent	Final concentration	Amount
NEM (12 mM)	2.4 mM	200 μ L
10% SDS (w/v)	1.5% (w/v)	150 μ L
Tris-HCl (pH 7.5) (1 M)	0.1 M	100 μ L
EDTA (50 mM)	5 mM	100 μ L
ddH ₂ O	N/A	450 μ L
Total	N/A	1 mL

Add 10% SDS, Tris-HCl, EDTA and H₂O directly to single-use NEM microfuge tube. Mix by vortexing.

Other solutions

Name	Reagents
100% acetone	Store at 20°C–25°C.
chloramphenicol (Cm)	30 mg/mL Cm in 100% ethanol. Store at –20°C.
dithiothreitol (DTT)	1 M DTT in H ₂ O. Store at –20°C.
10 mM Tris-HCl (pH 8.1)	Store at 20°C–25°C.
100% TCA (w/v)	Store at 4°C.
10% TCA (v/v)	Store at 4°C.
2× sample buffer	4% SDS (w/v), 0.125 M Tris-HCl (pH 6.8), 20% glycerol (v/v), 0.2% bromophenol blue (w/v). Store at 20°C–25°C.

STEP-BY-STEP METHOD DETAILS

Cell growth and *in vivo* cross-linking – Day 1

⌚ Timing: 7–9 h

E. coli cells are grown and induced to produce proteins of interest, which are then cross-linked *in vivo* using Cu²⁺(phenanthroline)₃ to trap protein-protein interactions.

Note: This protocol is an optimization of one described previously³ and modified slightly.²

Note: Use an appropriate number of biological replicates. We recommend using at least two biological replicates. We define a biological replicate as different colonies grown independently.

Note: To control for artifactual protein cross-linking, use an *E. coli* strain that produces a Cys-less variant of one or both of your proteins of interest. Treat this negative control culture exactly the same as the other cultures. This control should be performed each time a new protein of interest is tested.

1. Label flasks appropriately and add 10 mL of LB medium with selection.
2. Add 200 μ L of overnight culture to the corresponding flask.
3. Grow (250 rpm, 37°C) until *E. coli* reaches an optical density (OD) of either 60–80 Klett units (KU) using a Klett-Summerson transmittance filter colorimeter to measure bacterial culture density or the equivalent 0.6–0.8 absorbance at 600 nm (A₆₀₀) with a spectrophotometer.

Note: While cultures are growing, we recommend checking KU or OD after 1 h, then every 15–30 min.

Note: If a culture is growing slowly (relative to most cultures) after 1 h, add an additional 50–100 μ L of overnight culture. If a culture is growing too quickly, remove some of the culture and

replace it with selective LB medium. The goal is to have all the cultures growing at a similar rate so that cross-linking can be done all at once and not in batches (although sometimes batches are recommended, as indicated in step 5).

4. Once the cultures reach 60–80 KU, add 50 μL of 100 mM IPTG (0.5 mM) to induce protein production in *E. coli*.

Note: To induce protein production, we used a pET-derived plasmid (pET29b backbone) with kanamycin (50 $\mu\text{g}/\text{mL}$) resistance.

5. Incubate the cultures for 2 h (250 rpm, 37°C).

Note: For our proteins of interest, we determined that the maximum amount of proteins accumulated after a 2-h incubation with IPTG.

Note: We recommend determining the best induction conditions for your proteins by collecting samples at different times after IPTG addition. Some proteins may require less induction time or more (e.g., overnight for 16–20 h at 16°C induction to prevent formation of inclusion bodies).

Note: During this step, put 100% acetone on ice in a 4°C–12°C cold room.

6. After protein induction, pipet an equivalent optical density of *E. coli* (i.e., an approximately equal cell number) from each culture into a microfuge tube.
 - a. For each culture, pipet $(200/\text{KU} \times 500)$ μL , which corresponds to $(200/A_{600} \times 5)$ μL .
 - b. Cells from cultures induced at slightly different times (i.e., < 15 min apart) can be collected at the same time.
 - c. For cultures induced > 15 min after the initial batch, we recommend collecting cells in subsequent batches (i.e., each batch composed of cultures within 15 min of their predetermined induction time; 2 h for our proteins of interest). For each batch, collect cells (step 6) and proceed to step 7 immediately. Samples can be held at step 25 (see note) to make subsequent steps synchronous.
7. Sediment cells (2 min, 14,000–18,000 $\times g$) and remove the supernatant by pouring it off and gently tapping the inverted microfuge tube on a paper towel.

Note: You should now have approximately equal cell numbers in microfuge tubes. We next want to test for disulfide cross-linking of the single-Cys proteins of interest *in vivo*.

8. Take Cm solution and the necessary number of single-use tubes of 1,10-phenanthroline and CuSO_4 out of the -20°C freezer and place in a tube rack.
9. Add 1 mL of LB medium to the cell pellet in each microfuge tube.
10. Add 6.7 μL of Cm solution and 8.3 μL of 1,10-phenanthroline to each tube.
11. Quickly vortex each tube to resuspend the cells.
12. Sediment cells (1 min, 14,000–18,000 $\times g$) and remove the supernatant by pouring it off and gently tapping the inverted microfuge tube on a paper towel. The cell pellet will have a light pink hue (Figure 1).
13. Add 1 mL of 10 mM Tris-HCl (pH 8.1) and 8.3 μL of 1,10-phenanthroline to each tube.
14. Quickly vortex each tube to resuspend the cells.
15. Sediment cells (1 min, 14,000–18,000 $\times g$) and remove the supernatant by pouring it off and gently tapping the inverted microfuge tube on a paper towel.
16. Add 1 mL of 10 mM Tris-HCl (pH 8.1) to each tube.
17. Quickly vortex each tube to resuspend the cells.

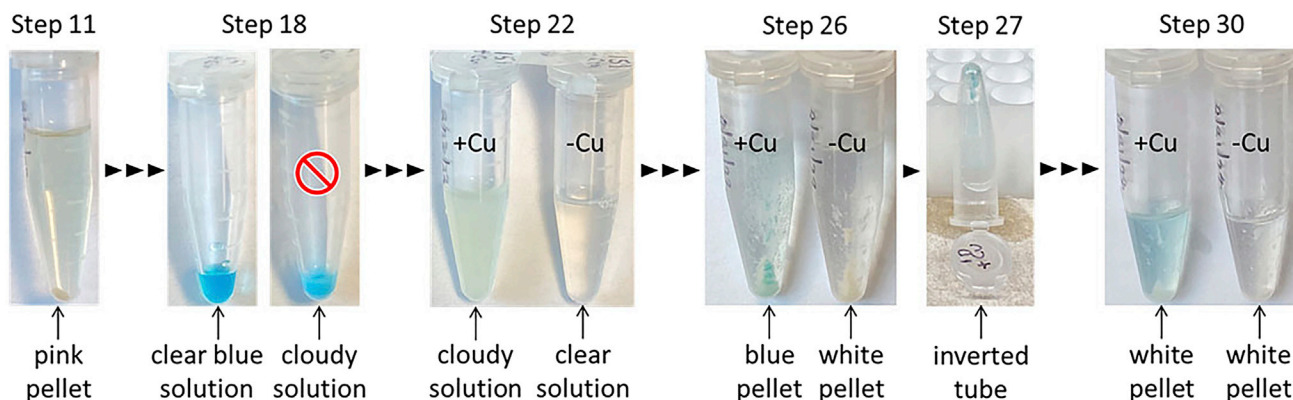


Figure 1. Appearance of samples at different steps of the protocol

Step 11 – sedimented cells are pink due to 2-phenanthroline treatment. Step 18 – $\text{Cu}^{2+}(\text{phenanthroline})_3$ solution must be clear blue, not cloudy. Step 22 – samples treated with $\text{Cu}^{2+}(\text{phenanthroline})_3$ (+Cu) become cloudy upon neocuproine addition, while those treated with 2-phenanthroline (-Cu) remain clear. Step 26 – TCA precipitation of +Cu samples yields a blue pellet after centrifugation, whereas precipitation of -Cu samples yields a white pellet. Step 27 – after very carefully discarding most of the supernatant, each tube is inverted against a rack on a paper towel, allowing the remaining supernatant to drain before gently tapping the tube on the paper towel. Step 30 – the acetone wash supernatant of +Cu samples is faint blue and the pellet is white after centrifugation, whereas the supernatant of -Cu samples is clear and the pellet remains white.

18. Pipet half of each cell suspension into another microfuge tube, so you have 2 tubes with 500 μL each.
 - a. Label the 2 tubes and include “-Cu” and “+Cu” to distinguish the samples that will receive 1,10-phenanthroline or $\text{Cu}^{2+}(1,10\text{-phenanthroline})_3$, respectively.
 - b. The -Cu sample will serve as a negative control.
 - c. Example: If you started with 8 cultures, you will now have 16 samples in tubes.
19. Make $\text{Cu}^{2+}(1,10\text{-phenanthroline})_3$ solution.
 - a. Add 2 volumes of 1,10-phenanthroline to a microfuge tube, then add 1 volume of CuSO_4 and mix thoroughly.
 - b. The $\text{Cu}^{2+}(1,10\text{-phenanthroline})_3$ solution will be clear blue and must not be cloudy (Figure 1).
 - c. Example: For 8 + Cu samples, you will need 50.4 μL of $\text{Cu}^{2+}(1,10\text{-phenanthroline})_3$ solution (i.e., $8 \times 6.3 \mu\text{L}/\text{sample}$), so mix 40 μL of 1,10-phenanthroline with 20 μL of CuSO_4 to ensure that you have enough.

△ CRITICAL: Make this solution right before you use it. DO NOT let it sit for longer than 15 min. Make sure the $\text{Cu}^{2+}(1,10\text{-phenanthroline})_3$ solution is not cloudy. If the solution is cloudy, thaw another single-use tube of 1,10-phenanthroline and remake the $\text{Cu}^{2+}(1,10\text{-phenanthroline})_3$ solution.

20. Add 4.2 μL of 1,10-phenanthroline (3 mM) to each -Cu sample and 6.3 μL of $\text{Cu}^{2+}(1,10\text{-phenanthroline})_3$ (1 mM) to each +Cu sample.
21. Quickly vortex each tube and centrifuge the tubes briefly to ensure that all the liquid is at the bottom.
22. Incubate the tubes at 37°C for 60 min.

Optional: A 60-min incubation is the maximum we have used to test for disulfide bond formation between two single-Cys proteins. The minimum we have used is 15 min. You can adjust this incubation time as needed or perform a time course with samples from the same culture.

Note: For a 60-min incubation, after 45 min remove single-use tubes of neocuproine from the freezer since they require about 15 min to thaw.

23. After the 60-min incubation, add 12.6 μL of neocuproine (12.5 mM) to each tube and quickly vortex to mix. This step terminates the cross-linking reaction. The +Cu samples should become white and cloudy (Figure 1).
24. Incubate the tubes at 37°C for 5 min.

Note: During this short incubation (or sooner), get ice for next steps.

25. Place tubes in a rack and add 500 μL of cold 10% TCA (5%), stored at 4°C, to each tube. Invert the tubes several times to gently mix the samples.
26. Place the tubes on ice for 30 min. Invert the tubes several times every 5–10 min during this step.

Note: If the cultures were induced in batches at step 5, the samples at this step can remain on ice longer than 30 min to allow subsequent steps to be performed synchronously. Longer TCA precipitation of proteins will not affect the samples. Make sure the samples remain on ice.

27. After the TCA precipitation of proteins, centrifuge the tubes (15 min, 14,000–18,000 $\times g$) at approximately 4°C–12°C in a refrigerated centrifuge or a microfuge located in a 4°C–12°C cold room (Figure 1).
28. For each tube, discard the supernatant VERY CAREFULLY as follows:
 - a. Gently place the tubes in a rack.
 - b. Place the rack on a paper towel.
 - c. Pour supernatant slowly out of each tube and invert the tubes on the paper towel, leaning against the rack for about 30 s (Figure 1).
 - d. GENTLY tap the inverted tube on the paper towel to remove excess liquid. DO NOT tap forcefully! The pellet is VERY delicate and easily lost.
29. Add 300 μL of ice-cold 100% acetone (which was placed on ice in a 4°C–12°C cold room during step 4) to each tube.

△ CRITICAL: If the acetone is not cold enough, the protein precipitate will dissolve and you will not recover much protein.

30. Invert the tubes 4–5 times to wash the pellets. The acetone in the +Cu samples will turn faint blue.
31. Centrifuge the tubes as in step 26 except for 5 min (Figure 1).
32. Discard the supernatant VERY CAREFULLY as in step 27.

Note: All samples should have small, white pellets around 1/10th the size of the pellets after step 26. Some of the pellets may be difficult to see since they may be spread along the side the tube.

33. Place the tubes in a rack, leaving the lids open. Allow the acetone to evaporate for 5 min at 20°C–25°C in a fume hood.

Note: DO NOT allow too much evaporation time. If the time exceeds 20 min, the pellets will be more difficult to resuspend.

34. During the evaporation, make the resuspension buffer (see recipe) by adding SDS, Tris-HCl, EDTA and H₂O to the single-use NEM tube. Quickly vortex the tube to mix thoroughly.
35. Add 50 μL of resuspension buffer to each tube and close the lid.
36. Set your pipette to 30 μL and resuspend each pellet in buffer by slowly pipeting up and down.

Note: DO NOT set the pipette to 50 μL since the sample will foam during resuspension due to the SDS in the buffer.

Note: Wash the wall of the tube by pipeting buffer above the top of the pellet.

Note: Use a timer during this step to make sure you take enough time to thoroughly resuspend each pellet. We recommend resuspending the pellet in each tube for 30–45 s, then repeating this 2 more times. If you are doing this correctly, it should take about 30 min to resuspend the pellets in 16 tubes from 8 cultures.

37. After resuspending the pellets, add 45 μL of 1.4 \times sample buffer to each tube. Quickly vortex each tube and invert once.

Note: Make enough 1.4 \times sample buffer for all the tubes. For 16 tubes, add 225 μL of H_2O to 525 μL of 2 \times sample buffer and mix by inversion. The amount of sample buffer is designed to bring the final concentrations of SDS, Tris-HCl, and glycerol into an acceptable range for subsequent SDS-PAGE, taking into account that resuspension buffer has SDS and Tris-HCl and that DTT will be added to some samples (see below).

Note: If too much residual TCA was in the tube, the sample will turn yellow or green. If the sample is yellow, add 1 μL of 10 N NaOH. If the sample is green, add 0.5 μL of 10 N NaOH. Vortex the tube to mix thoroughly.

38. Centrifuge the tubes briefly to ensure that all the liquid (about 100 μL) is at the bottom.
39. Label another set of tubes and divide the samples by pipeting 50 μL from each tube into a tube in the second set. For example, if you had 16 tubes after step 17, you should now have 32 tubes in total.
40. To one set of tubes, add "+ DTT" to the label and add 5 μL of 1 M DTT (100 mM). Quickly vortex each tube to mix thoroughly.

Note: You will now have 4 tubes for each culture that you started with: -Cu -DTT, -Cu +DTT, +Cu -DTT, and +Cu +DTT. We typically discard the -Cu -DTT negative control since the -Cu +DTT negative control is sufficient. The +Cu -DTT sample is used to quantify formation of the cross-linked complex, if one is observed. The +Cu +DTT sample tests for reversibility of disulfide cross-linking by the reducing agent DTT, which in our experience is variable, ranging from almost complete disappearance of the cross-linking complex to no change in its abundance.

41. Incubate the tubes at 37°C for 10 min.
42. Centrifuge the tubes briefly to ensure that all the liquid (about 50 μL) is at the bottom and store the tubes at -20°C .

▮▮ Pause point: From our experience, samples can be kept for approximately 3 weeks at -20°C prior to performing immunoblot analysis. After 3 weeks, the strength and sharpness of the signals deteriorates.

Immunoblot analysis of samples – Day 2

⌚ **Timing:** 4–5 h

Cross-linked samples are subjected to immunoblot analyses.

Note: The two proteins of interest should have different epitope tags for visualization. We recommend performing separate immunoblot analyses to detect each epitope tag (e.g., one with anti-penta-His antibody and one with anti-FLAG antibody). Disulfide cross-linking between the proteins of interest results in the same size complex on both immunoblots.

43. Perform SDS-PAGE.

Note: We recommend using 10% polyacrylamide gels in order to resolve large protein complexes (e.g., 50–90 kDa). We typically load 5–10 μ L of sample and run the gels for 2 h (30 min at 85 V and 1.5 h at 126 V) until the 34 kDa ladder band migrates to near the bottom of the SDS-PAGE gel.

Note: Samples do not need to be boiled before loading. We recommend taking the samples out of the freezer, letting them thaw at 20°C–25°C (this takes 5–7 min) and then vortexing gently before loading.

Optional: We load samples derived from the same starting culture in adjacent gel lanes as follows: negative control (-Cu +DTT), cross-linked proteins (+Cu -DTT), then cross-linked/reduced proteins (+DTT +Cu).

44. Electro-transfer the proteins from the gel to the blotting membrane.
45. Block the membrane (e.g., 1 h, 20°C–25°C, shaking at 100 rpm) and then incubate it with the primary antibody (e.g., overnight, 4°C–12°C cold room, shaking at 100 rpm).
- We use 5% milk in TBST as the blocking reagent.
 - After blocking, pour off blocking solution, then directly add solution with primary antibody. For the primary antibody incubation, we use 2% milk in TBST and either 1:10,000 anti-FLAG or 1:10,000 anti-His antibodies.

Image immunoblots – Day 3

⌚ Timing: 1 h

Proteins of interest are visualized.

46. Wash immunoblots.
- We wash with TBST.
 - Quickly rinse blot 3 times to remove primary antibody solution. Then wash 3 times with shaking for 10 min each (30 min total).
47. If only primary antibody is needed, develop and image. If secondary antibody is needed, incubate for minimum of 1 h in secondary antibody solution and wash in same manner as above.
48. Image immunoblots, choosing appropriate exposure times so that signals to be quantified are not saturated.

Note: We typically obtain images over a range of exposure times, including some in which some of the signals are saturated (e.g., protein of interest that has not been cross-linked), which may be necessary to visualize low-abundance cross-linked complexes.

EXPECTED OUTCOMES

Successful disulfide cross-linking results in a species, the cross-linked complex or “complex” for short, that produces a signal in the immunoblot analysis at the expected size for the combination of the two proteins of interest. Disulfide cross-linking using $\text{Cu}^{2+}(1,10\text{-phenanthroline})_3$ as an oxidizing reagent relies on close proximity between Cys residues. Thus, it is normal to see differences in the signal intensity of the complex for Cys residues located at different positions in the proteins of interest.

An example of cross-linking between two proteins (labeled Protein A and Protein B) is shown in [Figure 2](#). A Cys-less variant of Protein B is shown as a negative control. When a single-Cys variant of

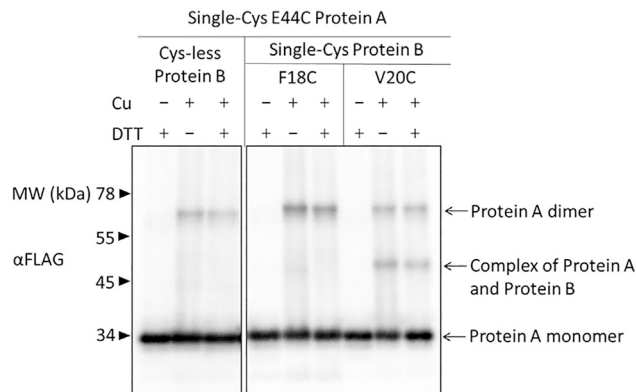


Figure 2. Example of disulfide cross-linking between two single-Cys membrane proteins

pET vector plasmids were used to produce single-Cys Protein A in combination with single-Cys Protein B or Cys-less Protein B as a negative control in *E. coli*. Samples collected after 2 h of IPTG induction and were treated for 60 min with Cu^{2+} (phenanthroline)₃ (+Cu) or with 2-phenanthroline (-Cu) as a negative control, then treated with TCA to precipitate proteins and resuspended in sample buffer with DTT (+) to reverse cross-links or without (-) to preserve cross-links, and finally subjected to immunoblot analysis with FLAG antibodies to visualize Protein A monomer, dimer, and complex with Protein B.

Protein A was produced with Cys-less Protein B, no complex was observed, only Protein A monomer and dimer were visible in the anti-FLAG immunoblot. In contrast, when single-Cys E44C Protein A and single-Cys V20C Protein B were produced, a complex between the two proteins was observed for the +Cu samples. On the other hand, little or no complex was observed when single-Cys F18C Protein B was produced in combination with single-Cys E44C Protein A. Taken together, the results imply that Protein B V20 is in close proximity to Protein A E44 when the two proteins interact. While it is tempting to infer that Protein B F18 is not in close proximity to Protein A E44 when the two proteins interact, weak or undetectable signals must be interpreted cautiously since residues may be proximal but in unfavorable orientations for cross-linking. Owing to these caveats, we typically do not quantify the signal intensity of complexes for comparison between different combinations of single-Cys proteins of interest. Rather, we make qualitative observations. However, for a given combination of single-Cys proteins of interest, it can be very useful to quantify the signal intensity of the complex in related samples [e.g., samples treated with Cu^{2+} (1,10-phenanthroline)₃ for different amounts of time¹ or samples prepared from *E. coli* producing a single-Cys protein of interest with an additional substitution in order to test for an effect on complex formation⁴]. The signal intensity of the complex can be quantified relative to the combined signal intensity of the complex, monomer, and dimer species in a sample. We call this the “cross-linked ratio” and we typically calculate it for the +Cu -DTT sample derived from a culture. Table 1 shows an example of how cross-linked ratios were calculated for the +Cu -DTT samples shown in Figure 2.

QUANTIFICATION AND STATISTICAL ANALYSIS

1. We use Bio-Rad Image Lab software to quantify immunoblot signals. Once you have a desirable immunoblot image (i.e., the complex, monomer, and dimer signals are not saturated), select the “Lane and Bands” tool. Manually input the number of lanes and resize the template that appears to an appropriate overall width. Center the frame of each lane, leaving all frames the same width. Do not overlap adjacent frames (Figure 3).
2. Once the lanes have been defined, select the “Band” tool and manually select each signal of interest (i.e., the complex, monomer, and dimer signals).
3. Once the bands have been defined, select the “Lane Profile” tool to adjust the band boundaries (i.e., approximately where the signal equals the background on each side of the intensity peak).

Table 1. Adjusted volume (Int) of the complex, monomer, and dimer used to calculate the complex ratio for samples

Protein B	Single-Cys E44C protein A signals			Complex ratio
	Complex	Monomer	Dimer	
Cys-less	44880	9155938	1653454	0.00413478
F18C	377640	8020080	3156240	0.0326849
V20C	1503020	7104980	1407840	0.1500643

4. Select the “Analysis Table” tool to get the adjusted volume intensity for the complex, monomer, and dimer signals.
5. Calculate the cross-linked ratio: (complex intensity)/(total intensity of complex, monomer, and dimer) to compare complex formation in related samples.

LIMITATIONS

Disulfide cross-linking using $\text{Cu}^{2+}(1,10\text{-phenanthroline})_3$ as an oxidant relies on close proximity (approximately 2 Å) of the single-Cys residues in the interacting proteins of interest. Thus, Cys residues located on opposite sides of interacting α -helices or β -sheets would probably not form a cross-link. To address this limitation, we suggest Cys scanning (i.e., testing single-Cys residues located at adjacent positions) of short stretches predicted to interact in each protein of interest.

Formation of dimer species can vary and seems to depend on the location of the single-Cys residue in a protein of interest. For instance, dimer formation tends to occur when the Cys residue is located in a flexible region. Since dimer formation is possible, we recommend testing for cross-linking of proteins with readily distinguishable sizes. For example, cross-linking of single-Cys His₆-tagged protein A (34 kDa) with single-Cys FLAG₂-tagged protein B (38 kDa) may result in cross-linked AB complex too similar in size to cross-linked AA and BB dimers, making it hard to resolve the different species on either anti-His or anti-FLAG immunoblots. If your two proteins of interest are similar in size, we recommend either adding a larger tag to one protein (e.g., MBP or GFP) or deleting part of one protein, while retaining function, in order to facilitate separation of different species.

TROUBLESHOOTING

Problem 1

Cloudy $\text{Cu}^{2+}(1,10\text{-phenanthroline})_3$ solution.

Potential solution

Cloudy solution (during step 19) occurs when 360 mM 1,10-phenanthroline stock solution is old (>3 months) or when 240 mM CuSO_4 stock solution is added slowly. Remake 1,10-phenanthroline stock solution. Add appropriate amount of 240 mM CuSO_4 stock solution and mix quickly by pipetting. Solution should be blue. If it is still cloudy, heat for 1–2 min at 37°C before using.

Problem 2

Low protein monomer yield.

Potential solution

Low protein yields are usually caused by losing sample during steps 27 and/or 31. When pouring out the supernatant after the TCA precipitation and acetone wash, make sure you do so slowly and with a steady hand so as not to disturb the loose, fluffy pellet. Always check after step 31 to make sure you see the pellets before continuing on with the next steps. If you suspect that you lost protein, we recommend redoing that sample.

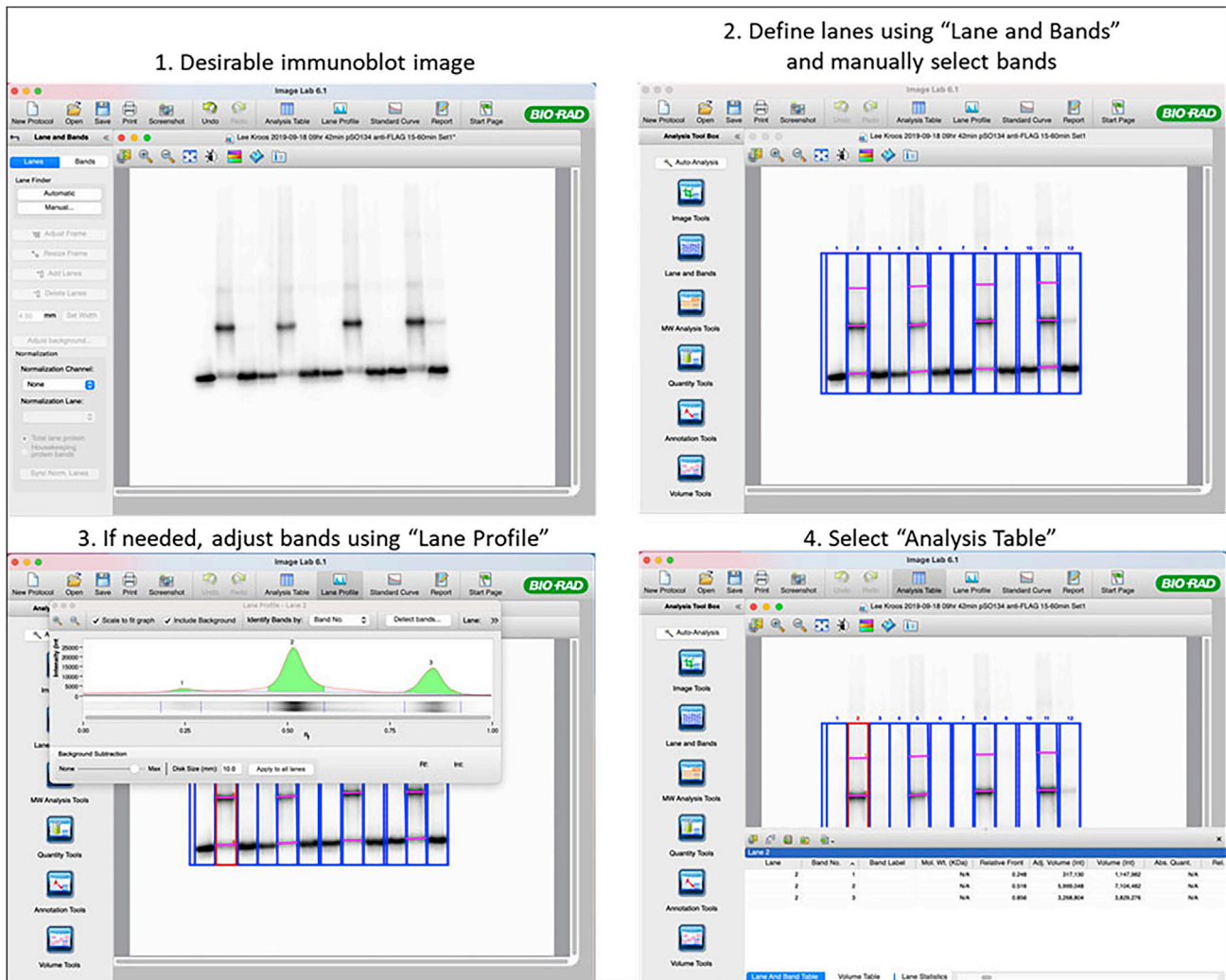


Figure 3. Analysis and quantification of crosslinking between proteins

After getting a desirable immunoblot image (top left), define the lanes and manually select bands of interest using the “Lanes and Bands” tool (top right). If needed, adjust the band boundaries using the “Lane Profile” tool (bottom left). Select the “Analysis Table” tool to get the adjusted volume intensity for the complex, monomer, and dimer signals (bottom right).

Problem 3

Variable protein monomer yield.

Potential solution

Confirm that plasmid sequence is correct for your single-Cys protein variants before you begin. Variable yield in the immunoblot could indicate errors in the protein sequence or epitope tag. Variable yield can also be caused by the single-Cys addition to your protein. Some protein variants will accumulate differently (see immunoblot imaging, step 48), potentially due to changes in stability. We recommend checking for protein accumulation by immunoblot before attempting to cross-link both proteins.

Problem 4

Visible loss of pellet after TCA and acetone washes.

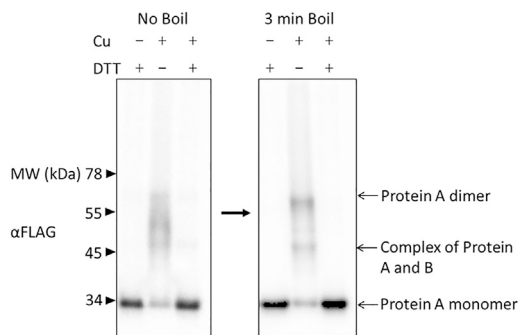


Figure 4. Troubleshooting smeared signals

If the +Cu sample exhibits smeared signals for the cross-linked complex and the dimer (left immunoblot image), a potential solution is to boil the samples for 3 min and repeat the immunoblot (right image).

Potential solution

Pellet is very fluffy and loose. Check the side of the microfuge tube as the pellet may be spread along the side the tube. If that is the case, move forward with the protocol and make sure to pipet the sides of the microfuge tube with resuspension buffer (step 35). If no pellet is visible in the microfuge tube, check the paper towel used in steps 27 and/or 31. If the pellet transferred to the paper towel, we recommend redoing that sample.

Problem 5

Smeared signal in the immunoblot analysis of the +Cu samples.

Potential solution

During immunoblot imaging (step 48), the +Cu samples may exhibit signals for the cross-linked complex and/or the dimer that have a smeared appearance (Figure 4). In order to improve the appearance of these samples, we recommend boiling them for 3 min and repeating the immunoblot analysis.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Lee Kroos (kroos@msu.edu).

Materials availability

Plasmids and *E. coli* strains are available from [lead contact](#) upon request.

Data and code availability

This study did not generate any unique datasets or code.

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AUTHOR CONTRIBUTIONS

S.O. was involved in conceptualization, formal analysis, investigation, methodology, resource provision, validation, visualization, writing the original draft, and reviewing and editing. L.K. was involved in conceptualization, funding acquisition, investigation, project administration, resource provision, supervision, visualization, and reviewing and editing.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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