In vivo Characterization of Endogenous Protein Interactomes in *Drosophila* Larval Brain, Using a CRISPR/Cas9-based Strategy and BioID-based Proximity Labeling

Ezgi Uçkun[1], Georg Wolfstetter[1], Johannes Fuchs[2] and Ruth H. Palmer[1], *

[1]Department of Medical Biochemistry and Cell Biology, Instititute of Biomedicine at the Sahlgrenska Academy, University of Gothenburg, SE-40530 Gothenburg, Sweden

[2]Proteomics Core Facility, Sahlgrenska Academy, University of Gothenburg, SE-40530 Gothenburg, Sweden

*For correspondence: ruth.palmer@gu.se

Abstract

Understanding protein-protein interactions (PPIs) and interactome networks is essential to reveal molecular mechanisms mediating various cellular processes. The most common method to study PPIs *in vivo* is affinity purification combined with mass spectrometry (AP–MS). Although AP–MS is a powerful method, loss of weak and transient interactions is still a major limitation. Proximity labeling (PL) techniques have been developed as alternatives to overcome these limitations. Proximity-dependent biotin identification (BioID) is one such widely used PL method. The first-generation BioID enzyme BirA*, a promiscuous bacterial biotin ligase, has been effectively used in cultured mammalian cells; however, relatively slow enzyme kinetics make it less effective for temporal analysis of protein interactions. In addition, BirA* exhibits reduced activity at temperatures below 37°C, further restricting its use in intact organisms cultured at lower optimal growth temperatures (*e.g.*, *Drosophila melanogaster*). TurboID, miniTurbo, and BirA*-G3 are next generation BirA* variants with improved catalytic activity, allowing investigators to use this powerful tool in model systems such as flies. Here, we describe a detailed experimental workflow to efficiently identify the proximal proteome (proximitome) of a protein of interest (POI) in the *Drosophila* brain using CRISPR/Cas9-induced homology-directed repair (HDR) strategies to endogenously tag the POI with next generation BioID enzymes.

Keywords: BirA, MiniTurbo, TurboID, Proximitome, Proteomics

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Background

There are numerous approaches to identify protein-protein interactions (PPI)s, including yeast two-hybrid (Y2H), affinity purification combined with mass spectrometry (AP–MS), and proximity labeling (PL). Conventional methods such as Y2H and AP–MS are commonly used to identify interacting protein partners. However, these methods rely on high affinity and stable protein interactions and are less effective at identifying weakly and/or transiently interacting partners (Rees *et al.*, 2015). In the past decade, PL methods coupled to MS have been developed as a powerful complementary approach and utilized to reveal PPI networks in spatial and temporal detail (Bosch *et al.*, 2021; Qin *et al.*, 2021). In general, PL enzymes are fused to a protein of interest (POI), where they convert a small molecule substrate to a reactive intermediate that, upon release, covalently labels proteins in close proximity (Xu *et al.*, 2021).

Proximity-dependent biotin identification (BioID) is one of the commonly employed PL techniques based on BirA, a 35 kDa biotin protein ligase from *Escherichia coli*. Wild-type BirA converts biotin to reactive biotinoyl-5'-AMP in the presence of ATP and labels exposed lysine residues of substrates with biotin. BioID-based PL utilizes a mutant form of BirA (BirA[R118G], referred to as BirA*), which has lower affinity for reactive biotinoyl-5'-AMP compared to wild-type BirA and thereby prematurely releases reactive biotin resulting in promiscuous biotinylation of neighboring proteins (Roux *et al.*, 2012; Sears *et al.*, 2019). Biotinylated proteins are then pulled down with affinity reagents and analyzed by MS to map the protein interactome. BirA* enzymes derived from *Aquifex aeolicus* (BioID2) and *Bacillus subtilis* (BASU) as well as engineered forms of *E. coli* BirA* (miniTurbo, TurboID, and BirA*G3) offer the advantage of increased enzymatic activity and/or smaller size, and have been successfully used for PL-strategies (Kim *et al.*, 2016; Branon *et al.*, 2018; Ramanathan *et al.*, 2018; Samavarchi-Tehrani *et al.*, 2020; Droujinine *et al.*, 2021).

One important consideration when using PL enzymes is the expression level, as it has been shown to have significant effects on the obtained interactome maps, especially in systems where expression cannot be tightly controlled, like endogenous protein expression (Shiraiwa *et al.*, 2020; Xu *et al.*, 2021). Overexpression alters the spatio-temporal occurrence of a POI and might interfere with its localization and/or physiological function, eventually resulting in biotin-labeling artifacts. As an alternative, CRISPR/Cas9-mediated HDR strategies allow modifying endogenous gene loci to express in-frame chimeras of the POI and a next generation PL enzyme under the control of endogenous regulatory elements.

Here, we provide a detailed protocol to generate endogenous BioID fusions in *Drosophila* and perform *in vivo* PL in larval brains of these flies, followed by liquid chromatography–tandem mass spectrometry cubed (LC–MS3) analysis.

Materials and Reagents

1. 1.5 mL microcentrifuge tube (Biotix, catalog number: MTL-0150-BC)
2. 2.0 mL microcentrifuge tube (Biotix, catalog number: MT-0200-BC)
3. Acetonitrile hypergrade (Merck, catalog number: 75-05-08)
4. Apple juice (any brand)
5. Bacto-agar (Sigma, catalog number: A5306)
6. cOmplete™, Mini, EDTA-free protease inhibitor cocktail (Roche, catalog number: 11836170001)
7. D-(+)-Biotin, 98+% (Alfa Aesar, catalog number: A14207)
8. Dithiothreitol (DTT) (Roche, catalog number: 11583786001)
9. *Drosophila* sorting brush (any brand)
10. Dynabeads™ MyOne™ Streptavidin C1 (Thermo Fisher Scientific, catalog number: 65002)
11. Embryo collection cages (Genesee Scientific, catalog number: 59-100)
12. Ethanol 96% vol (VWR Chemicals, catalog number: 83804.360)
13. Fly food (Nutri-Fly Bloomington formula (BF), Genesee Scientific, catalog number: 66-113)
14. Fly food vials (VWR, catalog number: 734-2264)
15. Formic acid (VWR Chemicals, HiPerSolv CHROMANORM for LC–MS, catalog number: 84865.290)

16. Goat anti-biotin HRP (Cell Signaling Technology, catalog number: 7075)
17. Methyl methanethiosulfonate (Sigma-Aldrich, catalog number: 64306-10mL)
18. Methyl-4-hydroxybenzoate (Sigma-Aldrich, catalog number: H5501-500G)
19. Petri dishes (VWR, catalog number: 390-1373)
20. Phosphate-buffered saline (PBS) tablets (Medicago, catalog number: 09-9400-100)
21. phosSTOP™ phosphatase inhibitor tablet (Roche, catalog number: 4906837001)
22. Pierce™ High pH Reversed-Phase Peptide Fractionation Kit (Pierce, catalog number: 84868)
23. Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, catalog number: 23225)
24. Rabbit 11H10 anti-α-tubulin (Cell Signaling Technology, catalog number: 2125)
25. rLys-C (Promega, catalog number: V1671)
26. Sucrose (Sigma, catalog number: S0389)
27. Tissue grinding pestles (Kisker Biotech, catalog number: 033522)
28. TMTpro™ 16plex Label Reagent Set (Thermo Fisher Scientific, catalog number: A44521)
29. Triethylammonium bicarbonate (Honeywell Fluka, catalog number: 17902-100mL)
30. Trifluoroacetic acid (Sigma-Aldrich, catalog number: 299537-50g)
31. Tris (2-carboxyethyl) phosphine (Thermo Scientific, catalog number: 77720)
32. Tris-Cl pH 7.4 (Alfa Aesar, catalog number: J60202.K2)
33. Pierce™ Trypsin protease (Pierce, catalog number: 90057)
34. Yeast paste (baker's yeast in tab water) (Kisker Biotech, catalog number: 789093)
35. Apple-juice agar plates (see Recipes)
36. 25% ethanolic Methyl-4-hydroxybenzoate (see Recipes)
37. 4× Laemmli Buffer (see Recipes)
38. RIPA buffer (see Recipes)
39. Wash buffer 1 (see Recipes)
40. Wash buffer 2 (see Recipes)
41. Wash buffer 3 (see Recipes)

Equipment

1. Acclaim™ PepMap™ 100 C18 HPLC Column (Thermo Fisher Scientific, catalog number: 164199)
2. Easy-nLC™ 1200 (Thermo Fisher Scientific, catalog number: LC140)
3. Heating block (Grant, model: QBD2)
4. Incubator (Termaks, catalog number: TS 8056)
5. In-house packed analytical column (ESI Source Solutions, catalog number: PTC3-75-50-SP)
6. Magnetic stand (Thermo Fisher Scientific, model: DynaMag-2)
7. Microcentrifuge (Thermo Fisher Scientific, model: Fresco 21)
8. Microcentrifuge (VWR, model: CT15RE)
9. Orbitrap Fusion™ Lumos™ Tribrid™ mass spectrometer interfaced with an Easy-nLC1200 liquid chromatography system (Thermo Fisher Scientific, catalog number: IQLAAEGAAPFADBMBHQ)
10. Reprosil-Pur C18 (Dr. Maisch, catalog number: R13.AQ.0001)
11. Rotator (Thermo Fisher Scientific, Labquake, catalog number: 13-687-12Q)
12. Ultrapure water system (Elga LabWater, Purelab)
13. Vacuum centrifuge (Genevac, miVac, catalog number: DUC-23050-B00)

Software

1. Mascot (Matrix Science, Version 2.5.1)
2. Proteome Discoverer™ (Thermo Fisher Scientific, Version 2.4, catalog number: OPTON-30945)

Procedure

A. A CRISPR-Cas9-mediated HDR strategy to endogenously tag a POI with BioID-enzymes

A detailed protocol for the generation of CRISPR components and screening strategies in *Drosophila* can be found in Housden *et al.* (2014).

1. Guide RNA design and cloning

   Select one or two suitable CRISPR target sites using the flyCRISPR Target Finder tool (http://targetfinder.flycrispr.neuro.brown.edu/) (Gratz *et al.*, 2014). The target site should ideally be located in close proximity (≤100 bp) to the intended hinge region between the endogenous coding DNA sequence (CDS) of the POI and the BioID-encoding insert. Guide sequences with estimated off-targets (annotated in the flyCRISPR Target Finder tool) should be avoided. We followed the flyCRISPR protocol for cloning guide plasmids (available at https://flycrispr.org/protocols/grna/).

   Note: If not possible otherwise, one off-target sequence on a different chromosome can be tolerated since the affected chromosome can be removed later during the screening process. A CRISPR off-target site might nonetheless decrease the number of positive candidates obtained.

2. dsDNA donor design and cloning

   Small, commonly used bacterial plasmids (*e.g.*, puc57, pBlueScript II, or pCRII Topo) can be efficiently employed as vector backbone. Gene-specific homology arms (from 0.5-1kb length; longer and shorter sequences can also be chosen but might affect HDR efficiency) are cloned from a genomic DNA source by standard procedures. Codon-optimized CDS of BioID-enzymes should be used for optimal codon usage in the targeted species. Plasmids for expression of next-generation BioID enzymes, miniTurbo (number 116905), and TurboID (number 116904) can be obtained from Addgene and used as template for amplification. Short and flexible amino acid linker sequences such as 1-4x[Glycine-Glycine-Serine-Glycine] or 1-4x[Glycine-Serine-Alanine-Threonine] between the POI and the BioID enzyme can be introduced to minimize potential effects on POI function caused by steric hindrance (http://parts.igem.org/Protein_domains/Linker, available @ igem.org, 22 April 2022). However, it should be considered that linker sequences likely increase the BioID labeling radius. Small protein tags, *e.g.*, HA (amino acid sequence Tyr-Pro-Tyr-Asp-Val-Pro-Asp-Tyr-Ala) or OLLAS: Ser-Gly-Phe-Ala-Asn-Glu-Leu-Gly-Pro-Arg-Leu-Met-Gly-Lys) can also be added to analyze expression and localization of the chimeric protein. Several strategies can be used to mask the CRISPR target site for Cas9 recognition in the donor vector: (1) changing Guanine(s) within the NGG PAM sequence, thereby introducing a silent mutation in the POI's CDS; (2) if changing the NGG sequence is not feasible, several nucleotides in the target sequence can be altered, introducing silent point mutations in the POI's CDS; (3) placing the CRISPR target site over the intended hinge region so that the recognized sequence will be split and separated by the BioID-encoding insert. The last approach is recommended if the NGG PAM following the target sequence is located in a non-protein-coding region where base-pair alterations could affect gene expression.

   Notes:

   a. *Sequence information of the miniTurbo and TurboID next generation BioID enzymes (Branon et al., 2018) can be obtained from plasmids deposited at Addgene #116905 and #116904, respectively. These can be employed as templates for amplification.*

   b. *Scarless gene editing provides a donor vector system that simplifies the candidate screening process (https://flycrispr.org/scarless-gene-editing/).*

   c. *AviTag^{TM} (Schatz, 1993; Cognet et al., 2005) can be used to incorporate artificial biotinylation sites in the POI or possible proximitome candidates. In case of no or few accessible lysine residues, this can serve as a BioID positive control (adding AviTag to the POI) or to enable detection of a proximitome candidate by MS.*

3. Plasmid injection into *Drosophila* embryos

   Injection of guide RNA and dsDNA donor plasmids in *Drosophila* embryos that express Cas9 in their germ line can either be done by following flyCRISPR injection protocols

(https://flycrispr.org/protocols/injection/) or using a *Drosophila* microinjection facility or commercial injection service.

Note: Several transgenic Drosophila stocks expressing Cas9 in their germ line (e.g., M{vas-Cas9}ZH-2A) are available for injection. It should be taken into account that the genetic background of the injection stock should a) be identical to the genetic background that was used to select the CRISPR/Cas9 guide RNA target sites, and b) not distort the following experimental analysis (certain traits, e.g., sleep and life span are highly impacted by genetic background). Furthermore, the source of the Cas9-transgene should be located on a different chromosome than the targeting region.

4. Screening for CRISPR-mediated HDR events

    Parental (P) male flies derived from the injected embryos are crossed individually to female virgin flies from a suitable balancer stock. P female flies are crossed "en masse" to suitable balancer males. After 2–3 days, egg-laying females can be identified and isolated in fresh culture vials. Isolation of fertile females can be continued on every other day. Excluding non-fertile females at this point significantly reduces consumables and work spent during the screening process. Two to five F1 males from each parental cross are isolated and crossed individually to balancer females. When larval progeny is visible in the F1 cross, the F1 males can be sacrificed for genomic DNA isolation and further genotyping using single fly PCR screening.

    Note: We follow a standard single fly DNA preparation protocol (available, e.g., from http://francois.schweisguth.free.fr/protocols/Single_fly_DNA_prep.pdf) with the following modifications: Proteinase K digestion is done in a PCR-cycler for 30 min at 50°C, followed by enzyme inactivation for 10 min at 85°C. We typically prepare 25 µL PCR mixes with 2.5 µL from the crude DNA preparation as template solution. Positive candidates should be analyzed by Sanger sequencing before further experiments are conducted.

B. *In vivo* labeling of the POI interactome in *Drosophila*

1. Place an apple-agar plate with a smear of yeast paste in an embryo collection cage.

2. Transfer 40–50 young (<7 days) female and male flies expressing the chimeric BioID-POIs to the embryo cages and allow egg laying for 2–8 h. If sufficient eggs are laid, remove plates from the cage and age at 25°C. If not, replace with a new apple-agar plate for another egg laying period.

    Note: As a background control, use an appropriate reference fly stock that closely matches the genetic background of your genetically engineered flies (e.g., w^{1118} or Canton-S).

3. After 22–23 h, remove hatched larvae from the plate using a *Drosophila* sorting brush and check again every hour for freshly hatched first instar L1 larvae. Transfer 50 L1 larvae from the same collection point into a *Drosophila* culture vial containing a sufficient amount of Bloomington formula (BF) fly food supplemented with 100 µM biotin. For each genotype/condition/replicate, prepare at least five vials.

    Notes:

    a. *Biotin stock is prepared with water and incorporated into fly food after cooking before pouring into the vials to make 100 µM final concentration.*

    b. *Synchronizing developmental timing at this step is important obtain the proximitome from a homogeneous population of age-matched third instar larvae.*

    c. *Higher concentrations of supplemented biotin resulted in lethality of control larvae that did not express any BioID-fusion protein (Uçkun et al., 2021).*

4. Collect 150 biotin-fed third instar larvae (72–120 h after egg laying; 48–96 h after hatching) for each genotype/condition/replicate.

C. Protein extraction from *Drosophila* larval brains

1. Dissect 150 larval brains (Hafer and Schedl, 2006) in ice-cold PBS and transfer to a microcentrifuge tube containing 300–400 µL PBS on ice.

    Notes:

    a. *Dissection is ideally performed by multiple persons to avoid sample decay and de-synchronizing of*

      developmental timing. If the dissection is performed by only one person, several rounds of dissection for one genotype/condition/replicate are required.

    b. *Dissected brains can be snap-frozen in liquid nitrogen and stored at -80°C until the required sample size is collected.*

2. After dissection is complete, remove the PBS and lyse dissected third instar larval brains directly in 700 µL of RIPA buffer supplemented with 1× cOmplete^{TM} Mini EDTA-free protease inhibitor cocktail, 1× phosSTOP^{TM} phosphatase inhibitor, and 1 mM DTT using a tissue grinding pestle.

3. Centrifuge samples at 21,500 × *g* at 4°C for 20 min and transfer supernatant to a new microcentrifuge tube.

4. Perform BCA assays to determine protein concentration for each sample.
   Notes:
   a. *Equal or greater than 1 mg of total protein for each sample/replicate/condition is desirable.*
   b. *BCA assay is performed according to the Pierce^{TM} BCA Protein Assay Kit user guide.*
   c. *Normalization of protein quantity is required prior to pull-down if different numbers of brains are lysed, and protein concentration varies between samples. This can be done by diluting more concentrated samples with RIPA buffer.*

5. Transfer 50 µL of lysate to a separate microcentrifuge tube for western blot analysis and validation of protein biotinylation; 50 µg protein is sufficient for western blot analysis of biotinylated proteins. Use specific antibodies to detect your POI or an optional protein tag as well as anti-Biotin-HRP/Streptavidin-HRP to confirm increased protein biotinylation. Use antibodies against housekeeping proteins (such as anti α-tubulin) as loading control.
   Note: Protocols for western blotting of biotinylated proteins with some examples are available in Roux et al. (2013).

D. Pull-down of biotinylated proteins

This pull-down protocol is adapted from Roux *et al*. (2013).

1. Prepare 1.5 mL microcentrifuge tubes for each sample/replicate and place on a magnetic stand. *Note: The following steps are performed at room temperature.*

2. Add 500 µL of RIPA buffer and 500 µL of 50 mM Tris-Cl pH 7.4 to each tube.

3. Homogenize Dynabeads^{TM} MyOne^{TM} Streptavidin C1 stock solution by gently tapping and add 300 µL to each tube. Place tubes in the magnetic stand and wait 3 min.
   Note: The quantity of streptavidin beads used will depend on the amount of protein in the sample; 300 µL of beads are sufficient for 1–1.5 mg of total protein. Use more beads for higher protein amounts.

4. Gently remove supernatant and add 700 µL of protein lysate corresponding to at least 1 mg of total protein from step C3.

5. Resuspend the lysate with beads and incubate on a rotator at 4°C overnight.

6. Place tubes in the magnetic stand and wait 3 min.

7. Transfer the supernatant to a new 1.5 mL microcentrifuge tube labeled as flow-through.
   Note: Flow-through labeled supernatant allows assessment of pull-down efficiency in western blotting, when compared to samples from step C5.

8. Resuspend beads with1.5 mL of wash buffer 1.

9. Place tubes on rotator at room temperature for 8 min.

10. Place tubes in magnetic stand and wait 3 min.

11. Remove supernatant and repeat steps D8–D10.

12. Resuspend beads with 1.5 mL of wash buffer 2 and repeat steps D9–D10.

13. Resuspend beads with 1.5 mL of wash buffer 3 and repeat steps D9–D10.

14. To remove detergent, wash beads four times with 1.5 mL 50 mM Tris-Cl pH 7.4 following the procedure described in steps D8–D10.

15. Remove 150 µL of resuspended beads to a new tube for western blot analysis and keep the remaining 1.35 mL of resuspended beads for MS analysis.

16. Spin both 150 µL and 1.35 mL aliquots of resuspended beads at 2,000 × *g* at room temperature for 5 min.

17. For western blot samples prepared in steps D15–D16, remove supernatant and add 100 µL of 1× LaemmLi buffer. Heat samples at 95°C for 5 min and centrifuge at $18,700 \times g$, 4°C for 10 min. The supernatant can be stored at -20°C for further western blot analysis.
18. For MS analysis, remove the supernatant from 1.35 mL samples and resuspend beads with 200 µL Tris-Cl pH 7.4.

E. Elution, reduction, alkylation, and tryptic digestion of biotinylated proteins

1. Pellet beads for 1 min using a magnetic stand and remove supernatant.
2. Wash beads twice with 1 mL of 50 mM triethylammonium bicarbonate (TEAB).
3. Add 100 µL of 50 mM TEAB to beads. Flip tubes gently five times so that all beads are in motion.
4. For proteolytic bead-elution, add 10 µL of 0.05 µg/µL rLys-C dissolved in the resuspension buffer provided with the enzyme directly to the sample.
5. Incubate for 3 h at 37°C in an incubator. Mix the beads every 30 min by flicking the sample tubes gently with one finger so that all beads are in motion. Do the same for each of the following steps. Do not mix beads by vortexing.
6. Reduction: Add 1 µL of 500 mM Tris (2-carboxyethyl) phosphine (TCEP) for a final concentration of 5 mM TCEP. Flip samples and incubate for 30 min at 37°C in an incubator.
7. Alkylation: Add 5 µL of 200 mM methyl methanethiosulfonate (MMTS) for a final concentration of 10 mM MMTS. Flip samples and incubate for 30 min at room temperature.
8. For further digestion of the samples, add 6 µL of 0.05 µg/µL trypsin (suspension in 50 mM TEAB). Flip sample tubes to ensure mixing of the samples and incubate at 37°C incubator overnight.
   Note: It is not necessary to use 50 mM TEAB. Water can be used. Buffers containing primary amines (e.g., Tris, ammonium bicarbonate), which inhibit TMT-labeling, should be avoided.
9. Pellet beads using the magnetic rack and transfer supernatant to new sample tubes.

F. Tandem mass tag (TMT) labeling with TMTpro™ 16plex

1. Allow TMT-reagents to equilibrate to room temperature.
2. Dissolve TMT-reagents in 130 µL of acetonitrile, vortex shortly, and dissolve for 5 min at room temperature.
3. Spin down TMT-reagents briefly and transfer 42 µL reagent to the respective sample, *e.g.*, 42 µL TMT-reagent 126 to control 1.
4. Mix sample on vortex, spin down briefly, and incubate for 1 h at room temperature.
5. Add 8 µL of 5% hydroxylamine (dilute 1:10 from 50% hydroxylamine provided in the TMT labeling kit) to each sample and incubate at room temperature for 15 min.
6. Pool all labeled samples. Wash each vial with 50 µL of 50% acetonitrile to recover remaining sample and add to the pool.
7. Dry samples in a vacuum centrifuge at 40°C. It might take several hours until sample is completely dry. Check occasionally.

G. High pH reversed phase fractionation

Note: Digested and labeled peptides are fractionated into 10 fractions in this step using a High pH Reversed-Phase Peptide Fractionation Kit. Analyzing fractions will result in more detected peptides via LC–MS, compared to injecting the pooled TMT-sample directly. Each column has a recommended capacity of 10–100 µg peptides.

1. Dissolve sample in 600 µL of 0.1% trifluoroacetic acid (TFA) and let stand at room temperature until step G7.
2. Prepare washing and elution buffers containing 8%, 10%, 12%, 14%, 16%, 18%, 20%, 22%, 25%, and 50% acetonitrile in 0.1% triethylamine (supplied in the high pH fractionation kit), according to the Table 1.

Table 1. Volumes of Acetonitrile and Triethylamine for preparation of washing and elution buffers.

Fraction No.	Acetonitrile (%)	Acetonitrile (μL)	Triethylamine (0.1%) (μL)
Wash buffer	3%	30	970
1	8%	80	920
2	10%	100	900
3	12%	120	880
4	14%	140	860
5	16%	160	840
6	18%	180	820
7	20%	200	800
8	22%	220	780
9	25%	250	750
10	50.0%	500	500

3. Use two columns and remove the protective white tip from the bottom of the fractionation column. Place each column into a 2.0 mL tube.
4. Centrifuge at $5,000 \times g$ for 2 min to remove storage buffer and pack the resin material.
5. Remove the red screw cap and add 300 μL of acetonitrile to the columns. Spin at $5,000 \times g$ for 2 min; repeat this step once and discard flow through.
6. Add 300 μL of 0.1% TFA to the columns. Spin at $5,000 \times g$ for 2 min; repeat this step once and discard flow through.
7. Transfer 300 μL sample to each of the two columns. Spin at $3,000 \times g$ for 2 min for following steps. Reapply sample on column and spin again.
8. Place column into a new 1.5 mL tube and add 300 μL water. Spin at $3,000 \times g$ for 2 min for all washing and elution steps.
9. Wash the column with 300 μL 3% acetonitrile in 0.1% triethylamine to remove unreacted TMT-reagent.
10. Place column into a new 1.5 mL tube and elute with 300 μL elution buffer containing 8% acetonitrile.
11. Repeat step 11 using a new tube for each elution buffer.
12. Dry samples in a vacuum centrifuge and dissolve sample in 3% acetonitrile, 0.1% formic acid for LC–MS3 analysis.

H. LC–MS3 analysis

1. Analyze each fraction on an Orbitrap Fusion™ Lumos™ Tribrid™ mass spectrometer interfaced with an Easy-nLC™ 1200 liquid chromatography system. The LC-system should be equipped with an Acclaim™ Pepmap™ 100 C18 trap column (100 μm × 2 cm, particle size 5 μm) and an in-house packed analytical column i.d. 75 μm, particle size 3 μm, Reprosil-Pur C18, Dr. Maisch, length 35 cm.
2. Separate peptides using a linear gradient from 5% to 33% B over 77 min followed by an increase to 100% solvent B for 3 min, and 100% B for 10 min at a flow of 300 nL/min. Solvent A is 0.2% formic acid, and solvent B is 80% acetonitrile, 0.2% formic acid.
3. Run the Orbitrap Fusion™ Lumos™ Tribrid™ mass spectrometer using SPS-MS3. The precursor ion mass spectra are acquired at 120,000 resolution, and MS/MS analysis is performed in a data-dependent multinotch mode where CID spectra of the most intense precursor ions are recorded in the ion trap with collision energy setting of 30 for 3 s ('top speed' setting). Precursors are isolated in the quadrupole with a 0.7 m/z isolation window, charge states 2 to 7 are selected for fragmentation, dynamic exclusion is set to

45 s and 10 ppm. MS3 spectra for reporter ion quantitation are recorded at 50,000 resolution with HCD fragmentation at collision energy 55 using synchronous precursor selection.

I. Proteomic Data Analysis

All data files are merged for identification and relative quantification using Proteome Discoverer™ 2.4 (Thermo Fisher Scientific). Data matching is performed against the *Drosophila melanogaster* database from Uniprot (Swissprot+TrEMBL) using Mascot version 2.5.1 (Matrix Science) as a search engine. The precursor mass tolerance is set to 5 ppm and fragment mass tolerance to 0.6 Da. Tryptic peptides were accepted with one missed cleavage, variable modifications of methionine oxidation and fixed cysteine alkylation, TMTpro-label modifications of N-terminal and lysine are selected. Percolator is used for the validation of Peptide-Spectrum-Matches (confidence threshold medium (<5%) and high (<1%)). TMT reporter ions are identified in the MS3 HCD spectra with 3 mmu mass tolerance. Only unique peptides for a given protein are considered for quantification of the proteins.

Recipes

1. **Apple-juice agar plates**
    Add 18 g of Bacto-agar to 800 mL of cold tap water and boil in a microwave oven. Add 20 g of sucrose and 200 mL of apple juice. Mix thoroughly and allow to cool to ~55°C. Add 5 mL of 25% ethanolic Methyl-4-hydroxybenzoate (Nipagin) stock solution, mix thoroughly, and pour into Petri dishes.

2. **25% ethanolic Methyl-4-hydroxybenzoate**
    Prepare 25% weight/volume (w/v) stock solution of Methyl-4-hydroxybenzoate in 96% ethanol

3. **4× Laemmli Buffer**
    200 mM Tris-Cl pH 6.8, 8% SDS, 40% glycerol, 0.4% bromophenol blue, and 50 mM DTT

4. **RIPA buffer**
    50 mM Tris-Cl, pH 7.4, 250 mM NaCl, 1 mM EDTA, 1 mM EGTA, and 0.5% Triton X-100

5. **Wash buffer 1**
    2% (w/v) SDS

6. **Wash buffer 2**
    0.1% (w/v) deoxycholic acid, 1% Triton X-100, 1 mM EDTA, 500 mM NaCl, 50 mM HEPES pH 7.5

7. **Wash buffer 3**
    0.5% (w/v) deoxycholic acid, 0.5% NP-40, 1 mM EDTA, 250 mM LiCl, 10 mM Tris-Cl pH 7.4

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Competing interests

The authors declare that they have no competing interests.

References

Bosch, J. A., Chen, C. L. and Perrimon, N. (2021). Proximity-dependent labeling methods for proteomic profiling in living cells: An update. *Wiley Interdiscip Rev Dev Biol* 10(1): e392.

Branon, T. C., Bosch, J. A., Sanchez, A. D., Udeshi, N. D., Svinkina, T., Carr, S. A., Feldman, J. L., Perrimon, N. and Ting, A. Y. (2018). Efficient proximity labeling in living cells and organisms with TurboID. *Nat Biotechnol* 36(9): 880-887.

Cognet, I., Guilhot, F., Gabriac, M., Chevalier, S., Chouikh, Y., Herman-Bert, A., Guay-Giroux, A., Corneau, S., Magistrelli, G., Elson, G. C., *et al*. (2005). Cardiotrophin-like cytokine labelling using Bir A biotin ligase: a sensitive tool to study receptor expression by immune and non-immune cells. *J Immunol Methods* 301(1-2): 53-65.

Droujinine, I. A., Meyer, A. S., Wang, D., Udeshi, N. D., Hu, Y., Rocco, D., McMahon, J. A., Yang, R., Guo, J., Mu, L., *et al*. (2021). Proteomics of protein trafficking by *in vivo* tissue-specific labeling. *Nat Commun* 12(1): 2382.

Gratz, S. J., Ukken, F. P., Rubinstein, C. D., Thiede, G., Donohue, L. K., Cummings, A. M. and O'Connor-Giles, K. M. (2014). Highly specific and efficient CRISPR/Cas9-catalyzed homology-directed repair in *Drosophila*. *Genetics* 196(4): 961-971.

Hafer, N. and Schedl, P. (2006). Dissection of larval CNS in *Drosophila melanogaster*. *J Vis Exp*(1): 85.

Housden, B. E., Lin, S. and Perrimon, N. (2014). Chapter Nineteen - Cas9-Based Genome Editing in *Drosophila*. In: Doudna, J. A. and Sontheimer, E. J. (Eds.). *Methods in Enzymology*. Academic Press, 415-439.

Kim, D. I., Jensen, S. C., Noble, K. A., Kc, B., Roux, K. H., Motamedchaboki, K. and Roux, K. J. (2016). An improved smaller biotin ligase for BioID proximity labeling. *Mol Biol Cell* 27(8): 1188-1196.

Qin, W., Cho, K. F., Cavanagh, P. E. and Ting, A. Y. (2021). Deciphering molecular interactions by proximity labeling. *Nat Methods* 18(2): 133-143.

Ramanathan, M., Majzoub, K., Rao, D. S., Neela, P. H., Zarnegar, B. J., Mondal, S., Roth, J. G., Gai, H., Kovalski, J. R., Siprashvili, Z., *et al*. (2018). RNA-protein interaction detection in living cells. *Nat Methods* 15(3): 207-212.

Rees, J. S., Li, X. W., Perrett, S., Lilley, K. S. and Jackson, A. P. (2015). Protein Neighbors and Proximity Proteomics. *Mol Cell Proteomics* 14(11): 2848-2856.

Roux, K. J., Kim, D. I. and Burke, B. (2013). BioID: a screen for protein-protein interactions. *Curr Protoc Protein Sci* 74: 19 23 11-19 23 14.

Roux, K. J., Kim, D. I., Raida, M. and Burke, B. (2012). A promiscuous biotin ligase fusion protein identifies proximal and interacting proteins in mammalian cells. *J Cell Biol* 196(6): 801-810.

Samavarchi-Tehrani, P., Samson, R. and Gingras, A. C. (2020). Proximity Dependent Biotinylation: Key Enzymes and Adaptation to Proteomics Approaches. *Mol Cell Proteomics* 19(5): 757-773.

Schatz, P. J. (1993). Use of peptide libraries to map the substrate specificity of a peptide-modifying enzyme: a 13 residue consensus peptide specifies biotinylation in *Escherichia coli*. *Biotechnology (N Y)* 11(10): 1138-1143.

Sears, R. M., May, D. G. and Roux, K. J. (2019). BioID as a Tool for Protein-Proximity Labeling in Living Cells. *Methods Mol Biol* 2012: 299-313.

Shiraiwa, K., Cheng, R., Nonaka, H., Tamura, T. and Hamachi, I. (2020). Chemical Tools for Endogenous Protein Labeling and Profiling. *Cell Chem Biol* 27(8): 970-985.

Uçkun, E., Wolfstetter, G., Anthonydhason, V., Sukumar, S. K., Umapathy, G., Molander, L., Fuchs, J. and Palmer, R. H. (2021). *In vivo* Profiling of the Alk Proximitome in the Developing *Drosophila* Brain. *J Mol Biol* 433(23): 167282.

Xu, Y., Fan, X. and Hu, Y. (2021). *In vivo* interactome profiling by enzyme-catalyzed proximity labeling. *Cell Biosci* 11(1): 27.