

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

Jhen-Wei Wu¹, Chueh-Wen Wang¹, Wei Yang Hong¹, Anna C. C. Jang^{1,*} and Yu-Chiuan Chang^{2,*}

¹Department of Biotechnology and Bioindustry Sciences, National Cheng Kung University, Tainan, Taiwan

²Institute of Biomedical Sciences, National Sun Yat-sen University, Kaohsiung, Taiwan

*For correspondence: ccjang@ncku.edu.tw; ycc@mail.nsysu.edu.tw

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.

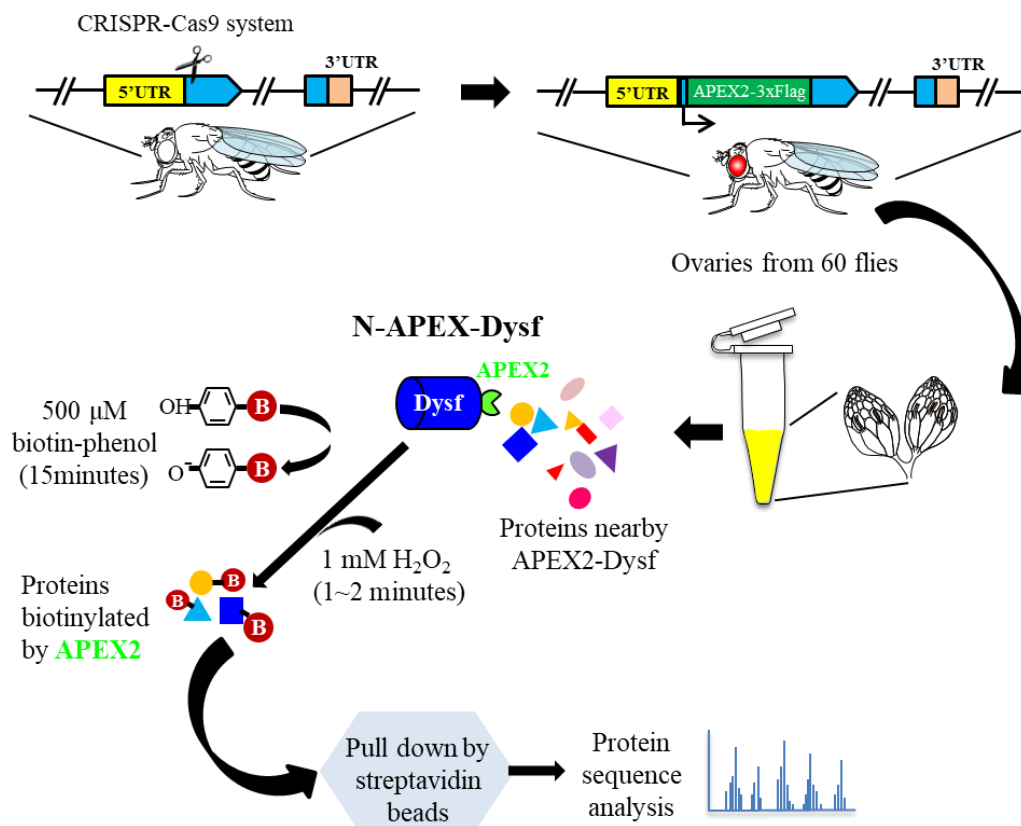
Key features

- 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

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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 (H₂O₂), 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: A1-238813)
9. Sodium azide (Merck, catalog number: S2002)
10. Sodium L-ascorbate (Merck, catalog number: A4034)
11. Hydrogen peroxide solution (H₂O₂) (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) (Biomann, 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 H₂O₂ (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 <i>Drosophila</i> 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 H₂O₂

| Reagent | Final concentration | Quantity or Volume |
|---|---------------------|--------------------|
| 30% H ₂ O ₂ (10M) | 100 mM | 10 µL |
| 10× PBS | 1× | 100 µL |
| H ₂ O | 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 ₂ O | 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 ₂ O | 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 μ L |
| 1 M Sodium azide | 10 mM | 10 μ L |
| 1 M Sodium ascorbate | 10 mM | 10 μ L |
| 10 \times PBS | 1 \times | 100 μ L |
| H ₂ O | n/a | 870 μ L |
| Total (optional) | n/a | 1 mL |

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

8. 25 \times Protease inhibitor cocktail

| Reagent | Final concentration | Quantity or Volume |
|--|---------------------|--------------------|
| cOmplete™, EDTA-free protease inhibitor cocktail | 25 \times | 1 tablet |
| H ₂ O | 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 ₂ O | 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 \times Protease inhibitor cocktail | 1 \times | 2 mL |
| H ₂ O | 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 \times PBS | 1 \times | 5 mL |
| H ₂ O | n/a | 44.25 mL |
| Total (optional) | n/a | 50 mL |

Equipment

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

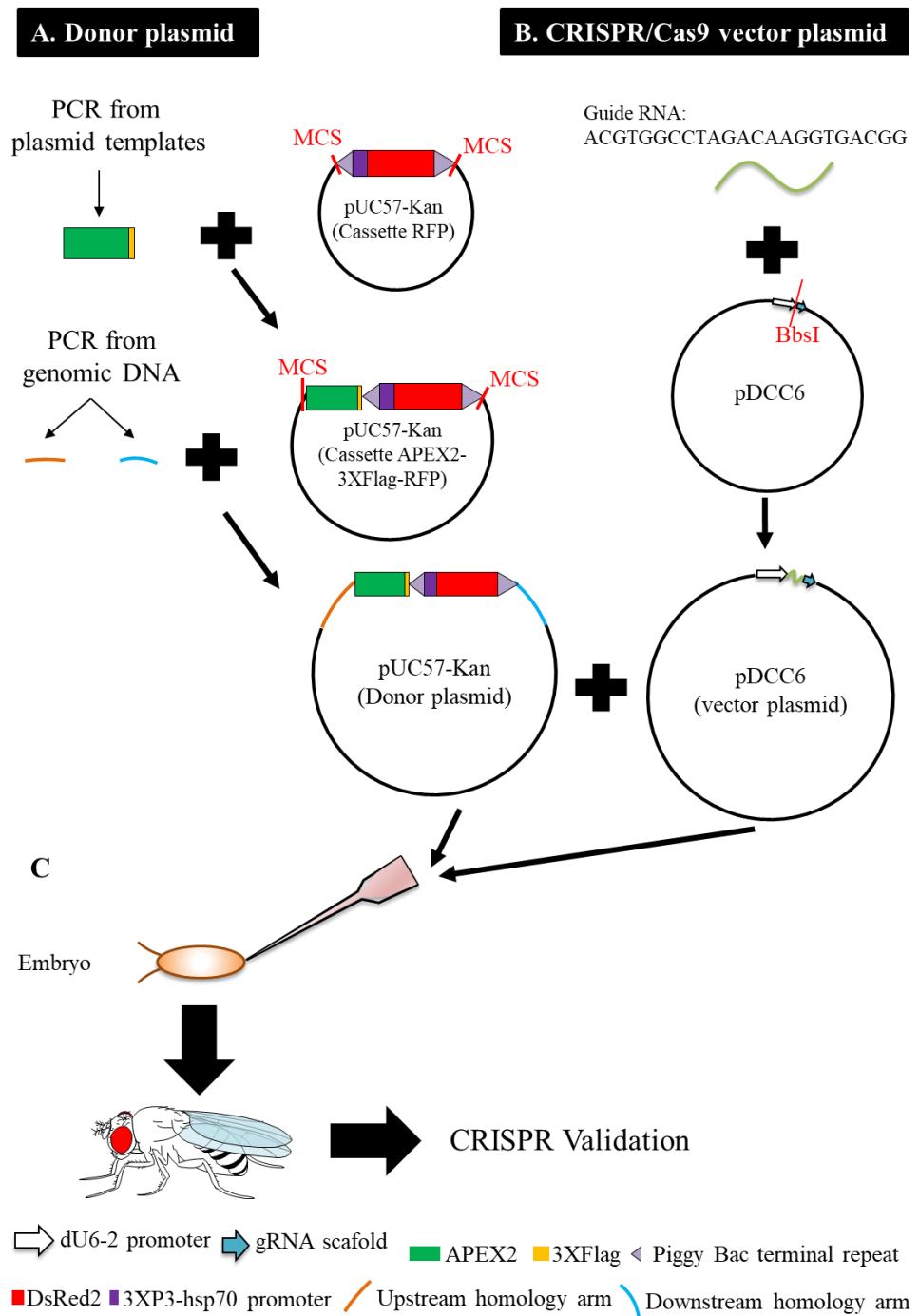


Figure 1. Workflow of generating the *dysf^W* allele using CRISPR/Cas9-mediated genome editing. A. APEX2, Piggy Bac terminal repeat, 3XFlag, homology arms, DsRed2, and 3XP3-hsp70 promoter were cloned into the pUC57-Kan plasmid as a donor plasmid. B. The guide RNA was cloned into the CRISPR/Cas9 vector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

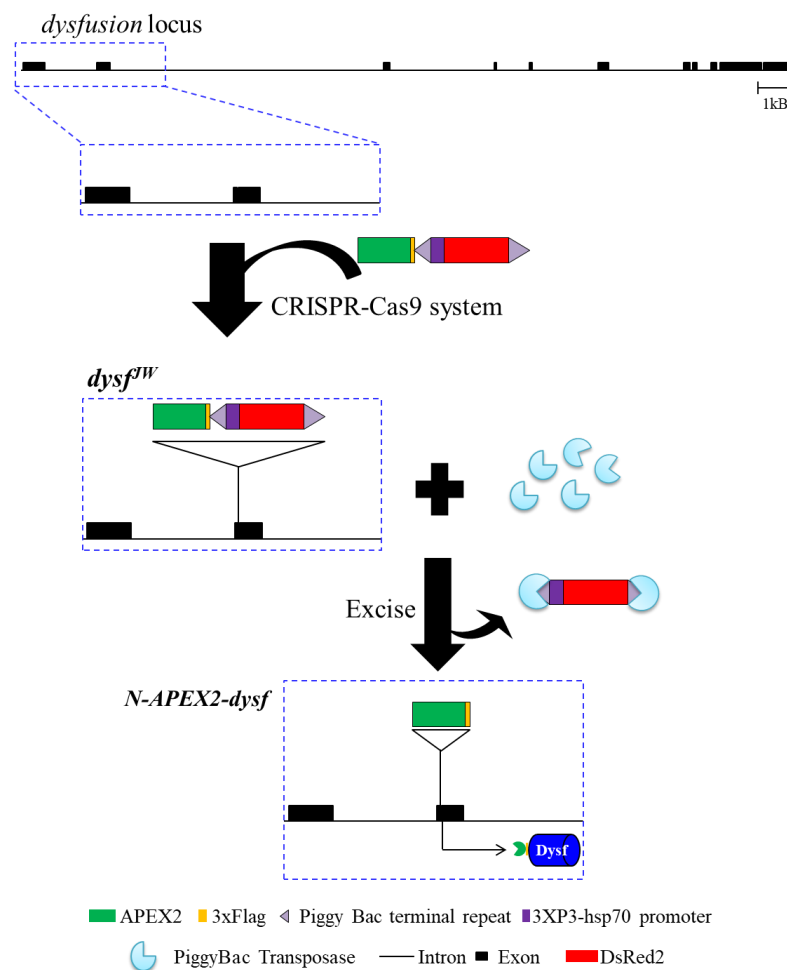


Figure 2. Generation of APEX2-3XFlag knock-in at the *dysf* locus. The APEX2-3XFlag-PBacDsRed fragment was inserted into the 5' end of the *dysf* coding region, creating *dysf^{flW}*. To generate an APEX2 knock-in tag, *dysf^{flW}* flies were crossed with BL8285 to excise the PBacDsRed fragment.

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 H₂O₂ with 790 μL of dissection medium. Then, add this premix into the samples from step B3, achieving a final concentration of 1 mM H₂O₂, 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 H₂O₂.

- 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.

C. Ovary sample lysis

1. Homogenize ovary samples using a plastic pestle with 600 μ L 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 \times *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 μ L aliquots of streptavidin magnetic beads and wash them twice with 1 mL of RIPA lysis buffer.

D. Streptavidin-based enrichment

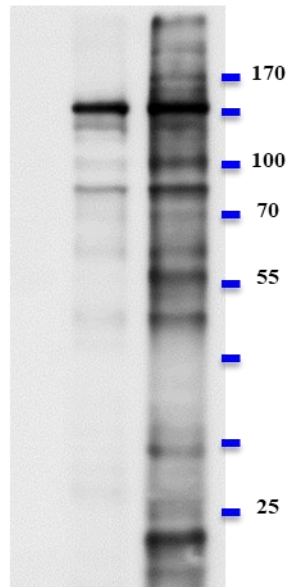
1. For each sample, take 50 μ L aliquots of streptavidin magnetic beads. Subsequently, incubate 550 μ L of each lysate sample with 50 μ L of streptavidin magnetic beads for 3 h at 4 °C on a rotator set at 10 rpm. To facilitate rotation, an additional 500 μ L of RIPA buffer is added to each sample.
2. Pellet the beads using a magnetic rack and wash each bead sample five times with 500 μ L 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 \times 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 \times TBST for 2 h at room temperature.
5. Subsequently, gently rock the membrane in 10 mL of 0.3 μ g/mL streptavidin-HRP in 1 \times TBST at room temperature for 2 h. Then, wash the membrane with 1 \times 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.

N-APEX2-Dysf

| | | |
|---|---|---|
| Biotin phenol treatment | + | + |
| H ₂ O ₂ treatment | - | + |



Blotting with streptavidin^{HRP}

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 H₂O₂ (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.

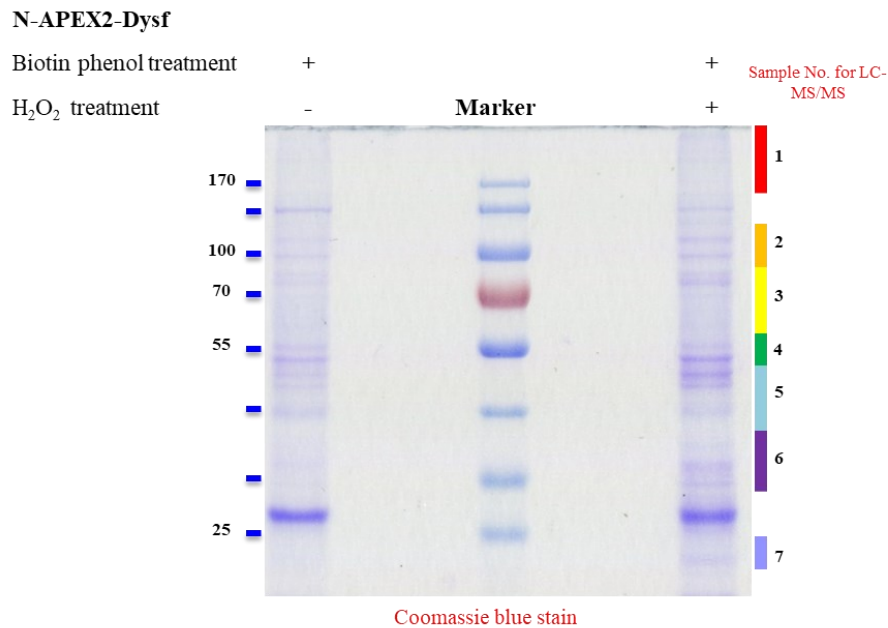


Figure 4. Coomassie blue staining for streptavidin-based enrichment. SDS-PAGE was stained with Coomassie blue to show biotinylated proteins. Color bars indicate sample fractions that were subjected to proteomic analysis. Figure from Wu et al. [7]

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.

References

1. Bosch, J. A., Chen, C. and Perrimon, N. (2020). Proximity-dependent labeling methods for proteomic profiling in living cells: An update. *WIREs Dev Biol.* 10(1): e392. <https://doi.org/10.1002/wdev.392>
2. Hung, V., Udeshi, N. D., Lam, S. S., Loh, K. H., Cox, K. J., Pedram, K., Carr, S. A. and Ting, A. Y. (2016). Spatially resolved proteomic mapping in living cells with the engineered peroxidase APEX2. *Nat Protoc.* 11(3): 456–475. <https://doi.org/10.1038/nprot.2016.018>
3. Rhee, H. W., Zou, P., Udeshi, N. D., Martell, J. D., Mootha, V. K., Carr, S. A. and Ting, A. Y. (2013). Proteomic Mapping of Mitochondria in Living Cells via Spatially Restricted Enzymatic Tagging. *Science (1979)*. 339(6125): 1328–1331. <https://doi.org/10.1126/science.1230593>
4. Chen, C. L., Hu, Y., Udeshi, N. D., Lau, T. Y., Wirtz-Peitz, F., He, L., Ting, A. Y., Carr, S. A. and Perrimon, N. (2015). Proteomic mapping in live *Drosophila* tissues using an engineered ascorbate peroxidase. *Proc Natl Acad Sci USA.* 112(39): 12093–12098. <https://doi.org/10.1073/pnas.1515623112>
5. Mannix, K. M., Starble, R. M., Kaufman, R. S. and Cooley, L. (2019). Proximity labeling reveals novel interactomes in live *Drosophila* tissue. e1101/542795. <https://doi.org/10.1101/542795>
6. Chakraborty, A., Lin, W. C., Lin, Y. T., Huang, K. J., Wang, P. Y., Chang, I. F., Wang, H. I., Ma, K. T., Wang, C. Y., Huang, X. R., et al. (2020). SNAP29 mediates the assembly of histidine-induced CTP synthase filaments in proximity to the cytokeratin network. *J Cell Sci.* 133(9): e240200. <https://doi.org/10.1242/jcs.240200>
7. Wu, J. W., Wang, C. W., Chen, R. Y., Hung, L. Y., Tsai, Y. C., Chan, Y. T., Chang, Y. C. and Jang, A. C. (2022). Spatiotemporal gating of Stat nuclear influx by *Drosophila* Npas4 in collective cell migration. *Sci Adv.* 8(29): eabm2411. <https://doi.org/10.1126/sciadv.abm2411>
8. Prasad, M., Jang, A. C., Starz-Gaiano, M., Melani, M. and Montell, D. J. (2007). A protocol for culturing *Drosophila melanogaster* stage 9 egg chambers for live imaging. *Nat Protoc.* 2(10): 2467–2473. <https://doi.org/10.1038/nprot.2007.363>