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Detecting Native Protein–Protein Interactions by APEX2 Proximity Labeling in *Drosophila* Tissues

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Abstract

Enzyme-catalyzed proximity labeling is a potent technique for the discernment of subtle molecular interactions and subcellular localization, furnishing contextual insights into the protein of interest within cells. Although ascorbate peroxidase2 (APEX2) has proven effective in this approach when overexpressed, its compatibility with endogenous proteins remains untested. We improved this technique for studying native protein–protein interactions in live *Drosophila* ovary tissue. Through CRISPR/Cas9 genome editing, APEX2 was fused with the endogenous *dysfusion* gene. By pre-treating the tissue with Triton X-100 to enhance biotin-phenol penetration, we successfully identified multiple proteins interacting with the native Dysfusion proteins that reside on the inner nuclear membrane. Our protocol offers a comprehensive workflow for delineating the interactome networks of ovarian components in *Drosophila*, aiding future studies on endogenous protein–protein interactions in various tissues of other animals.

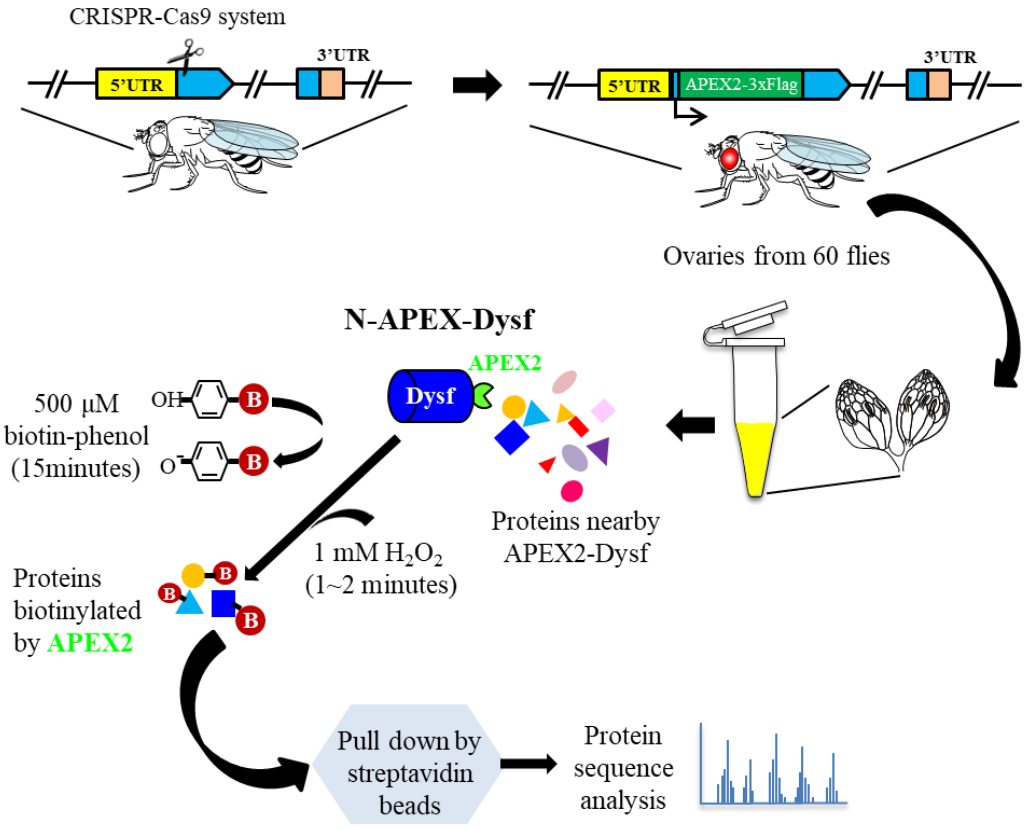
Key features

- Elucidating Protein-protein interactions provides deeper insights into the regulation of gene expression in molecular network and complex signaling pathways, advancing protein engineering and drug design.
- This protocol utilizes CRISPR/Cas9 knock-in technology to tag endogenous proteins with the APEX2 to label nearby proteins within a 20 nm radius in *Drosophila melanogaster*.
- We optimize APEX2-proximity labeling by using Triton X-100 pre-treatment to enhance biotin-phenol penetration into *Drosophila* ovaries, enabling endogenous proteins enrichment under native conditions.

Keywords: Proximity labeling, APEX2, *Drosophila*, Oogenesis, Pull-down assay, CRISPR/Cas9

This protocol is used in: Science Advances (2022), DOI: 10.1126/sciadv.abm2411

Graphical overview



Workflow of proximity labeling with APEX2 tagging endogenous proteins in *Drosophila* ovary

Background

Proximity labeling is a powerful technique applied to mark adjacent proteins, providing valuable insights into the spatial arrangement of a given protein of interest within cells [1]. This approach excels over conventional assays because it can detect subtle or temporary protein–protein interactions (PPI) and pinpoint the precise localization within intact cellular compartments. Ascorbate peroxidase2 (APEX2) is engineered to enhance proximity labeling techniques for uncovering intricate subcellular proteomes in vivo [2]. By utilizing hydrogen peroxide (H_2O_2), APEX2 catalyzes biotin-phenol (BP) into biotin-phenoxyl radical, a highly reactive and short-lived species (lasting less than 1 ms), which quickly forms covalent bonds with nearby proteins within a 20 nm radius [3]. The resulting biotinylated proteins can be purified with streptavidin beads and then subjected to liquid chromatography-tandem mass spectrometry (LC-MS/MS) for identification [3]. Although APEX2 has shown efficacy in cell culture systems and *Drosophila* tissues when overexpressed, the compatibility with endogenous proteins tagged with this enzyme has not been tested [2–6]. While ectopic expression facilitates research on PPI, it may hinder embryonic development or lead to misinterpretation due to aberrant cellular behavior; a preferable method for unveiling PPI is to purify proteins expressed under native conditions.

In this protocol, we improved APEX2-based proximity labeling in live *Drosophila* ovary tissue to study PPI in the natural state. By using CRISPR/Cas9 genome editing, we generated a transgenic fly line that carries the APEX2 gene inserted at the N-terminal of endogenous *dysfusion (dysf)* locus. To enhance protein biotinylation catalyzed by APEX2, fly ovary tissues were pre-treated with Triton X-100 to facilitate the penetration of BP into tissues. After a

sequence of protein enrichment and LC-MS/MS analysis, we identified numerous proteins that were labeled by APEX2-tagged Dysf proteins [7].
Altogether, our protocol provides a proximity-labeling workflow for mapping the interactome networks of *Drosophila* ovary. The level of detail in this protocol shall empower future researchers to explore endogenous PPI in living tissues of other multicellular organisms.

Materials and reagents

Biological materials

1. *Drosophila melanogaster*

Reagents

1. Schneider's *Drosophila* medium (Thermo Fisher Scientific, catalog number: 21720024)
2. Fetal bovine serum (FBS) (Thermo Fisher Scientific, catalog number: A5670701)
3. 100× Penicillin-Streptomycin (Thermo Fisher Scientific, catalog number: 15070063)
4. Streptavidin-horseradish peroxidase (streptavidin-HRP) (Life Technologies, catalog number: S-911)
5. cOmplete™, EDTA-free protease inhibitor cocktail (Merck, catalog number: 4693132001)
6. (3aS,4S,6aR)-hexahydro-N-[2-(4-hydroxyphenyl)ethyl]-2-oxo-1H-thieno[3,4-d]imidazole-4-pentanamide (Biotin-phenol, BP) (Iris Biotech, catalog number: LS-3500)
7. Pierce™ Streptavidin magnetic beads (Thermo Fisher Scientific, catalog number: 88816)
8. Trolox (Merck, catalog number: Al-238813)
9. Sodium azide (Merck, catalog number: S2002)
10. Sodium L-ascorbate (Merck, catalog number: A4034)
11. Hydrogen peroxide solution (H_2O_2) (Merck, catalog number: 31642)
12. Phosphate buffered saline (PBS) (Merck, catalog number: P3813)
13. Bio-Rad protein assay dye reagent concentrate (Bio-Rad, catalog number: 5000006)
14. Tris base (cyrusbioscience, catalog number: 101-77-86-1)
15. Glycine (cyrusbioscience, catalog number: 101-56-40-6)
16. NaCl (cyrusbioscience, catalog number: 101-7647-14-5)
17. SDS (cyrusbioscience, catalog number: 101-151-21-3)
18. Triton X-100 (JT Baker®, X198-07)
19. Bovine serum albumin (BSA) (Bioman, catalog number: ALB001)
20. Dimethyl sulfoxide (DMSO) (Merck, catalog number: D2650)

Solutions

1. 10× PBS stock
2. 1 M Tris-HCL pH 8.0
3. 5 M NaCl
4. 10% SDS
5. Dissection medium (see Recipes)
6. 500 mM Biotin-phenol (see Recipes)
7. 100 mM H_2O_2 (see Recipes)
8. 1 M Sodium ascorbate (see Recipes)
9. 500 mM Trolox (see Recipes)
10. 1 M Sodium azide (see Recipes)
11. Quencher solution (see Recipes)
12. 25× Protease inhibitor cocktail (see Recipes)

13. 20% Triton X-100 (see Recipes)
14. RIPA lysis buffer (see Recipes)
15. 0.3% PBST (see Recipes)

Recipes

1. Dissection medium

Reagent	Final concentration	Quantity or Volume
Schneider's *Drosophila* medium	500 mM	445 mL
FBS	10%	50 mL
100× Penicillin-Streptomycin	1×	5 mL
Total	n/a	500 mL

2. 500 mM Biotin-phenol

Reagent	Final concentration	Quantity or Volume
Biotin-phenol	500 mM	100 mg
DMSO	n/a	Up to 550 µL
Total	n/a	550 µL

a. The stock needs to be shaken by vortexing.
b. Aliquot the stock solution into small volumes and store at -80 °C to prevent repeated freeze-thaw cycles.

3. 100 mM H_2O_2

Reagent	Final concentration	Quantity or Volume
30% H_2O_2(10M)	100 mM	10 µL
10× PBS	1×	100 µL
H_2O	n/a	890 µL
Total	n/a	1 mL

Do not store this stock.

4. 1 M Sodium ascorbate

Reagent	Final concentration	Quantity or Volume
Sodium ascorbate	1 M	0.59 g
H_2O	n/a	Up to 5 mL
Total (optional)	n/a	5 mL

5. 500 mM Trolox

Reagent	Final concentration	Quantity or Volume
Trolox	500 mM	12.5145 mg
DMSO	n/a	Up to 100 µL
Total (optional)	n/a	100 µL

Do not store this stock.

6. 1 M Sodium azide

Reagent	Final concentration	Quantity or Volume
Sodium azide	1 M	0.65 g
H_2O	n/a	Up to 10 mL
Total (optional)	n/a	10 mL

Aliquots can be stored at -20 °C or below for several months.

7. **Quencher solution**

Reagent	Final concentration	Quantity or Volume
500 mM Trolox	5 mM	10 µL
1 M Sodium azide	10 mM	10 µL
1 M Sodium ascorbate	10 mM	10 µL
10× PBS	1×	100 µL
H_2O	n/a	870 µL
Total (optional)	n/a	1 mL

a. Make this solution immediately before it is to be used to quench the biotinylation reaction.
b. Do not store this solution.

8. **25× Protease inhibitor cocktail**

Reagent	Final concentration	Quantity or Volume
cOmplete™, EDTA-free protease inhibitor cocktail	25×	1 tablet
H_2O	n/a	Up to 2 mL
Total (optional)	n/a	2 mL

9. **20% Triton X-100**

Reagent	Final concentration	Quantity or Volume
Triton X-100	20%	10 mL
H_2O	n/a	40 mL
Total (optional)	n/a	50 mL

10. **RIPA lysis buffer**

Reagent	Final concentration	Quantity or Volume
1 M Tris-HCL pH 8.0	50 mM	2.5 mL
5 M NaCl	150 mM	1.5 mL
10% SDS	0.1%	0.5 mL
20% Triton X-100	1%	2.5 mL
25× Protease inhibitor cocktail	1×	2 mL
H_2O	n/a	41 mL
Total (optional)	n/a	50 mL

11. **0.3% PBST**

Reagent	Final concentration	Quantity or Volume
20% Triton X-100	0.3%	0.75 mL
10× PBS	1×	5 mL
H_2O	n/a	44.25 mL
Total (optional)	n/a	50 mL

Equipment

1. Standard equipment for fly incubation
2. Micro refrigerated centrifuge (Kubota, model: 3740)
3. Magnetic stand (G-Biosciences, catalog number: 786-888)
4. Multi-mixer overhead mixer shaker Mischer Rotator (NanoEnTek, model: SLRM-3)
5. Ultrasonic processor (ChromTech, model: UP-500)
6. xMark™ microplate absorbance spectrophotometer (BIO-RAD, catalog number: 1681150)

7. Mini Trans-Blot electrophoretic transfer cell (BIO-RAD, catalog number: 1703930)
8. Mini-PROTEAN® Tetra Cell (BIO-RAD, catalog number: 1658001)

Software and datasets

1. NCBIprot (20180429)

Procedure

A. Transgenic fly generation

1. The *N-APEX2-dysf* knock-in fly was produced by WellGenetics Company (Taiwan) using CRISPR/Cas9-mediated genome editing through the following steps.
   a. Guide RNA construction: The pDCC6 vector was digested with BbsI and ligated with the annealed guide RNA (ACGTGGCCTAGACAAGGTGACGG). This was followed by transformation, colony PCR selection, and sequencing.
   b. Donor plasmid construction: The APEX2 and 3XFlag sequences were amplified and cloned into the pUC57-Kan (Cassette RFP) vector using a sequence- and ligation-independent method (named Cassette APEX2-3XFlag-RFP). The two homology arms (upstream and downstream) of *dysf* were amplified from genomic DNA and then cloned into Cassette APEX2-3XFlag-RFP.
   c. Microinjection: The constructs (donor plasmid and CRISPR/Cas9 vector plasmid) were injected into embryos according to standard procedures.
   d. Selection and verification: Transgenic offspring were identified by genomic PCR and sequencing, and then crossed with balancer flies to establish a stable line. These steps are illustrated in Figure 1.
2. As a result of homology-dependent repair, the fragment of APEX2-3XFlag-PBacDsRed was inserted into the 5' end of *dysf* coding region after embryo microinjection, resulting in a new null allele, $dysf^{dW}$. In order to generate an APEX2 knock-in tag in the *dysf* locus, $dysf^{dW}$ was crossed with BL8285 (w^{1118}; *CyO*, *P{Tub-PBac\T}2/wg*$^{Sp\text{-}1}$; *l(3)**/TM6B, Tb*^{1}) to excise the PBacDsRed fragment. After precise excision, one GTTAAA sequence was left as a linker peptide to bridge 3XFlag and *dysf*, as illustrated in Figure 2.

B. APEX2 reaction in *Drosophila* ovary

1. Fatten 60 *N-APEX2-dysf* genotype female flies, from 3 to 5 days old, with wet yeast for 14–16 h at 29 °C. Subsequently, carefully dissect their ovaries with fine-tip forceps in dissection medium [8].
   Note: Quickly and gently tear the ovary, ensuring the process is brief to minimize tissue damage.
2. After dissection, pre-treat ovaries with 1 mL of 0.3% PBST for 15 min and then wash with 1 mL of 1× PBS once.
   Note: Freshly prepare 0.3% PBST to enhance the penetration of biotin-phenol.
3. After pre-treatment, incubate all ovaries in 200 µL of dissection medium supplemented with 500 µM BP for 15 min at 25 °C.
   Critical: Prewarm the medium to 25 °C to facilitate the dissolution of BP. Ensure that the solution fully dissolves in the dissection medium.
4. Prepare a premix by combining 10 µL of freshly prepared 100 mM H_2O_2 with 790 µL of dissection medium. Then, add this premix into the samples from step B3, achieving a final concentration of 1 mM H_2O_2, and incubate at room temperature for 1–2 min.
   a. For the negative control group, we recommend using fruit fly ovaries without APEX2 or ovaries untreated with BP or H_2O_2.

b. The biotinylation time varies depending on the expression level of the APEX2-tagged target protein and cell types, typically ranging from 30 s to 2 min [4,5,7].

5. Rinse the ovaries three times, each for 1 min, with 1 mL of fresh quencher solution. Subsequently, carefully remove as much supernatant as possible.
   Pause point: The ovaries can be stored at -80 °C for several months.

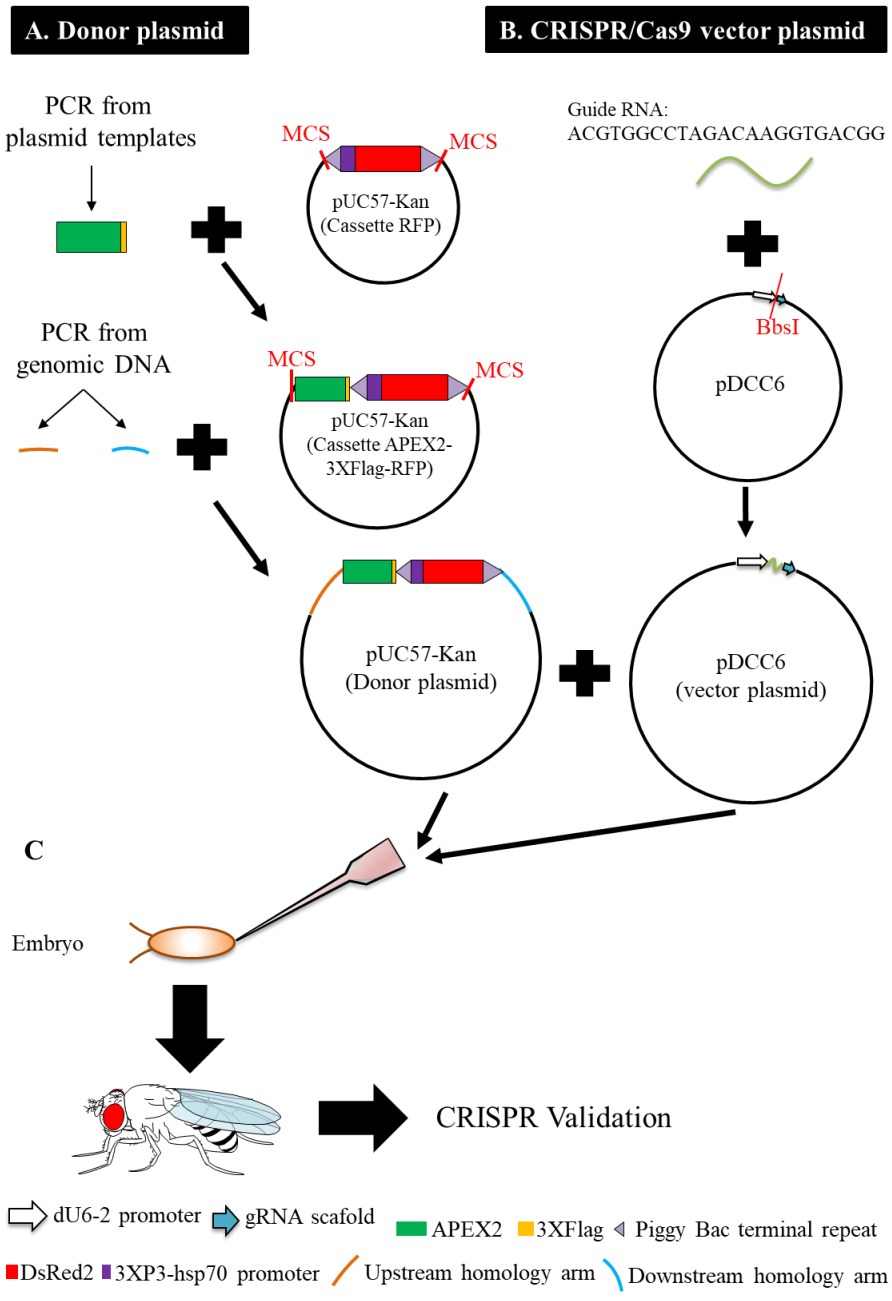


Figure 1. Workflow of generating the *dysf*^{IW} allele using CRISPR/Cas9-mediated genome editing. A. APEX2, Piggy Bac terminal repeat, 3XFlag, homology arms, DsRed2, and 3XP3-hsp70 promoter were cloned into the pUC57-Kan plasmid as a donor plasmid. B. The guide RNA was cloned into the CRISPR/Cas9 vector plasmid (pDCC6). C. Both plasmids were co-injected into a *Drosophila* embryo following the standard protocol.

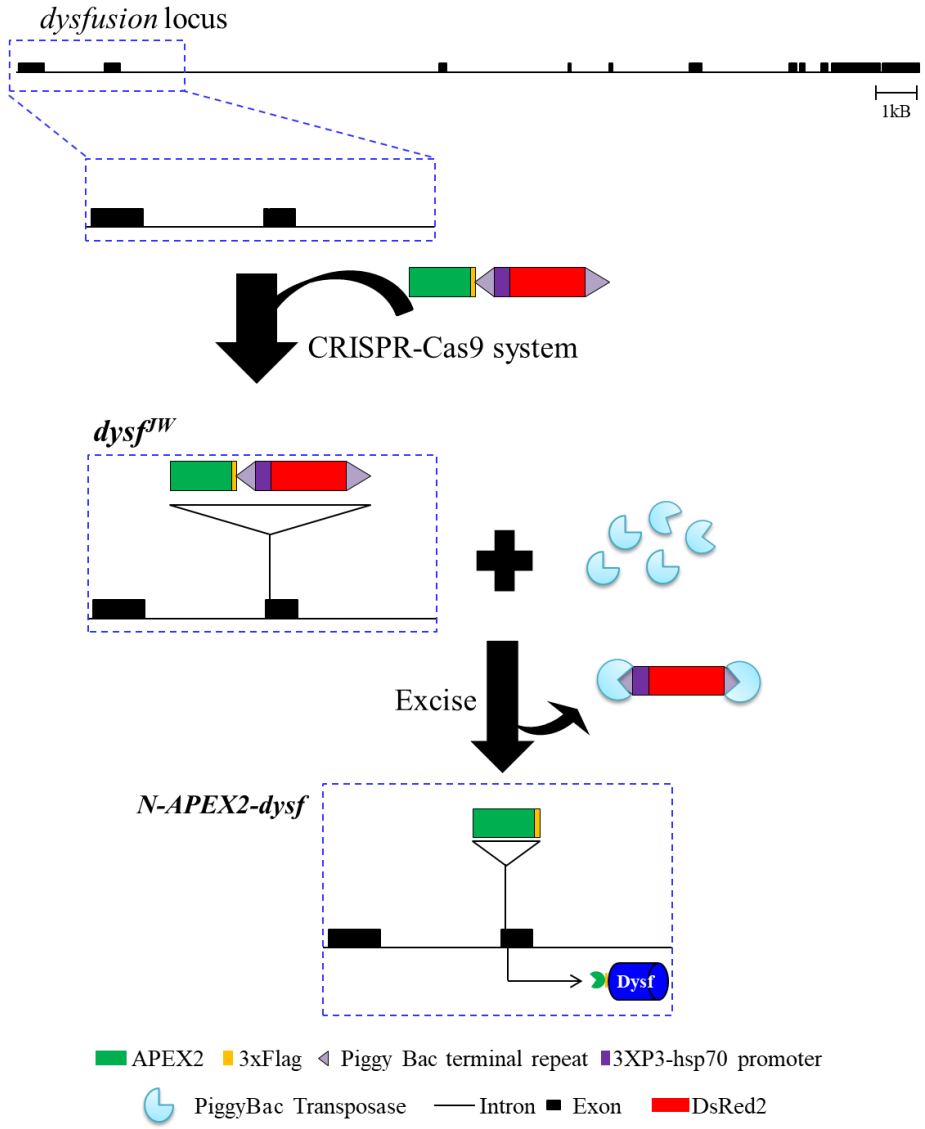


Figure 2. Generation of APEX2-3XFlag knock-in at the *dysf* locus. The APEX2-3XFlag-PBacDsRed fragment was inserted into the 5' end of the *dysf* coding region, creating *dysf*^{IW}. To generate an APEX2 knock-in tag, *dysf*^{IW} flies were crossed with BL8285 to excise the PBacDsRed fragment.

C. Ovary sample lysis

1. Homogenize ovary samples using a plastic pestle with 600 µL of RIPA lysis buffer and then sonicate for 10 cycles at 30% amplitude. Each cycle comprises 5 s of sonication followed by a 5 s rest on ice between pulses.
2. Centrifuge the lysate at 15,000× *g* for 10 min at 4 °C. Transfer the supernatant to a new tube and ensure to maintain the lysate on ice throughout the entire process.
3. Quantify the protein content in each clarified whole-cell lysate using the Bio-Rad Protein assay dye reagent concentrate. If needed, dilute the clarified lysate beforehand to ensure that the concentrations fall within the linear range of the assay. Take 50 µL aliquots of streptavidin magnetic beads and wash them twice with 1 mL of RIPA lysis buffer.

D. Streptavidin-based enrichment

1. For each sample, take 50 µL aliquots of streptavidin magnetic beads. Subsequently, incubate 550 µL of each lysate sample with 50 µL of streptavidin magnetic beads for 3 h at 4 °C on a rotator set at 10 rpm. To facilitate rotation, an additional 500 µL of RIPA buffer is added to each sample.
2. Pellet the beads using a magnetic rack and wash each bead sample five times with 500 µL of RIPA lysis buffer per wash to eliminate nonspecific binders. Keep the wash buffers on ice throughout the procedure.

E. Western blot analysis

1. To conduct the western blot analysis, start by preparing and boiling the samples in 1× protein loading buffer. Then, cool them on ice and briefly spin to minimize condensation.
2. Next, load and run the samples on an 8% (w/v) SDS gel, typically loading 15% of the streptavidin-based enrichment for analysis.
3. After gel electrophoresis, transfer the samples to a PVDF membrane using standard equipment and protocols.
4. Once transferred, block the membrane with 5% (w/v) BSA in 1× TBST for 2 h at room temperature.
5. Subsequently, gently rock the membrane in 10 mL of 0.3 µg/mL streptavidin-HRP in 1× TBST at room temperature for 2 h. Then, wash the membrane with 1× TBST four times for 5 min each before proceeding to membrane development.
6. If the streptavidin affinity pulldown is effective, it should result in the depletion of biotinylated proteins in the flowthrough fraction, while simultaneously enriching them in the bound fraction, as illustrated in Figure 3.

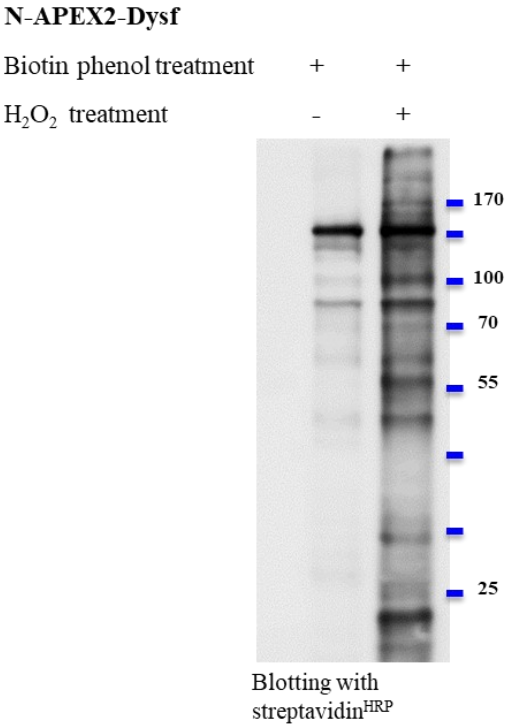


Figure 3. Streptavidin-based enrichment of biotin-tagged proteins. Western blot probed with streptavidin-HRP reveals proteins labeled by APEX2 in the co-treatment of biotin-phenol and H_2O_2 (right panel). Figure from Wu et al. [7]

F. Liquid chromatography-tandem mass spectrometry (LC-MS/MS)

1. Perform electrophoresis on the remaining samples from step E2 using an 8% Tris-glycine mini gel.
2. Apply Coomassie Blue staining to visualize proteins (Figure 4) and then carefully excise the stained bands from the gel using a new razor blade.

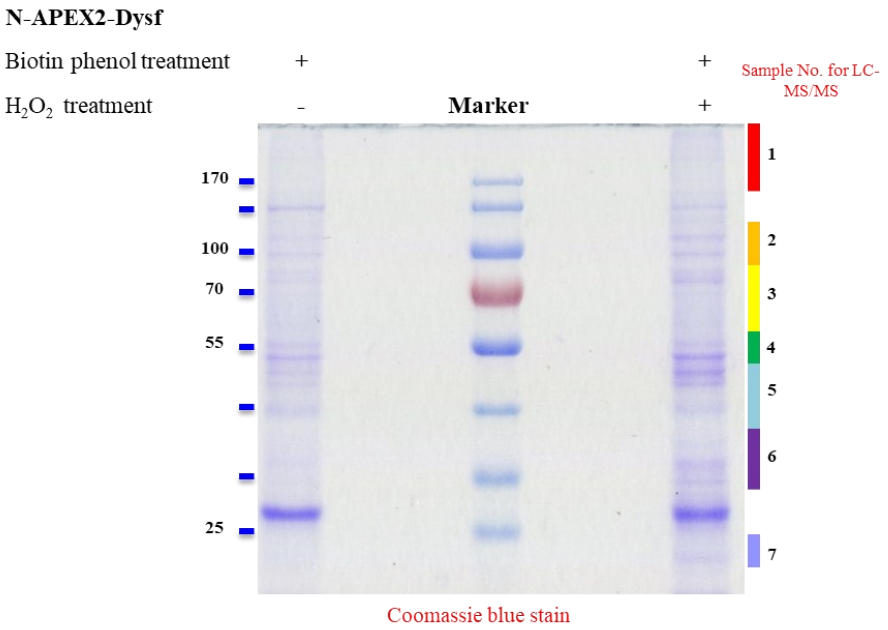


Figure 4. Coomassie blue staining for streptavidin-based enrichment. SDS-PAGE was stained with Coomassie blue to show biotinylated proteins. Color bars indicate sample fractions that were subjected to proteomic analysis. Figure from Wu et al. [7]

3. Submit the excised samples to a reputable spectrometry facility for in-gel tryptic digestion of proteins followed by liquid chromatography and mass spectrometry analysis, adhering to their established protocols.
4. Analyze the data generated by mass spectrometry from the samples.

Validation of protocol

This protocol has been employed and validated in the following research article:

- Wu et al. [7]. Spatiotemporal gating of Stat nuclear influx by *Drosophila* Npas4 in collective cell migration. Sci Adv. (Figures 6A and S5).

Acknowledgments

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Competing interests

The authors declare no competing interests.

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